

# Effect of Oxygen on Thermal Degradation of 5-Methyl-5,6,7,8-Tetrahydrofolic Acid

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## ABSTRACT

Previous studies of thermal degradation of folic acid have not given consistent rate expressions or activation energies. The present study employed a model system which eliminated oxygen mass transfer limitations to examine degradation as a result of heating. A pseudo first-order reaction rate constant ( $k_{365^\circ\text{K}}$ ) and an activation energy ( $E_a$ ) for thermal degradation due to an oxidative mechanism were estimated to be  $0.154 \pm 0.044 \text{ min}^{-1}$  and  $16.3 \text{ kcal/mol}$ , respectively. In the presence of  $\text{N}_2$ , these parameters were estimated to be  $0.094 \pm 0.009 \text{ min}^{-1}$  and  $23.3 \text{ kcal/mol}$ , respectively. While elimination of oxygen will not ensure folic acid retention, the overall degradation rate will be reduced significantly.

## INTRODUCTION

FOLIC ACID and its derivatives have been shown to be among the most labile vitamins by numerous investigators (Herbert, 1968; Ford, 1967; Malin, 1977). Herbert (1968) estimated that as much as 95% of the initial folic acid in food could be lost through oxidation during heating. Ford (1967) concluded that variation in stability of folic acid in milk was directly related to presence of oxygen. Although the primary inactivation process is oxidative, the mechanisms of these reactions and their susceptibility of heat are not yet clear.

Previous studies of thermal degradation of folic acid and its predominant naturally occurring form, 5- $\text{CH}_3$ -5,6,7,8-tetrahydrofolic acid (5- $\text{CH}_3$ -THF) have not produced consistent rate expressions or activation energies (Dick et al., 1948; Garret, 1966; Shah et al, 1976; Chen and Cooper, 1979; Ruddick et al., 1980). Reaction rate expressions have ranged from zero to first order in folic acid. Rates measured at  $100^\circ\text{C}$  vary by a factor of over 100 and reported activation energies range from 7.1 to 58 kcal/mol. Most of these studies were conducted in vessels containing folic acid solution and a head space of air. It has been suggested that discrepancies in earlier work resulted from limitations in mass transfer of oxygen into the solution and decreased oxygen solubility at higher temperatures.

The present study employed a model solution of 5- $\text{CH}_3$ -THF in phosphate buffer to study degradation as a result of heating in both the presence and absence of oxygen. Loss of oxygen during heating was also monitored in a phosphate buffer solution containing excess 5- $\text{CH}_3$ -THF. An experimental technique which eliminated the gas-liquid interface was utilized to avoid the potential for mass transfer and allow for unambiguous determinations of reaction rates and activation energies.

## MATERIALS & METHODS

### Sample preparation

Twenty milligram samples of crystalline d,1-N-5-methyl-tetrahydrofolic acid (barium salt, Sigma Chemical Co., St. Louis, MO, 90%

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purity) were weighed out under nitrogen in a glove bag using a Cahn Electrobalance (E. H. Sargent and Co., Paramount, CA). Stock solutions were prepared by dissolving crystalline 5- $\text{CH}_3$ -THF in 100 mL  $\text{N}_2$  or  $\text{O}_2$ -saturated 0.1M phosphate buffer (pH 7.0) at  $0^\circ\text{C}$ , to give a final concentration of  $3.36 \times 10^{-5}$  5- $\text{CH}_3$ -THF (20  $\mu\text{g/mL}$ ). All samples were prepared with doubly distilled, deionized water and stored in ice-water and diffuse light throughout the experiment to minimize degradation.

Stock solution for the study of oxygen loss during heating in a phosphate buffer solution with excess 5- $\text{CH}_3$ -THF was prepared by weighing 6.0 mg of 5- $\text{CH}_3$ -THF as described previously and diluting to 20 mL with phosphate buffer to yield a final concentration of  $5.04 \times 10^{-4}\text{M}$  (300  $\mu\text{g/mL}$ ). Oxygen concentration of the phosphate buffer was decreased to  $2.1 \times 10^{-5}\text{M}$  to establish a molar ratio of 24:1, [5- $\text{CH}_3$ -THF] to  $[\text{O}_2]$ .

