[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON, SEATTLE 5, WASHINGTON]

The Structure of "Active Formaldehyde" (N^5,N^{10}-Methylene Tetrahydrofolic Acid)[1]

BY M. J. OSBORN,[2] P. T. TALBERT[3] AND F. M. HUENNEKENS

RECEIVED SEPTEMBER 2, 1959

A method is described for the chemical synthesis and chromatographic isolation of "active formaldehyde" from formaldehyde and tetrahydrofolate. The authenticity of the synthetic material is confirmed by its quantitative oxidation to "active formate" *via* the TPN-linked hydroxymethyl tetrahydrofolic dehydrogenase. The following chemical evidence is presented to support the assignment of the structure, N^5,N^{10}-methylene tetrahydrofolate (I), to "active formaldehyde": (a) the stability to oxidizing agents, (b) the dissociation at different *p*H values in the presence and absence of hydroxylamine, (c) the *p*H dependence of the rate of formation from formaldehyde and tetrahydrofolate and (d) the chemical synthesis *via* reduction of N^5,N^{10}-methenyl tetrahydrofolate with sodium borohydride.

In enzymic systems tetrahydrofolic acid serves as the carrier, or coenzyme, of one-carbon (C_1)[4] fragments at the oxidation levels of both formate and formaldehyde.[5-11] The adduct between formate and tetrahydrofolate may assume several different forms, *i.e*, N^{10}-formyl tetrahydrofolate, N^5-formyl tetrahydrofolate or N^5,N^{10}-methenyl tetrahydrofolate. Similarly, an adduct between formaldehyde and tetrahydrofolate, "active formaldehyde," has been detected as an intermediate in several enzymic reactions, but the exact structure of this compound is less well-established.

The reversible *enzymic* synthesis of "active formaldehyde" can be accomplished by several systems, notably serine hydroxymethylase,[12-19] hydroxymethyl tetrahydrofolic dehydrogenase[7,15,20-22] and formaldehyde activating enzyme[23]; the appropriate reactions are indicated by equations 1-3, respectively.

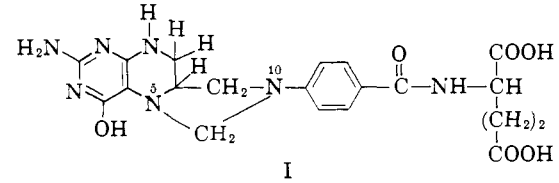
serine + tetrahydrofolate $\rightleftharpoons$ "active formaldehyde" + glycine (1)

"active formaldehyde" + TPN^+ $\rightleftharpoons$ (N^5,N^{10}-methenyl tetrahydrofolate)$^+$ + TPNH (2)

HCHO + tetrahydrofolate $\rightleftharpoons$ "active formaldehyde" (3)

In reaction 3 the absorption spectrum of the enzymically synthesized product[23] differed from that of tetrahydrofolate and was shown to be authentic "active formaldehyde" by specific enzymic assays utilizing the hydroxymethyl tetrahydrofolic dehydrogenase and the serine hydroxymethylase systems. In addition, recent evidence (reviewed in ref. 11) also suggests that "active formaldehyde" participates as the C_1 donor in the biosynthesis of the methyl groups of methionine, choline and thymine.

The *chemical* synthesis of "active formaldehyde" by the simple admixing of formaldehyde and tetrahydrofolate, according to equation 3, was first reported by Kisliuk[24] and Jaenicke[15] and has since been studied in detail by Blakley[25,26] and by Kisliuk.[27] From diverse lines of chemical and enzymic evidence. Kisliuk,[27] Greenberg and Jaenicke,[7] Blakley,[26,28] and our laboratory[9,22,23] have proposed that "active formaldehyde" has the bridge structure, N^5,N^{10}-methylene tetrahydrofolate (I), although the N^5-hydroxymethyl structure has also been considered.[25,26]



I

N^5,N^{10}-Methylene tetrahydrofolic acid

(1) Supported by grants from the United States Public Health Service (CY-3310) and the Life Insurance Medical Research Fund. Paper IX in the series "Folic Acid Coenzymes and Active One-Carbon Units." (For paper VIII, see L. D. Kay, M. J. Osborn, Y. Hatefi and F. M. Huennekens, *J. Biol. Chem.*, **235**, 195 (1960)). A preliminary account of this work has appeared elsewhere (F. M. Huennekens and M. J. Osborn, IVth International Congress of Biochemistry, Vienna, September, 1958, Abstracts, Section 4, p. 54).

(2) Department of Microbioloby, New York University.

(3) Postdoctoral Fellow of the National Heart Institute, National Institutes of Health. Department of Chemistry, Howard University, Washington, D.C.

(4) The following abbreviations are used: TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide; GSSG, oxidized glutathione.

(5) A. D. Welch and C. A. Nichol, *Ann. Rev. Biochem.*, **21**, 633 (1952).

(6) W. Sakami in "Amino Acid Metabolism," ed. by W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, Md., 1955, p. 658.

(7) G. R. Greenberg and L. Jaenicke in "The Chemistry and Biology of Purines," ed. by G. E. W. Wolstenholme and C. M. O'Connor, Little, Brown and Co., Boston, 1957, p. 204.

(8) R. L. Blakley in "Current Trends in Heterocyclic Chemistry," ed. by A. Albert, G. M. Badger and C. W. Shoppee, Academic Press, Inc., New York, N. Y., 1958, p. 140.

(9) F. M. Huennekens, M. J. Osborn and H. R. Whiteley, *Science*, **128**, 120 (1958).

(10) B. E. Wright, Symposium on Vitamins in Metabolism, IVth International Congress of Biochemistry, Vienna, 1958, Pergamon Press, New York, N.Y., p. 266.

(11) F. M. Huennekens anh M. J. Osborn, *Adv. Enzymol.*, **21**, 369 (1959).

(12) R. L. Kisliuk and W. Sakami, THIS JOURNAL, **76**, 1456 (1954); *J. Biol. Chem.*, **214**, 47 (1955).

(13) R. L. Blakley, *Biochem. J.*, **58**, 448 (1954); *ibid.*, **61**, 315 (1955); *Nature*, **174**, 652 (1954).

(14) S. Deodhar, W. Sakami and A. L. Stevens, *Federation Proc.*, **14**, 201 (1955).

