Oxidation of Tetrahydrofolic Acid by Air

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Oxidation of tetrahydrofolic acid by oxygen in air in aqueous solution has been examined by using high-performance liquid chromatography to characterize and quantitate the reaction products. At pH 4, 7, and 10, the major product was (*p*-aminobenzoyl)glutamate, indicating that oxidation leads predominantly to cleavage of the tetrahydrofolate. The major products containing the pterin ring were pterin at pH 4 and 6-formylpterin at pH 7 and 10. 7,8-Dihydrofolate was only detected in the reaction at pH 10. Mechanisms are proposed to account for these reactions.

The naturally occurring reduced forms of folic acid are unstable, and considerable losses of vitamin activity occur during the course of food processing and storage (Archer and Tannenbaum, 1979). Although various studies have shown that the primary inactivation process is oxidative (Burton et al., 1967, 1970; Ford et al., 1969), the extent and mechanism of loss of the various folic acid derivatives in foods are not yet clear.

Numerous studies have been performed to identify and quantitate the products of oxidation of tetrahydrofolate in aqueous solution (Blakley, 1957; Zakrzewski, 1966; Futterman and Silverman, 1957; O'Dell et al., 1947; Chippel and Scrimgeour, 1970; Blair and Pearson, 1974). Most have met with limited success because of the multiplicity of products and poor analytical methods. Chippel and Scrimgeour (1970) investigated the anaerobic oxidation of tetrahydrofolate by ferricyanide. They found that tetrahydrofolate was oxidized, depending on the reaction conditions, by ferricyanide to (*p*-aminobenzoyl)glutamate, formaldehyde, dihydrofolate, folate, and a number of pterins (2-amino-4-hydroxypteridines), including dihydroxanthopterin, 6-formyldihydropterin, and pterin. Products were separated and quantitated by using classical chromatographic techniques that did not allow precursor–product relationships to be established. Blair and Pearson (1974) have examined the kinetics of oxidation of tetrahydrofolate by air. They found by measuring initial rates that the reaction was first order in both oxygen and tetrahydrofolate and that the rate of the reaction depended markedly on the state of ionization of the tetrahydrofolate. The identity and amounts of the reaction products were far from clear, however, since they were only examined by thin-layer chromatography.

In order to investigate the complex chemistry of tetrahydrofolate oxidation reactions, we have developed methods for the rapid, efficient analysis of folate and pterin derivatives, using high-performance liquid chromatography (Reed and Archer, 1976; Archer and Reed, 1980). Analysis of microgram samples of these derivatives which can be injected sequentially at 5–10-min intervals has thus become possible. We have recently used these methods to investigate the interaction of nitrous acid, folate, and tetrahydrofolate, reactions that are of potential importance in foods (Reed and Archer, 1979). We present here an analysis of the products of oxidation of tetrahydrofolate by air and suggest mechanisms to account for their formation. This information is important to an understanding of the stability of naturally occurring folates in food.

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MATERIALS AND METHODS

Folic acid, dihydrofolic acid, tetrahydrofolic acid, pterin, xanthopterin, and (*p*-aminobenzoyl)glutamate were purchased from Sigma Chemical Co. (St. Louis, MO); 6-formylpterin was prepared by the method of Waller et al. (1950).

High-performance liquid chromatography of the various folic acid derivatives and pterins has been previously described in detail (Reed and Archer, 1976; Archer and Reed, 1980). Briefly, we used an LDC Model 705 pump, an LDC ultraviolet monitor (254 nm) (Laboratory Data Control, Riviera Beach, FL), and a Valco sample injection valve (Valco Instruments, Houston, TX). Folic acid derivatives and (*p*-aminobenzoyl)glutamate were seperated by using a weak anion-exchange column (137 cm, Al-Pellionex-WAX, Reeve Angel, Clifton, NJ) eluted with 0.025 M sodium dihydrogen phosphate, pH 4.8, at 1.8 mL/min, and pterin derivatives were separated by using a high-efficiency cation-exchange column (30.5 cm, DC-1A, Durrum Chemical Corp., Palo Alto, CA) eluted with 0.01 M citrate, pH 6.0, at 0.44 mL/min. Under these conditions, typical elution times were as follows: (*p*-aminobenzoyl)glutamate, 1.9 min; tetrahydrofolate, 2.4 min; folate, 4.5 min; dihydrofolate, 6.4 min; xanthopterin, 4.5 min; 6-formylpterin, 6.5 min; pterin, 9 min; and dihydroxanthopterin, 14.5 min. Quantitation of chromatographic peak areas was performed by using a computing integrator. Molar absorptivities at 254 nm (the wavelength of the chromatographic detector) and at the pH of the chromatographic eluant buffer were determined by comparison with reported spectra (Blakley, 1969).