### Elimination of gas-liquid interface

A technique to study thermal degradation of 5- $\text{CH}_3$ -THF was developed which eliminated the gas-liquid interface, thus avoiding potential mass transfer limitations. Septum-capped 2.5 mL glass vials were overfilled with 5- $\text{CH}_3$ -THF solution. Twenty-two gauge hypodermic needles (Becton, Dickinson & Co., Rutherford, NJ) were inserted through each septum to allow for overfilling and loss of gases during heating. All manipulations were conducted in a nitrogen-filled and flushed glove bag.

### Exposure to heat

The reaction vials were placed in a wire basket inside a constantly stirred Magni-Whirl water bath and heated to either  $40^\circ$ ,  $60^\circ$ ,  $80^\circ$  or  $92^\circ\text{C}$  for various times. A pilot tube with a thermocouple was used to determine heating and cooling lags; oxygen concentration was monitored throughout the reaction. The reaction was quenched by cooling the vials in ice-water. Duplicate vials were removed at regular intervals. A galvanic cell electrode, developed and described by Johnson et al. (1964), was successfully used to monitor oxygen concentration.

### 5- $\text{CH}_3$ -THF determination

5- $\text{CH}_3$ -THF concentration was determined using high performance liquid chromatography (Clifford and Clifford, 1977). Ten microliter samples were withdrawn through reaction vial septums and injected directly into the HPLC system (Waters Associates Model ALC 202 liquid chromatograph pump, Waters Associates, Milford, MA; Waters Model U6K injector; Gilson Model III ultraviolet detector, Gilson Medical Electronics, Middletown, WI; Waters  $\mu$ -Bondapak  $\text{C}_{18}$  column). An isocratic solvent system of 40% methanol-water at ambient temperature was used. The solvent was buffered with 0.005 M tetrabutyl ammonium phosphate.

### Calculations

Reaction rate constants were determined using a first-order model after preliminary data analysis showed that a first-order model adequately described the data ( $r^2 = 0.99$ ). Activation energies were determined using Arrhenius equation:

$$\log \frac{k_1}{k_2} = \frac{-E_a}{2.303R} \left( \frac{1}{T_1} - \frac{1}{T_2} \right)$$

where  $k$  is reaction rate constant,  $T$  is absolute temperature (Kelvin),  $R$  is the gas constant, and  $E_a$  is activation energy.

## RESULTS & DISCUSSION

### Kinetic Studies in an oxygen-saturated system

Degradation of 5-CH<sub>3</sub>-THF in an oxygen-saturated phosphate buffer (pH 7.0) at various temperatures is given in Fig. 1. At least 75% destruction occurred during each heat treatment, although the last points for the 40°C treatment are not included so that data at higher temperatures might be shown.

Linear regression of log 5-CH<sub>3</sub>-THF concentration versus time indicated that a pseudo first order kinetic model adequately described degradation of 5-CH<sub>3</sub>-THF at temperatures between 40–92°C in excess O<sub>2</sub> ( $r^2 = 0.99$ ). The reaction is referred to as 'pseudo first order' because oxygen is present in an abundant supply throughout the reaction. Even at 92°C, where O<sub>2</sub> is least soluble, the molar ratio of [O<sub>2</sub>]:[5-CH<sub>3</sub>-THF] is 9.5:1.0.