(15) L. Jaenicke, *ibid.*, **15**, 281 (1956).

(16) F. M. Huennekens, Y. Hatefi and L. D. Kay, *J. Biol. Chem.*, **224**, 435 (1957).

(17) N. Alexander and D. M. Greenberg, *ibid.*, **214**, 821 (1955); *ibid.*, **220**, 775 (1956).

(18) B. E. Wright, *Biochim. Biophys. Acta*, **16**, 165 (1955).

(19) K. G. Scrimgeour, M. J. Osborn and F. M. Huennekens, in preparation.

(20) L. Jaenicke, *Biochim. Biophys. Acta*, **17**, 588 (1955).

(21) Y. Hatefi, M. J. Osborn, L. D. Kay and F. M. Huennekens, *J. Biol. Chem.*, **227**, 637 (1957).

(22) M. J. Osborn and F. M. Huennekens, *Biochim. Biophys. Acta*, **26**, 646 (1957).

(23) M. J. Osborn, E. N. Vercamer, P. T. Talbert and F. M. Huennekens, THIS JOURNAL, **79**, 6565 (1957).

(24) R. L. Kisliuk, *Federation Proc.*, **15**, 289 (1956).

(25) R. L. Blakley, *Biochim. Biophys. Acta*, **23**, 654 (1957).

(26) R. L. Blakley, *Nature*, **182**, 1719 (1958).

(27) R. L. Kisliuk, *J. Biol. Chem.*, **227**, 805 (1957).

(28) R. L. Blakley, *Biochem. J.*, **58**, 448 (1954).

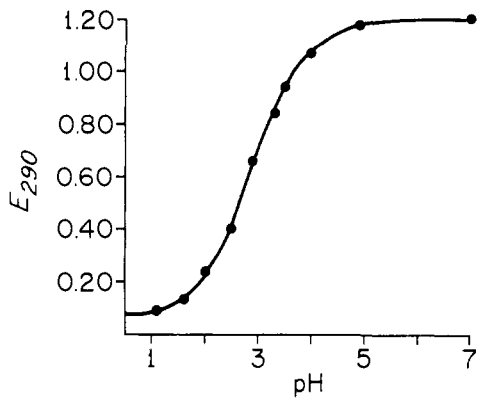


Fig. 1.—Spectrophotometric titration of N-methyl *p*-aminobenzoylglutamate.

The present communication describes an improved method for the chemical synthesis and purification of "active formaldehyde." Studies on the chemical properties of "active formaldehyde" and model compounds related to the N^5- and N^{10}-positions of tetrahydrofolate, provide additional support for the assignment of structure (I) to the material.

Experimental

Materials.—Chemicals were obtained from the commercial sources: TPNH, Sigma Chemical Co.; GSSG, Schwarz Laboratories, Inc.; *p*-aminobenzoylglutamate, Nutritional Biochemicals, Inc.; sodium borohydride, Metal Hydrides, Inc.; 2-mercaptoethanol and acetyl acetone, Eastman Kodak Co.; monomethylol dimethylhydantoin, E. I. duPont de Nemours and Co. DL-tetrahydrofolate was prepared by hydrogenation of folic acid according to the method of O'Dell, *et al.*,[29] as modified by Hatefi, *et al.*[30]; N^5-formyl tetrahydrofolate (folinic acid) was generously provided by Dr. E.L.R. Stokstad of Lederle Laboratories; N^5,N^{10}-methenyl tetrahydrofolate and N^{10}-formyl tetrahydrofolate were prepared from folinic acid by previously published methods.[31] Thiazolidine carboxylic acid and N,N-diphenyl imidazolidine were synthesized by the interaction of formaldehyde with cysteine[32] and with N,N-diphenylethylene diamine,[33] respectively. N-methyl *p*-aminobenzoylglutamate was prepared by treatment of *p*-iodobenzoylglutamate with methylamine at an elevated temperature.[34] Solka-floc was obtained from the Brown Co., and washed prior to use according to the directions of Campbell, *et al.*[35] GSSG reductase was isolated from yeast.[36] Hydroxymethyl tetrahydrofolic dehydrogenase was partially purified from chicken liver acetone powder through step 2 of a previously described procedure,[22] except that the 30–45% ammonium sulfate fraction was used.

Formaldehyde Estimation.—Bound formaldehyde in "active formaldehyde" was estimated by the acetyl acetone method,[37] using the solid compound, monomethylol dimethylhydantoin, as the primary standard for formaldehyde.

***pK* Determination of the Amino Group of N-Methyl *p*-Aminobenzoylglutamate.**—In a silica cuvette 0.1 ml. of $10^{-3}M$ N-methyl *p*-aminobenzoylglutamate was added to 1.4 ml. of various buffer solutions in the *p*H range 1–7 and the optical density determined at 290 mμ against a cuvette containing only buffer. The *p*H of each mixture was measured with a Beckman *p*H meter, Model G. A plot of the optical density (E_{290}) *versus* *p*H is shown in Fig. 1; the *pK* was estimated to be 2.9 from the inflection point of the curve.

Absorption Spectra.—A Beckman Spectrophotometer, Model DU, was used to measure changes in optical density at a single wave length while the Cary recording spectrophotometer, Model 11M, was employed to measure complete spectra over the region 220–400 mμ.

Paper Chromatography of "Active Formaldehyde" and Tetrahydrofolate.—Paper chromatography was used to distinguish "active formaldehyde" from tetrahydrofolate, but considerable degradation of both compounds was encountered. Ascending chromatography was carried out on sheets of Whatman No. 1 paper using the following solvent systems: (1) 0.1 *M* phosphate buffer, *p*H 8, (2) 1 *M* sodium formate–2% formic acid and (3) 70% ethanol–30% H_2O. All solvent systems contained 0.2% mercaptoethanol. R_f values of 0.53, 0.62 and 0.32 were observed for tetrahydrofolate in solvent systems 1, 2 and 3; the corresponding values for "active formaldehyde" were 0.27, 0.20 and 0.25. The R_f value for "active formaldehyde" in system 1 is identical to that reported by Kisliuk.[27]

Hydroxymethyl Tetrahydrofolic Dehydrogenase Assay.—The components added to a Corex cuvette of 1 cm. optical path were: 0.1 μmole of "active formaldehyde," 0.3 μmole of TPN, 10 μmoles of 2-mercaptoethanol, 50 μmoles of bicarbonate buffer, *p*H 9.5, and water to make 1.2 ml. The optical blank contained all components except TPN. Enzyme (0.03 ml.) was added to start the reaction and the changes in optical density at 340 mμ (ΔE_{340}) were determined as a function of time. The extinction coefficient (ϵ) for TPNH is 6.2×10^6 cm.²/mole. The rate was further corrected for a control omitting "active formaldehyde." In the above assay, "active formaldehyde" could be replaced by 0.12 μmole of DL-tetrahydrofolate plus either 2.5 μmoles of L-serine (reaction 1) or 2.5 μmoles of formaldehyde (reaction 3).