Products of the oxidation reactions were identified by comparing chromatographic retention times and UV spectra of isolated chromatographic peaks with authentic standards. Under the chromatographic conditions used, 7,8-dihydropterin, dihydroxanthopterin, and 6-formyldihydropterin eluted together as one peak. To illustrate the formation and decomposition of these intermediates, quantitation of the combined dihydropterins was based on the absorption of 7,8-dihydropterin at 254 nm and the pH of the eluant buffer.

Tetrahydrofolic acid was dissolved in citrate–phosphate–borate buffer (0.02 M in each component) adjusted to either pH 4, 7, or 10. Samples containing 10–20 mL of tetrahydrofolate solution were placed in flasks in a constant temperature bath at 30 °C; air was bubbled into the solution through a sparger at a constant flow rate. When these reaction mixtures were monitored by means of an oxygen electrode (Rank Bros., Bottisham, Cambs., England), we observed that the oxygen concentration remained constant throughout the experiments. The reaction mix-

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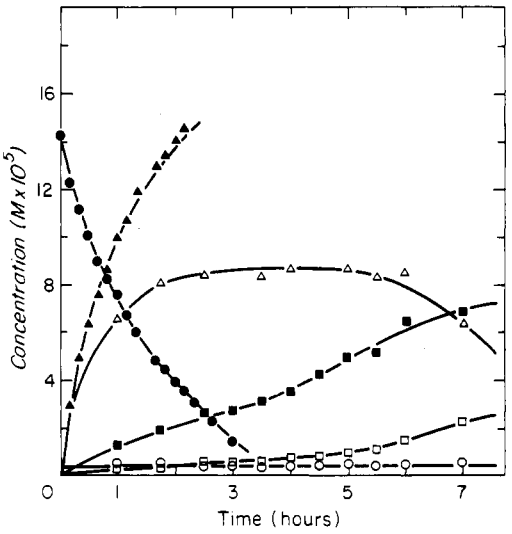

Figure 1. Time course of product formation for the reaction of tetrahydrofolate and oxygen in air at pH 4: (●) tetrahydrofolate, (△) dihydropterins, (▲) (*p*-aminobenzoyl)glutamate, (□) 6-formylpterin, (■) pterin, (○) xanthopterin.

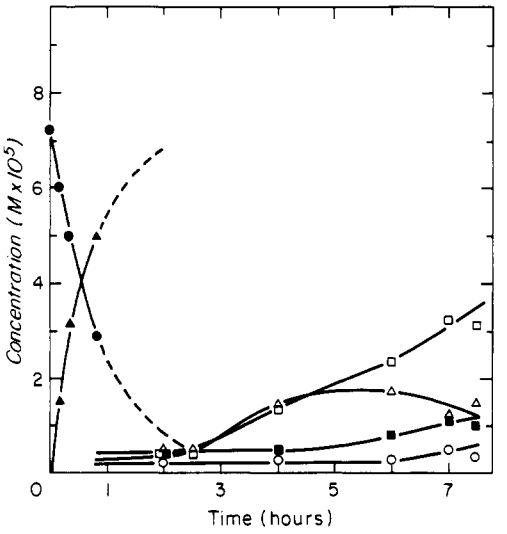

Figure 2. Time course of product formation for the reaction of tetrahydrofolate with oxygen in air at pH 7: (●) tetrahydrofolate, (△) dihydropterins, (▲) (*p*-aminobenzoyl)glutamate, (□) 6-formylpterin, (■) pterin, (○) xanthopterin.

tures were therefore never limiting in oxygen and the kinetics were pseudo first order. Samples were withdrawn at various time periods, deaerated immediately with nitrogen gas, and kept on ice until analyzed. Control experiments were performed in which nitrogen instead of air was bubbled through the reaction mixtures. No reaction occurred in these controls.

RESULTS

Using high-performance anion and cation exchange chromatography, we followed the appearance of products in the degradation of tetrahydrofolate by oxygen in air at pH 4, 7, and 10. Others have shown that formaldehyde is formed in some of these degradation reactions (Chippel and Scrimgeour, 1970); we did not analyze reaction mixtures for this product.

Figure 1 shows that at pH 4 the rate of loss of tetrahydrofolate ($t_{1/2}$ = 60 min) was equal to the rate of formation of (*p*-aminobenzoyl)glutamate. (The final (*p*-aminobenzoyl)glutamate concentration was about 16 × 10^{-5} M, which was a little higher than the initial concentration of tetrahydrofolate. This discrepancy is most likely caused by slight inaccuracies in the reported molar absorptivity values.) The dihydropterins appeared a little more slowly. The concentration of dihydropterins was almost constant from the second to the sixth hour of reaction. Thus the rates of formation and degradation of the dihydropterins were approximately equal. Possible precursors of the dihydropterins include quinonoid dihydrofolate and 6-formyltetrahydropterin (see Discussion) that we were unable to detect chromatographically. The maximum concentration of dihydropterins was roughly 60% of the initial concentration of tetrahydrofolate. Upon completion of the oxidation of the dihydropterins, the rate of formation of pterin and 6-formylpterin increased, although pterin was produced slowly throughout the early part of the reaction. At the termination of the experiment, the yield of pterin products was roughly 100% based on the initial concentration of tetrahydrofolate.