Oxygen concentration was monitored throughout the course of heating and decreased to saturation concentration at each temperature, where it remained during the course of the experiment. Loss of oxygen during heating in phosphate buffer solution with excess 5-CH<sub>3</sub>-THF (24:1 ratio of [5-CH<sub>3</sub>-THF] to [O<sub>2</sub>]) was also examined. Linearity of the semilog plot of percent O<sub>2</sub> remaining vs heating time over five half-lives (Fig. 2) indicates that loss of oxygen in phosphate buffer with 5-CH<sub>3</sub>-THF at 92°C is adequately described by a first order kinetic model ( $r^2 = 0.99$ ), with a reaction rate constant of  $0.288 \pm 0.007 \text{ min}^{-1}$ . The calculated pseudo first order rate constants for the degradation of 5-CH<sub>3</sub>-THF have been reported by both Chen and Cooper (1979) and Ruddick et al. (1980). Chen and Cooper (1979), who did not report the oxygen concentration in their solutions, found rates of 0.0043 to 0.0323  $\text{min}^{-1}$  over a range of 49°C to 100°C. Ruddick et al. (1980) utilized an oxygen-saturated system and reported values of 0.018 to 0.108  $\text{min}^{-1}$  over a temperature range 40–100°C.

Various investigators have studied oxidation of 5-CH<sub>3</sub>-THF. However, disagreement exists concerning the oxidation products. Donaldson and Keresztesy (1962) and others (Zakrewski,

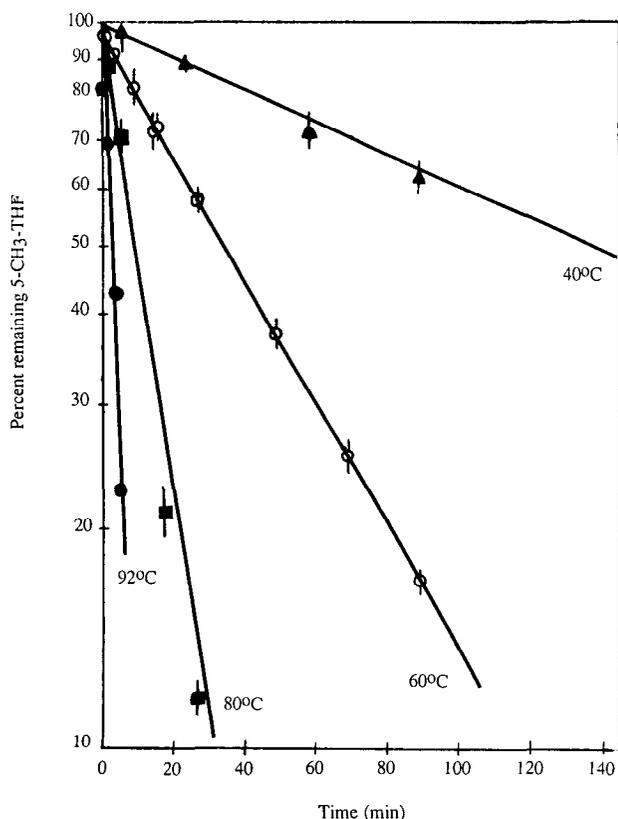


Fig. 1—Degradation of 5-CH<sub>3</sub>-THF in the presence of oxygen.

1966; Blakely, 1969; Blair et al., 1975) reported that 5-CH<sub>3</sub>-THF is readily oxidized to a mixture of 5-CH<sub>3</sub>-5,6-DHF and 4a-hydroxy-5-CH<sub>3</sub>-4a,5,6,7,-THF, while Jongejan et al. (1979) reported a pyrazino-s-triazine structure as the final oxidation product. Blair et al. (1975) reported that 5-CH<sub>3</sub>-5,6-DHF and 4a-OH-5-CH<sub>3</sub>-4a,5,6,7,-THF were relatively stable to further oxidation. However, others (Blakely, 1969; Stokstad and The-nen, 1972) have noted that DHF is readily oxidized by air to p-aminobenzoylglutamate and a pterin. Reed and Archer (1980) utilized high performance liquid chromatography to show that oxidation of tetrahydrofolic acid led predominantly to cleavage of the molecule and formation of p-aminobenzoylglutamate and 6-formylpterin at pH 7.