It was expedient to perform the assays for "active formaldehyde" at *p*H 9.5 since the equilibrium for reaction 2 lies far to the right under these conditions. Furthermore, at this *p*H there is little interference by mixtures of formaldehyde and tetrahydrofolate (*cf.* Table I). At *p*H 7.5 the reduction of TPN was faster when purified synthetic "active formaldehyde" was used as the substrate than when the material was generated *in situ* from the chemical interaction of tetrahydrofolate and a 10-fold molar excess of formaldehyde. The difference in rate of TPNH formation was amplified when the dehydrogenase reaction was carried out at *p*H 9.5; the mixture of tetrahydrofolate and formaldehyde was almost completely inert while "active formaldehyde" was still fully active.

TABLE I

COMPARISON OF "ACTIVE FORMALDEHYDE" WITH FORMALDEHYDE PLUS TETRAHYDROFOLATE IN THE HYDROXYMETHYL TETRAHYDROFOLIC DEHYDROGENASE SYSTEM[a]

Substrate	*p*H	TPNH formation E_{340} per min.
"Active formaldehyde"	7.5	0.157
Formaldehyde + tetrahydrofolate	7.5	.060
"Active formaldehyde"	9.5	.310
Formaldehyde + tetrahydrofolate	9.5	.015

[a] Assay system for hydroxymethyl tetrahydrofolic dehydrogenase, employing 0.38 μmole of "active formaldehyde" or 0.38 μmole of tetrahydrofolate plus 3.5 μmoles of formaldehyde. E_{340} refers to the change in optical density at 340 mμ.

Chemical Synthesis and Isolation of "Active Formaldehyde".—Twenty mg. of DL-tetrahydrofolate was suspended in 2.0 ml. of 0.025 *M* formaldehyde and brought into solution by the dropwise addition of 5 *N* NaOH to *p*H 5. After standing at room temperature for 10–15 minutes, the mixture was cooled to 2° and the *p*H adjusted to 9.5 by the dropwise addition of 1 *N* NaOH. The solution was adsorbed onto a 1 × 10 cm. column of washed Solka-floc and the

(29) B. L. O'Dell, J. M. Vanderbilt, E. S. Bloom and J. J. Pfiffner, THIS JOURNAL, **69**, 250 (1947).

(30) Y. Hatefi, P. T. Talbert, M. J. Osborn and F. M. Huennekens, *Biochemical Preparations, Vol. VII*, 89 (1960).

(31) J. C. Rabinowitz and W. E. Pricer, Jr., THIS JOURNAL, **78**, 4176 (1956).

(32) S. Ratner and H. T. Clarke, *ibid.*, **59**, 200 (1937).

(33) R. L. Blakley, *Biochem. J.*, **65**, 331, 342 (1957).

(34) D. B. Cosulich and J. M. Smith, Jr., THIS JOURNAL, **70**, 1922 (1948).

(35) P. N. Campbell, T. S. Work and E. Mellanby, *Biochem. J.*, **48**, 106 (1951).

(36) E. Racker in "Methods in Enzymology," Vol. II, ed. by S. P. Colowick and N. O. Kaplan, Academic Press, Inc., New York, N. Y., 1955, p. 723.

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

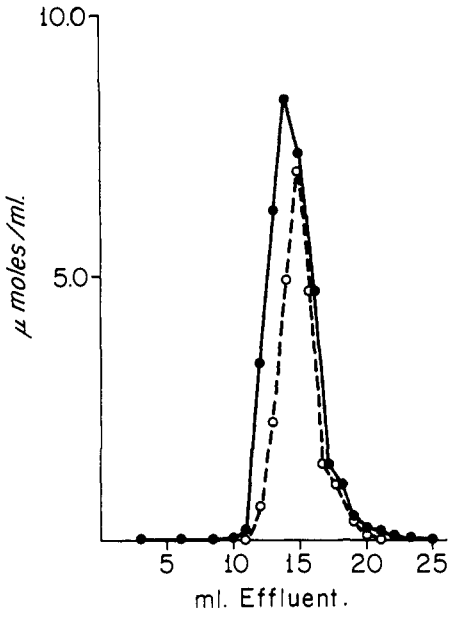


Fig. 2.—Chromatography of "active formaldehyde" on a powdered paper column. "Active formaldehyde" was synthesized chemically from formaldehyde and tetrahydrofolate and chromatographed on a 1 × 10 cm. column of Solkafloc, as described in the Experimental section. The fractions were analyzed for total pteridine (solid line, closed circles) by light absorption at 295 mμ (assuming $\epsilon = 25 \times 10^6$ cm.^{2} mole^{-1}) and for "active formaldehyde" (dashed line, open circles) by enzymic assay with the hydroxymethyl tetrahydrofolic dehydrogenase at *p*H 9.5 (see Experimental section).

column was eluted with a mixture of 40% ethanol–60% bicarbonate buffer ($5 \times 10^{-2} M$, *p*H 9.3) containing $10^{-2} M$ mercaptoethanol. One ml. samples of the effluent were collected with an automatic fraction collector. The elution profile for a typical column is illustrated in Fig. 2. The first fractions of the single asymmetrical peak contained unreacted tetrahydrofolate plus some "active formaldehyde," while the small amount of material in the trailing fractions was identified by absorption spectrum as dihydrofolate (λ_{max} at 283 mμ 7.5). The center fractions represented 30–50% of the total pteridine material and was largely "active formaldehyde" (85–100% pure) as judged by enzymic assay in the hydroxymethyl tetrahydrofolic dehydrogenase system. Typical data for selected fractions from separate columns (Expts. A-C) are presented in Table II. In all succeeding experiments cited in this paper, only "active formaldehyde" of purity greater than 90% was used. The chromatographically purified material was also free from excess formaldehyde: a typical sample which contained 1.12 μmole/ml. of enzymically active "active formaldehyde" (corresponding to 2.24 μmoles/ml. of *total* "active formaldehyde") was found to have 2.26 μmoles/ml. of formaldehyde as estimated by the acetyl acetone method. It is evident, however, that *quantitative* enzymic assay in the hydroxymethyl tetrahydrofolic dehydrogenase system, is preferable to spectroscopic criteria or analysis for bound formaldehyde for determining both the authenticity and amount of "active formaldehyde."