### Kinetic studies in an oxygen-free system

Degradation of 5-CH<sub>3</sub>-THF in a nitrogen-saturated phosphate buffer (pH 7.0) at various temperatures is illustrated in Fig. 3. At least 80% degradation of the 5-CH<sub>3</sub>-THF was achieved during each treatment. Linear regression of log 5-CH<sub>3</sub>-THF concentration versus time indicated that a first order kinetic model described the degradation of 5-CH<sub>3</sub>-THF at temperatures between 40–92°C in the absence of oxygen ( $r^2 = 0.99$ ). Calculated first order rate constants for the reaction are given in Table 1.

Little is known about the degradation of 5-CH<sub>3</sub>-THF in the absence of oxygen. Only one group of investigators (Ruddick et al., 1980) examined the thermal degradation reaction in limited oxygen systems. In their study, it was concluded that degradation of 5-CH<sub>3</sub>-THF was highly dependent on the concentration of dissolved oxygen. Heiss et al. (1980) found that oxygen diffusion rates and solubility were of great importance in determining the shelf life of packaged foods.

An additional experiment was conducted to determine if the degradation of 5-CH<sub>3</sub>-THF in the absence of oxygen might be a simple oxidation-reduction catalyzed by metal ions. Ethylenediaminetetraacetic acid was added to N<sub>2</sub>-flushed, phosphate-buffered 5-CH<sub>3</sub>-THF solution and heated as previously described. There was no difference in reaction rate, which would indicate the absence of metal ions as catalysts.

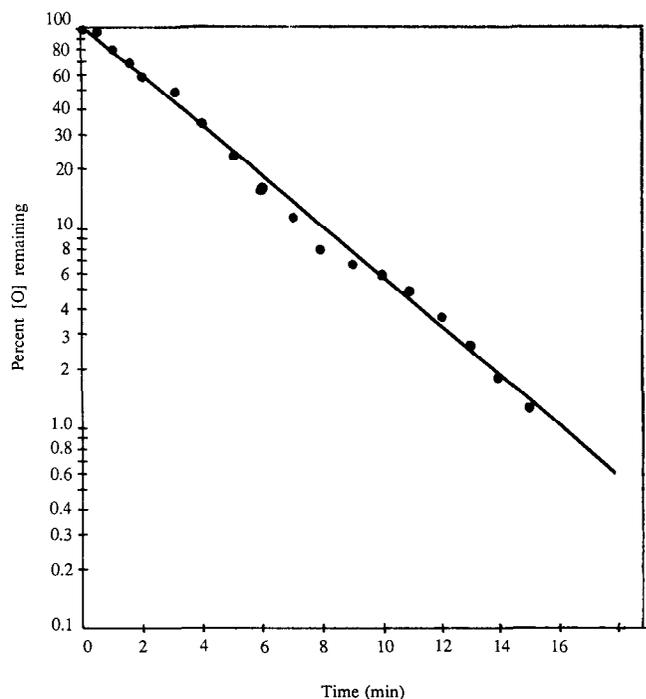
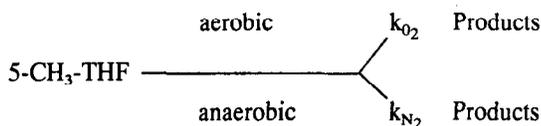


Fig. 2—Loss of oxygen during heating in phosphate buffer with excess 5-CH<sub>3</sub>-THF.

## THERMAL DEGRADATION OF 5-CH<sub>3</sub>-THF...

Because the degradation reaction was found to be pseudo first order overall in 5-CH<sub>3</sub>-THF (Fig. 1 and 3) and pseudo first order in oxygen (Fig. 2), the reaction rate constant for the oxidative reaction alone ( $k_{O_2}$ ) can be obtained by subtracting the rate constant for the reaction in nitrogen ( $k_{N_2}$ ) from the rate constant obtained for the reaction in the oxygen saturated system ( $k_{O_2 + N_2}$ ). These values are given in Table 1. Total loss of 5-CH<sub>3</sub>-THF can be represented as:



$$\frac{d[5\text{-CH}_3\text{THF}]}{dt} = (k_{O_2} [O_2] [5\text{-CH}_3\text{-THF}] + k_{N_2} [5\text{-CH}_3\text{-THF}])$$

In excess oxygen  $k'_{O_2} = k_{O_2}[O_2]$ , and

$$\begin{aligned}
 \frac{d[5\text{-CH}_3\text{-THF}]}{dt} &= -(k'_{O_2} [5\text{-CH}_3\text{-THF}] + k_{N_2} [5\text{-CH}_3\text{-THF}]) \\
 &= -(k'_{O_2} + k_{N_2}) [5\text{-CH}_3\text{-THF}]
 \end{aligned}$$

### Degradation of 5-CH<sub>3</sub>-THF

For a second order reaction, the specific rate constant of the reaction can be determined from the pseudo-first order rate constants obtained under conditions of excess of one reactant. For oxidation of 5-CH<sub>3</sub>-THF at 92°C, the specific rate constant under excess oxygen is:

$$k = \frac{k}{[O_2]} = \frac{0.155 \text{ min}^{-1}}{3.20 \times 10^{-4} \text{ M}} = 480 \pm 130 \text{ M}^{-1} \text{ min}^{-1}$$

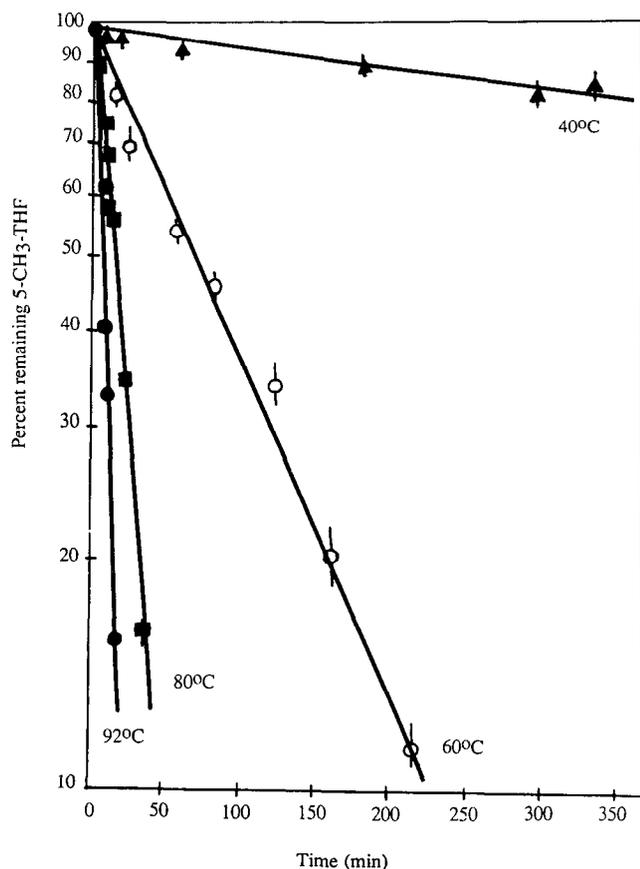


Fig. 3—Degradation of 5-CH<sub>3</sub>-THF in the presence of nitrogen.

The specific rate constant under excess 5-CH<sub>3</sub>-THF is:

$$\begin{aligned}
 k &= \frac{k}{[5\text{-CH}_3\text{-THF}]} = \frac{0.288 \text{ min}^{-1}}{5.04 \times 10^{-4} \text{ M}} \\
 &= 570 \pm 130 \text{ M}^{-1} \text{ min}^{-1}
 \end{aligned}$$

where  $3.20 \times 10^{-4} \text{ M}$  is the maximum solubility of  $[O_2]$  in phosphate buffer at 92°C, and  $5.04 \times 10^{-4} \text{ M}$  is the concentration of 5-CH<sub>3</sub>-THF used to study the pseudo first order oxygen loss reaction. Since the two calculated specific rate constants are similar, the assumption of a second order kinetic model for oxidative degradation of 5-CH<sub>3</sub>-THF is justified.