Results and Discussion

Spectroscopic Criteria for the Chemical Synthesis of "Active Formaldehyde."—In agreement with the results of other investigators,[7,13,15,24] it has been found that tetrahydrofolate and formaldehyde react together chemically to yield "active formaldehyde".[38] When a dilute solution ($7 \times 10^{-5} M$) of tetrahydrofolate was treated with a 20- to 50-fold excess of formaldehyde at *p*H 7.5, the spectrum[30] of the former (λ_{max} at 298 mμ; $\epsilon = 22 \times 10^6$ cm.^{2} mole^{-1}) was gradually replaced over a 10–15 minute period by that of a new species having an absorption maximum at 295 mμ ($\epsilon = 25 \times 10^6$ cm.^{2} mole^{-1}).[39] The effect of tetrahydrofolate and formaldehyde concentration upon the rate of adduct formation will be reported in another communication.[19] The above product was similar to that reported by Blakley[13] (λ_{max} at 294 mμ, $\epsilon = 32 \times 10^6$ cm.^{2} mole^{-1}), except the extinction coefficient was

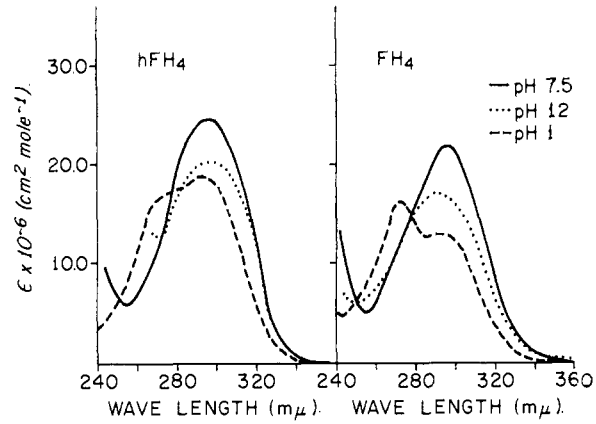


Fig. 3.—Absorption spectra of "active formaldehyde" and tetrahydrofolate. Spectra were determined at *p*H values of 1 (10^{-1} *M* HCl), 7.5 (10^{-1} *M* phosphate buffer), and 12 (10^{-2} *M* NaOH). hFH_4 and FH_4, refer to "active formaldehyde" and tetrahydrofolate.

TABLE II

ENZYMIC ACTIVITY OF CHEMICALLY SYNTHESIZED "ACTIVE FORMALDEHYDE"[a]

Expt.	Fraction no.	½ Pteridine, μmole	"Active formaldehyde," μmole	"Active formaldehyde" content, %
A	7	0.157	0.056	36
	9	.184	.135	74
	10	.113	.110	98
	11	.130	.129	100
	12	.109	.100	92
B	8	.099	.087	90
	9	.078	.064	94
C	8	.121	.063	52
	9	.129	.112	87
	10	.093	.081	87
	11	.057	.049	87
	12	.044	.035	80

[a] The amount of pteridine was calculated from the optical density reading at 295 mμ assuming that $\epsilon = 25 \times 10^6$ cm.^{2} mole^{-1}. Since only one-half of this material would be expected to be enzymically active (owing to the asymmetric center at the C^6-position), the spectrophotometric value is divided by two. "Active formaldehyde" was determined by the amount of TPNH formation in the hydroxymethyl tetrahydrofolic dehydrogenase assay at *p*H 9.5.

(38) It has been demonstrated spectroscopically[9] that formaldehyde can form dissociable adducts with model compounds representative of the various reactive centers (N^5, N^{10}, N^2) in tetrahydrofolate and that addition of varying amounts of formaldehyde to tetrahydrofolate results in adducts whose absorption maximum falls between 290 and 300 mμ. Thus, it is not evident whether the simple admixing of formaldehyde and tetrahydrofolate can be considered to give a quantitative yield of "active formaldehyde."

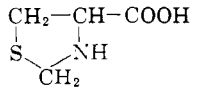
(39) The extinction coefficient is calculated on the basis that all of the tetrahydrofolate has been converted only to "active formaldehyde."

somewhat lower and similar to the product synthesized by the formaldehyde activating enzyme (λ_{max} at 293 mμ, $\epsilon \approx 26 \times 10^6$ cm.^{2} mole^{-1}).

The absorption spectra of "active formaldehyde" and tetrahydrofolate at *p*H values of 1, 7.5 and 12 are compared in Fig. 3. Although the spectrum of "active formaldehyde" is quite similar to that of tetrahydrofolate in neutral or basic solution, the two compounds can be readily distinguished by their spectra in acid solution. A further discussion of the absorption spectra of pteridines and model compounds, and of the various adducts between C_1 units and tetrahydrofolate, will be presented in a subsequent paper.[40]

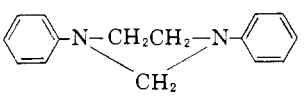
Stability of "Active Formaldehyde."—In contrast to tetrahydrofolate, the reduced pyrazine ring of "active formaldehyde" is quite stable to chemical oxidation. Treatment of "active formaldehyde" with oxidants (*e.g.* MnO_2, H_2O_2 or O_2), which rapidly destroy tetrahydrofolate or N^{10}-formyl tetrahydrofolate, produced no change in the spectrum of "active formaldehyde," nor was its activity reduced in the hydroxymethyl tetrahydrofolic dehydrogenase system. Other investigators[7,13,27] have also called attention to the stability of "active formaldehyde" to oxidation. The well-known resistance to oxidation of N^5-substituted tetrahydropteridine derivatives (*e.g.*, N^5-formyl tetrahydrofolate and N^5,N^{10}-methenyl tetrahydrofolate), in contrast to derivatives in which the N^5-atom is free (*e.g.* tetrahydrofolate and N^{10}-formyl tetrahydrofolate), thereby provides evidence that the formaldehyde group in "active formaldehyde" must be attached at least to the N^5-position, *i.e.*, that the adduct must be either the N^5-hydroxymethyl or the N^5,N^{10}-methylene derivative of tetrahydrofolate.