The Arrhenius equation was used to describe the temperature dependence of the rate constants. Figure 4 is an Arrhenius plot for both the reaction in the presence and absence of  $O_2$ . Estimated  $E_a$  values and reference reaction rate constants at 92°C are given in Table 2.

The activation energy values obtained are in the range of those generally found for thermal degradation of other vitamins (10–30 kcal/mol, Lund, 1977). The temperature dependence of the oxidative reaction alone is slightly less than that of the additive combination of oxidative and nonoxidative reactions.

Two other studies reported values for  $E_a$  for thermal degradation of 5-CH<sub>3</sub>-THF. Chen and Cooper (1979) found an  $E_a$  of 9.5 kcal/mol, and Ruddick et al. (1980) reported an  $E_a$  of 7.1 kcal/mol for thermal degradation of 5-CH<sub>3</sub>-THF in a phosphate buffer at temperatures from 40–100°C. Both of these values appear suspiciously low, more typical of a physical process, such as diffusion. In the study of Ruddick et al. (1980), oxygen was bubbled through the solution containing folic acid. Under these conditions, there could be mass transfer limitations of oxygen into the solution, resulting in a temperature dependence which is consistent with diffusion limitations. In our study, direct measurement of oxygen concentration indicated that oxygen was in large excess compared to concentration of folic acid. Ruddick et al. (1980) could not verify a

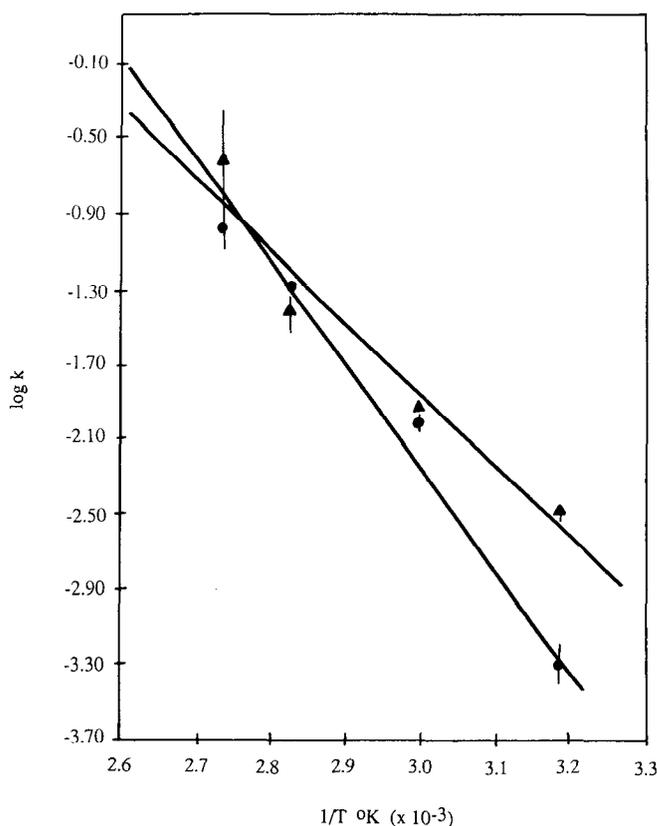


Fig. 4—Arrhenius plot for degradation of 5-CH<sub>3</sub>-THF (▲ Oxygen; ● Nitrogen).

Table 1—Reaction rate constants for 5-CH<sub>3</sub>-THF degradation in the presence of excess O<sub>2</sub> and N<sub>2</sub>

Temperature (°C)	K <sup>a</sup> (O <sub>2</sub> + N <sub>2</sub> ) <sup>b</sup> (min <sup>-1</sup> )	K <sup>c</sup> N <sub>2</sub> (min <sup>-1</sup> )	K <sup>d</sup> O <sub>2</sub> (min <sup>-1</sup> )
40	0.004 ± 0.0002	0.0005 ± 0.00001	0.004 ± 0.00005
60	0.020 ± 0.0005	0.009 ± 0.0004	0.011 ± 0.0001
80	0.081 ± 0.010	0.046 ± 0.003	0.035 ± 0.009
92	0.249 ± 0.050	0.094 ± 0.009	0.155 ± 0.044

<sup>a</sup> All k values are given ± the 95% confidence interval.