The bridge compound, N^5,N^{10}-methylene tetrahydrofolate, would be expected, a *priori*, to be relatively stable to dissociation into formaldehyde and tetrahydrofolate by analogy with other cyclic, 5-membered formaldehyde adducts, such as thiazolidine carboxylic acid[32] (II) and N,N-diphenyl imidazolidine[33] (III).



II

Thiazolidinecarboxylic acid



III

N,N-Diphenylimidazolidine

Experiments carried out previously[41] in connection with the hydroxymethyl tetrahydrofolic dehydrogenase have revealed that the enzymic reduction of "active formate," *via* the reversal of reaction 2, yielded largely free tetrahydrofolate rather than "active formaldehyde." Thus, "active formaldehyde" is apparently an unstable adduct at *p*H 7.5. The dissociation of "active formaldehyde" as a function of *p*H was examined, therefore, with the synthetic material. The decrease in optical density at 290 mμ (*cf.* Fig. 3) as "active formaldehyde" is converted to tetrahydrofolate, provided a satisfactory method for following the rate of dissociation. As seen in Table III, the dissociation of "active formaldehyde" is markedly sensitive to *p*H. In the presence of 2-mercaptoethanol, the material rapidly decomposed to tetrahydrofolate at both acidic and neutral *p*H values (Expt. A). The dissociation was essentially complete within 15 minutes at *p*H values of 7.0 and below. At *p*H 9.5, however, "active formaldehyde" appeared to be completely stable, showing no significant change in its absorption spectrum over this time interval, either in the presence or absence of mercaptoethanol. In confirmation of this observation, solutions of "active formaldehyde" which had been stored in the cold at *p*H 9.5 for periods of over one month showed no decrease in activity when assayed with the hydroxymethyl tetrahydrofolic dehydrogenase.

TABLE III

STABILITY OF "ACTIVE FORMALDEHYDE"[a]

Expt.	*p*H	Additions	E_{290} Without mercaptoethanol	E_{290} With mercaptoethanol
A	1.1	None	−0.500	
	4.0	None	− .460	−0.306
	7.0	None	− .340	− .200
	9.5	None	− .010	− .010
B	7.0	H_2NOH	− .430	
	9.5	H_2NOH	− .030	
C	4.0	None		− .210
	4.0	HCHO	− .010	− .115

[a] The experimental cuvette contained 0.15 μmole of "active formaldehyde" in 2.0 ml. of buffer at the indicated *p*H. The buffers used were: *p*H 1.1, 0.1 *M* KCl–HCl; *p*H 4.0, 0.1 *M* acetate; *p*H 7.0, 0.05 *M* phosphate; *p*H 9.5, 0.1 *M* phosphate. Ten μmoles of mercaptoethanol, 2.5 μmoles of formaldehyde, or 30 μmoles of H_2NOH were added as indicated. E_{290} refers to the change in optical density at 290 mμ over a 16-minute period (expts. A and B) or a 10-minute period (expts. C).

The effect of *p*H on the stability of "active formaldehyde" was also evident in the sensitivity of the adduct to cleavage by formaldehyde binding agents, such as hydroxylamine. At *p*H 9.5, "active formaldehyde" was stable to attack by H_2NOH, while at *p*H 7.0 and below, the adduct was almost instantaneously decomposed by this reagent (Table III, Expt. B).

As expected from equation 3, the dissociation of "active formaldehyde," even at acidic and neutral *p*H values, was repressed by the addition of excess ($10^{-3}M$) formaldehyde (Expt. C). The stabilizing effect of formaldehyde at low *p*H values was partially overcome by the addition of mercaptoethanol at a final concentration of $5 \times 10^{-3}M$, *i.e.*, a five-fold excess over the formaldehyde. This effect is probably referable to the action of thiols in lowering the effective concentration of free formaldehyde, through formation of thiohemiacetals and thioacetals.

Although the stability of "active formaldehyde" in *alkaline* solution is entirely consistent with the N^5,N^{10}-bridge structure, the relative ease of dissociation in acid solution is less easily reconciled to this formulation. Mechanisms for these reactions have been presented elsewhere.[42] It should be noted that the bound formaldehyde of the cyclic

(40) P. T. Talbert, J. G. Ozols, M. J. Osborn and F. M. Huennekens, in preparation.

(41) M. J. Osborn, Ph.D. Thesis, University of Washington, 1958.

(42) F. M. Huenekens, H. R. Whitleley and M. J. Osborn, Symposium on Mechanism of Enzyme Action, Gatlinburg, Tenn., April, 1959; *J. Cell. Comp. Physiol.*, **54**, Supplement 1, 109 (1959).

model compounds, II and III, does not estimate as free formaldehyde with the acetyl acetone reagent at *p*H 6, in contrast to "active formaldehyde," which releases its formaldehyde under these conditions. It is possible, however, that the 5-membered ring is more strained in "active formaldehyde" than in the model compounds, thus enhancing dissociation.

Association Constant for "Active Formaldehyde" Formation.—Quantitative information on the stability of "active formaldehyde" was obtained by measuring the equilibrium constant for reaction 3. "Active Formaldehyde" *formation* was studied by measurement of the increase in light absorption at 290 mμ following the addition of formaldehyde to a solution of tetrahydrofolate in 0.1 *M* acetate buffer at *p*H 4.3, the *p*H optimum (see below) for the chemical synthesis of "active formaldehyde." Mercaptoethanol was omitted from the reaction mixture in order to avoid competing reactions between the thiol and formaldehyde. Since adduct formation was very rapid at the *p*H employed, no detectible oxidation of tetrahydrofolate occurred in the absence of the reducing agent. The results are shown in Table IV. Four separate determinations yielded an average value of the equilibrium constant (K) for "active formaldehyde" formation of $1.3 \times 10^4\ M^{-1}$ at *p*H 4.3 and 22°. A value of 2.1×10^4 for the formation of "active formaldehyde" at *p*H 7.2 has been reported previously.[26] The relatively large value (*ca.* 10^4) for the association constant[43] is consistent with the formaldehyde being bound to two nitrogen atoms of tetrahydrofolate rather than one.