<sup>b</sup> First order rate constant for the combined oxidative and nonoxidative degradation of 5-CH<sub>3</sub>-THF.

<sup>c</sup> First order rate constant for the nonoxidative degradation of 5-CH<sub>3</sub>-THF.

<sup>d</sup> First order rate constant for oxidative degradation of 5-CH<sub>3</sub>-THF in excess oxygen.

Table 2—Estimated values of E<sub>a</sub> and reference reaction rate constants at 92°C for the thermal degradation of 5-CH<sub>3</sub>-THF in 0.1M phosphate buffer in the presence and absence of O<sub>2</sub> over the temperature range of 40–92°C

Environment	(k <sub>365°K</sub> ) (min <sup>-1</sup> )	E <sub>a</sub> ± 95% C.I. (kcal/mol)
O <sub>2</sub>	0.155	16.3 ± 0.85
N <sub>2</sub>	0.094	23.2 ± 0.93

large excess of oxygen because of interference of phosphate with the measurement of oxygen. Consequently, oxygen diffusion could have been the rate-limiting step in the degradation of folic acid. This reported value for activation energy (7.1 kcal/mol) is consistent with activation energies reported for physical processes such as diffusion (generally from 1-10 kcal/mol). In the present study, the gas-liquid interface was eliminated to avoid any possible limitation due to oxygen mass transfer. The resulting E<sub>a</sub> values, typical of thermal degradation of vitamins, would support the assumption of an interaction of the two processes in past studies. This illustrates the care that should be taken in such experiments.

## SUMMARY & CONCLUSIONS

THERMAL DEGRADATION rates of 5-CH<sub>3</sub>-THF in a 0.1M phosphate buffer (pH 7.0) heated in the presence and absence of oxygen over a temperature range of 40–92°C were measured. A pseudo first order reaction rate constant (k<sub>365°K</sub>) and an Arrhenius activation energy (E<sub>a</sub>) for thermal degradation due to an oxidative mechanism, in the presence of 3.20 × 10<sup>-4</sup> M [O<sub>2</sub>], were estimated to be 0.154 ± 0.044 min<sup>-1</sup> and 16.3 kcal/mol, respectively. A first order reaction rate constant (K<sub>365°K</sub>) and an activation energy for the reaction in the presence of N<sub>2</sub> were estimated to be 0.094 ± 0.009 min<sup>-1</sup> and 23.2 kcal/mol, respectively.

Loss of oxygen in a 0.1M phosphate buffer solution (pH 7.0) with excess 5-CH<sub>3</sub>-THF, heated to 92°C, was also monitored. A pseudo first order reaction rate constant was estimated to be 0.288 ± 0.007 min<sup>-1</sup> in the presence of 5.04 × 10<sup>-4</sup> M [5-CH<sub>3</sub>-THF]. The specific rate constant for the reaction was calculated to be in the range of 480–570 M<sup>-1</sup> min<sup>-1</sup>

at 92°C, and the activation energy 16.3 kcal/mol. Parameters describing thermal degradation of 5-CH<sub>3</sub>-THF in 0.1M phosphate buffer indicated that the loss of this vitamin by both oxidative and nonoxidative mechanisms might be significant during thermal processing. Elimination of oxygen will not ensure retention of folic acid but the overall rate of degradation will be reduced significantly.

The parameters calculated in this study were in the range expected for chemical reactions. Suspiciously low E<sub>a</sub> values reported for degradation of folic acid or its derivatives by other investigators may be due to oxygen diffusion limitations in their systems. Elimination of the gas-liquid interface in the present study effectively removed possible limitations due to mass transfer of oxygen and allowed for an unambiguous determination of the chemical kinetic model for degradation of 5-CH<sub>3</sub>-THF in the presence of oxygen.

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