TABLE IV

ASSOCIATION CONSTANT FOR "ACTIVE FORMALDEHYDE" SYNTHESIS[a]

	Initial concentration[b]		Final concentration[b]			
Expt.	Formaldehyde	Tetrahydrofolate	"Active formaldehyde"	Formaldehyde	Tetrahydrofolate	$K \times 10^4$
A	83.0	5.9	5.35	77.6	0.55	1.3
B	33.0	5.9	4.67	28.3	1.23	1.3
C	16.7	5.9	3.14	13.6	2.76	0.8
D	8.35	5.9	2.70	5.65	3.10	1.5
					Av.	1.3×10^4

[a] The experimental cuvette contained 0.177 μmole of tetrahydrofolate in 2.9 ml. of 0.05 *M* sodium acetate, *p*H 4.3. After an initial optical density reading at 290 mμ, 0.1 ml. of formaldehyde was added to give a final concentration as indicated in the table and the increase in light absorption at 290 mμ was measured over a 5-minute period. The amount of "active formaldehyde" formed was determined from the total change in optical density ($\Delta\epsilon = 5.85 \times 10^6$ cm.^{2} mole^{-1} at 290 mμ for the conversion of tetrahydrofolate to "active formaldehyde"). [b] All concentrations were $10^{-5}\ M$.

***p*H Dependence of "Active Formaldehyde" Synthesis.**—Direct evidence for the participation of both the N^5- and N^{10}-positions of tetrahydrofolate in the *chemical* synthesis of "active formaldehyde" was obtained from an investigation of the effect of *p*H on the rate of this process. The reaction rate was determined spectrophotometrically, *i.e.*, by the increase in light absorption at 290 mμ following the addition of formaldehyde to a solution of tetrahydrofolate in a buffer ($5 \times 10^{-2} M$) of the desired *p*H. The results are shown in Fig. 4. In order to confirm that the product formed at the different *p*H values was actually "active formaldehyde," a parallel series of reactions was carried out in which the amount of product was assayed by transfer of an aliquot to the hydroxymethyl tetrahydrofolic dehydrogenase system at *p*H 9.5.

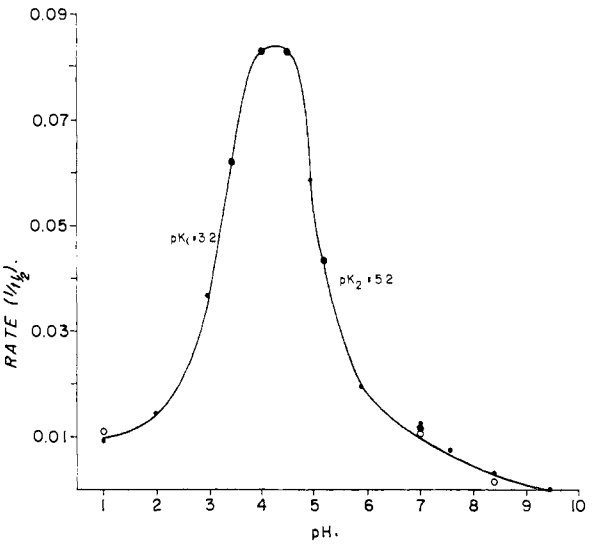


Fig. 4.—*p*H dependence of the chemical synthesis of "active formaldehyde." The experimental cuvette initially contained 0.075 μmole of tetrahydrofolate and 10 μmoles of mercaptoethanol in 1.5 ml. of buffer of the desired *p*H. 2.5 μmoles of formaldehyde was added to start the reaction and the rate of formation of "active formaldehyde" was measured by the increase in light absorption at 290 mμ over a 10-minute period. Optical density readings were corrected for separate blank cuvettes omitting tetrahydrofolate and formaldehyde. Since the difference in extinction coefficient between tetrahydrofolate and "active formaldehyde" (and, hence, the total increase in optical density) varies with *p*H (*cf.* Fig. 3), the rate of "active formaldehyde" formation at any *p*H value is expressed as the reciprocal of the time required for the reaction to reach 50% completion. This rate is linear over this period at all *p*H values. The buffers used were: *p*H 1.1–2.0, 0.1 *M* KCl–HCl; *p*H 3.0–6.0, 0.05 *M* citrate; *p*H 7.0–9.5, 0.05 *M* phosphate. Open circles, closed circles, and triangles represent three separate experiments.

The *p*H dependence of the chemical rate of "active formaldehyde" synthesis followed a smooth, bell-shaped curve with a *p*H optimum in the range of 4.0 to 4.5, and with inflection points at approximately *p*H 3.2 and 5.2. The shape of the curve may be interpreted as indicating the participation of *two* prototropic groups in the interaction of tetrahydrofolate and formaldehyde, whereupon the inflection points of the curve correspond to the *pk* values of the groups involved. Although no ionization constants are available for tetrahydrofolate itself, approximate *pk* values may be assigned to the various groups in tetrahydrofolate on the basis of titration data for related model compounds. Thus, Pohland, *et al.*,[44] have given a *pk* value in the region

(43) "Active formaldehyde" is unique among "active" metabolites (*i.e.*, adducts between a mobile metabolic group and a coenzyme) in that it has a *negative* free energy of formation from its components.

(44) A. Pohland, E. H. Flynn, R. G. Jones and W. Shive, THIS JOURNAL, **73**, 3247 (1951).

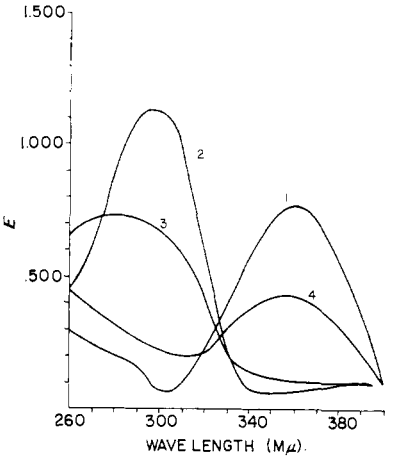


Fig. 5.—Spectrophotometric study of the reduction of N^5,N^{10}-methenyl tetrahydrofolate by borohydride. The experimental cuvette initially contained 0.074 μmole of N^5,N^{10}-methenyl tetrahydrofolate in 2.0 ml. of 0.1 *M* phosphate buffer, *p*H 7.0. The blank cuvette was identical except for omission of N^5,N^{10}-methenyl tetrahydrofolate. An initial spectrum of N^5,N^{10}-methenyl tetrahydrofolate (curve 1) was taken 1 minute after its addition to the buffer. At 3 minutes, 0.02 ml. of 0.1 *M* $NaBH_4$ was added to both cuvettes and the spectrum of the reduction product (curve 2) was taken at 4 minutes. At 6.5 minutes, 0.05 ml. of purified hydroxymethyl tetrahydrofolic dehydrogenase was added; no change in the spectrum of curve 2 was observed. At 10.5 minutes, 0.06 μmole of TPN, 40 μmoles of GSSG and 0.04 ml. of purified GSSG reductase were added, and the resulting spectral changes were followed over a 15-minute period. Curve 3 represents the final spectrum after no further changes had occurred. At this time, 0.1 ml. of 4.0 *N* HCl was added in order to convert N^{10}-formyl tetrahydrofolate to N^5,N^{10}-methenyl tetrahydrofolate. Curve 4 represents the spectrum 10 minutes after acidification.

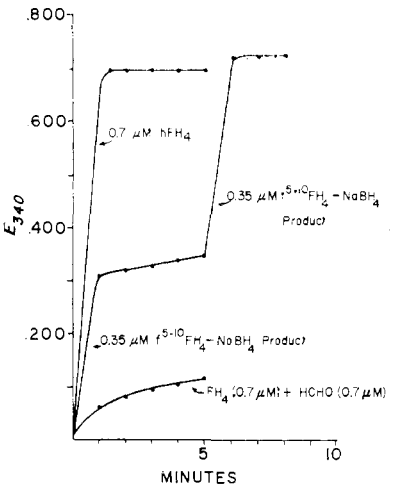


Fig. 6.—Reactivity of the borohydride reduction product in the hydroxymethyl tetrahydrofolic dehydrogenase system. Standard spectrophotometric assay system for hydroxymethyl tetrahydrofolic dehydrogenase at *p*H 9.5. As indicated by the different curves, the substrate was: (a) 0.7 μmole of authentic "active formaldehyde," (b) two separate additions (0.35 μmole each) of N^5,N^{10}-methenyl tetrahydrofolate reduced by borohydride and (c) 0.7 μmole of tetrahydrofolate mixed with 0.7 μmole of formaldehyde. hFH_4, FH_4, $f^{5-10}FH_4$, refer to "active formaldehyde," tetrahydrofolate and N^5,N^{10}-methenyl tetrahydrofolate.

of 5.1–5.6 to the reduced N^5-atom from a study of the model compounds, 2-amino-4-hydroxy-6-methyl tetrahydropteridine and 2,5,6-trimamino-4-hydroxy pyrimidine and their formyl derivatives. Similarly, N-methyl *p*-aminobenzoylglutamate, may be taken as a model of the N^{10}-atom of tetrahydrofolate. The *pk* value of the amino nitrogen of *p*-aminobenzoylglutamate is reported[45] to be 2.61, while a spectrophotometric titration of the N-methyl derivative (see Experimental section) yielded a *pk* value of 2.9. From these data, the N^5- and N^{10}-atoms of tetrahydrofolate may be assumed to have approximate *pk* values of 5.3 and 2.9, respectively, and these values correspond to the two ionizing groups observed in "active formaldehyde" synthesis. Mechanisms[42] for the chemical synthesis of "active formaldehyde" must take into account, therefore, the fact that, at the *p*H optimum, the N^5-atom is charged and the N^{10}-atom uncharged.

Synthesis of "Active Formaldehyde" by Chemical Reduction of N^5,N^{10}-Methenyl Tetrahydrofolate.—It has been shown previously[22] that N^5,N^{10}-methenyl tetrahydrofolate is the initial product of the enzymic oxidation of "active formaldehyde" in reaction 2. In order to test the hypothesis that the ring structure of N^5,N^{10}-methenyl tetrahydrofolate is retained upon reduction to "active formaldehyde," a study was made of the possible interconversion of "active formaldehyde" and "active formate" by chemical means.

All attempts to effect the chemical oxidation of "active formaldehyde" to a formyl derivative of tetrahydrofolate were unsuccessful. With mild oxidants (MnO_2, Ag_2O) no reaction was observed, while under more drastic conditions (MnO_4^-) the reduced pyrazine ring was oxidized to a variety of products as judged by changes in absorption spectrum. On the other hand, chemical reduction of "active formate" to the formaldehyde level proved to be more feasible. Greenberg and Jaenicke[7] mentioned the use of borohydride for the reduction of N^{10}-formyl folate to the hydroxymethyl derivative of folate. As shown in Fig. 5, treatment of N^5,N^{10}-methenyl tetrahydrofolate with small amounts of $NaBH_4$ in alkaline solution caused the characteristic absorption spectrum of the former compound (curve 1) to be replaced by a new spectrum with an absorption maximum at 295 mμ (curve 2). The reaction was extremely rapid and was specific for N^5,N^{10}-methenyl tetrahydrofolate; no change in spectrum was observed when either N^5-formyl tetrahydrofolate or N^{10}-formyl tetrahydrofolate was treated with $NaBH_4$. The reduction product of N^5,N^{10}-methenyl tetrahydrofolate was identical with authentic "active formaldehyde," with respect to its absorption spectra in neutral and acidic solutions and its activity in the hydroxymethyl tetra-

(45) In folic acid the 2-amino group has a *pK* value of 3.2, while the glutamate carboxyl groups have values of 3.8 (S. F. Mason in "Chemistry and Biology of Pteridines," ed. by G. E. W. Wolstenholme and M. P. Cameron, Little, Brown and Co., Boston, Mass., 1954, p. 81). It is unlikely, however, that these ionizing groups play any role in the chemical formation of "active formaldehyde."

hydrofolic dehydrogenase system. This latter point is also demonstrated in Fig. 5. When the borohydride reduction product (curve 2) was treated with TPN and hydroxymethyl tetrahydrofolic dehydrogenase (containing cyclohydrolase[22]), the absorption spectrum changed to that of curve 3. The decrease in light absorption at 300 mμ and the increase in the region of 260 mμ are indicative of N^{10}-formyl tetrahydrofolate[46] formation, although the spectrum is obscured by the contribution due to the unreacted "active formaldehyde." Only 50% of the "active formaldehyde" would be reactive, due to the asymmetric carbon atom at the C^6-position. The appearance of the absorption band at 355 mμ, characteristic of N^5,N^{10}-methenyl tetrahydrofolate, after acidification of the cell contents (curve 4), confirmed that the product of the *enzymic* oxidation was N^{10}-formyl tetrahydrofolate. The above sequence of reactions is summarized in equation 4: steps (a) and (d) are non-enzymic, while (b) and (c) are enzymic.

The product of the borohydride reduction of N^5,N^{10}-methenyl tetrahydrofolate was more conclusively identified as "active formaldehyde" by a detailed examination of its reactivity in the hydroxymethyl tetrahydrofolic dehydrogenase system at *p*H 9.5. As shown in Fig. 6, synthetic "active formaldehyde" was an effective substrate for TPN reduction, and the amount of TPNH produced (0.35 μmole) was equal to the theoretical maximum, *i.e.*, 50% of the "active formaldehyde"

(46) At the *p*H employed, the cyclohydrolase-catalyzed equilibrium of N^5,N^{10}-methenyl tetrahydrofolate and N^{10}-formyl tetrahydrofolate lies far in the direction of the latter compound (see ref. 22).

$$\text{N}^5,\text{N}^{10}\text{-methenyl tetrahydrofolate} \xrightarrow[(a)]{\text{NaBH}_4} \text{"active formaldehyde"} \xrightarrow[(b)]{\text{TPN}} \text{N}^5,\text{N}^{10}\text{-methenyl tetrahydrofolate} \xrightarrow[(c)]{\text{H}_2\text{O}} \text{N}^{10}\text{-formyl tetrahydrofolate} \xrightarrow[(d)]{\text{H}^+} \text{N}^5,\text{N}^{10}\text{-methenyl tetrahydofolate} \quad (4)$$

added (0.70 μmole). Under the same conditions, the borohydride reduction product was also quantitatively oxidized by TPN: 0.17 μmole of TPNH were formed after the addition of 0.35 μmole of borohydride-treated N^5,N^{10}-methenyl tetrahydrofolate to the reaction mixture, and the addition of a second, equal increment of the borohydride product led to the formation of a second equal increment of TPNH. A mixture of tetrahydrofolate and formaldehyde was, however, almost completely inactive, since the rate of adduct formation is low at this *p*H. It could be concluded, therefore, that "active formaldehyde" was formed *quantitatively* by the chemical reduction of N^5,N^{10}-methenyl tetrahydrofolate. Since $NaBH_4$ reduction was specific for N^5,N^{10}-methenyl tetrahydrofolate, additional evidence is provided for the contention that the N^5,N^{10}-bridge structure is retained during reduction to the formaldehyde level. A hydride-ion mechanism has been presented recently[42] for the enzymic or chemical interconversion of "active formaldehyde" and "active formate."

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, ILLINOIS INSTITUTE OF TECHNOLOGY]

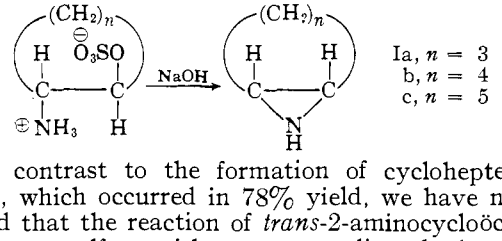
Chemistry of Ethylenimine. VII. Cycloöctenimine or 9-Azabicyclo[6.1.0]nonane[1]

BY D. V. KASHELIKAR AND PAUL E. FANTA[2]

RECEIVED JANUARY 29, 1960

Reaction of *trans*-2-aminocycloöctyl hydrogen sulfate with aqueous sodium hydroxide gives a mixture of cycloöctanone and the expected *cis*-cycloöctenimine. Formation of the two products is rationalized in terms of two alternative, energetically nearly equivalent transition states analogous to those proposed for the Hofmann elimination of cycloöctytrimethylammonium hydroxide. Pyrolysis of N-(*p*-nitrobenzoyl)-cycloöctenimine (IIc) in toluene yields *cis*-N-(*p*-nitrobenzoyl)-3-cycloöctenylamine (V) and an isomeric oxazoline (VII). Formation of V from IIc is sterically and mechanistically analogous to the formation of *cis*-cycloöctene by the pyrolysis of cycloöctyldimethylamine oxide.

Previous papers in this series have described the preparation of cyclopentenimine (Ia),[3] cyclohexenimine (Ib)[4] and cycloheptenimine (Ic)[5] by the treatment of the corresponding *trans*-2-aminocycloalkyl hydrogen sulfates with aqueous sodium hydroxide. Since these small carbocycles cannot accommodate a three-membered ring fused *trans*, the imines were assigned the *cis* configuration and the closure of the aziridine ring therefore must have occurred in each instance with inversion at the substituted carbon atom.



In contrast to the formation of cycloheptenimine, which occurred in 78% yield, we have now found that the reaction of *trans*-2-aminocycloöctyl hydrogen sulfate with aqueous sodium hydroxide gives a crude, volatile reaction product from which cycloöctenimine was isolated in only 33% yield, accompanied by 14% of cycloöctanone.

An analogous difference in the mode of an elimination reaction on going from the seven- to the eight-membered ring was reported by Cope,[6] who

(1) This research was supported by National Science Foundation Grant NSF-G6220 which provided a postdoctoral stipend for D. V. Kashelikar.

(2) To whom inquiries regarding this paper should be sent.

(3) P. E. Fanta, *J. Chem. Soc.*, 1441 (1957).

(4) O. E. Paris and P. E. Fanta, THIS JOURNAL, **74**, 3007 (1951).

(5) P. B. Talukdar and P. E. Fanta, *J. Org. Chem.*, **24**, 555 (1959).

(6) A. C. Cope, R. A. Pike and C. F. Spencer, THIS JOURNAL, **75**, 3212 (1953).