At pH 7, the rate of loss of tetrahydrofolate ($t_{1/2}$ = 40 min) was also equal to the rate of formation of (*p*-aminobenzoyl)glutamate (Figure 2). The appearance of the dihydropterins, however, was delayed for several hours in contrast to their early formation at pH 4. Other possible products formed early in this reaction include tetrahydropterins, particularly 6-formyltetrahydropterin (see Discussion), that were not detectable by our current techniques. The maximum concentration of dihydropterins was only about 25% of the initial concentration of tetrahydrofolate. 6-Formylpterin was the major product at pH 7, with smaller amounts of pterin and xanthopterin. After roughly 8 h of reaction, the yield of pterins was 85%, based on the initial concentration of tetrahydrofolate. As at pH 4, no dihydrofolate or folate was observed as products.

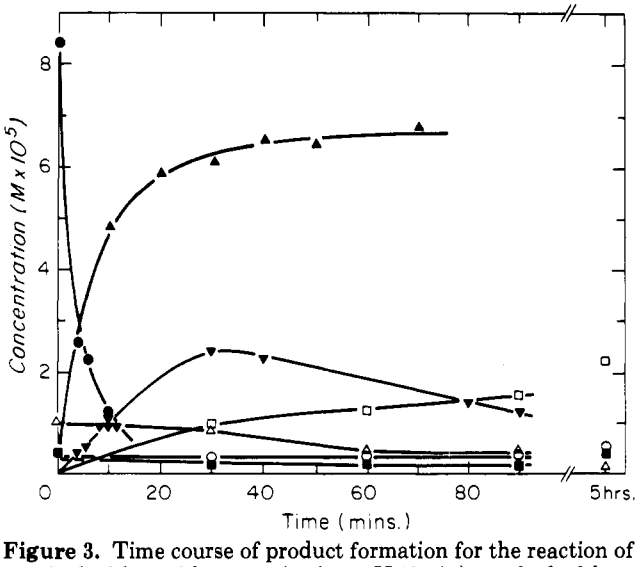

Figure 3. Time course of product formation for the reaction of tetrahydrofolate with oxygen in air at pH 10: (●) tetrahydrofolate, (▼) dihydrofolate, (△) dihydropterins, (▲) (*p*-aminobenzoyl)-glutamate, (□) 6-formylpterin, (■) pterin, (○) xanthopterin.

In a separate experiment at pH 7, we observed that oxidation of dihydrofolate was much slower ($t_{1/2}$ = 4.5 h) than oxidation of tetrahydrofolate under the same conditions. (*p*-Aminobenzoyl)glutamate and 6-formylpterin were the exclusive products of this reaction.

At pH 10, the rate of oxidation of tetrahydrofolate in air was extremely rapid ($t_{1/2}$ = 2 min, Figure 3). By the time the first sample was taken, dihydropterins had already formed. The rate of formation of (*p*-aminobenzoyl)glutamate was somewhat slower ($t_{1/2}$ = 5 min). Other major products were 6-formylpterin and dihydro-

Table I. Yields of Pterins in the Oxidation of Tetrahydrofolate by Air[a]

product	pH 4 (8 h)	pH 7 (7 h)	pH 10 (4.5 h)
6-formylpterin	18	57	78
xanthopterin	3	8	11
dihydropterins	30	16	3
pterin	49	19	8

[a] Results expressed as percent of total pterins produced. Total yield of pterin products (as percent of initial tetrahydrofolate): pH 4, 103%; pH 7, 85%; pH 10, 53%. All reactions performed in borate-citrate-phosphate buffer, 30 °C.

folate. No folic acid was seen as a product. At the termination of the experiment, pterin products comprised only 53% of the initial concentration of tetrahydrofolate. An unidentified compound that eluted slightly earlier than dihydrofolate on the weak anion exchange column was also seen as a product at pH 10.

Table I shows a comparison of the yields of the individual pterins expressed as a proportion of the total yield of pterin products at pH 4, 7, and 10. It is clear that the major pterin product was pterin itself at pH 4 and 6-formylpterin at pH 7 and pH 10. The proportion of the minor product xanthopterin in the pterin products also increased as the pH was raised from 4 to 10.

DISCUSSION

At pH 4, 7, and 10, (*p*-aminobenzoyl)glutamate was the major product of oxidation of tetrahydrofolate, a result that has been reported in several studies (Blakley, 1957; Futterman and Silverman, 1957; Zakrewski, 1966; Chippel and Scrimgeour, 1970). The rate of formation of (*p*-aminobenzoyl)glutamate at pH 4 and pH 7 was identical with the rate of loss of the tetrahydrofolate peak in the liquid chromatograms. This result indicates that cleavage of the side chain across either the C6–C9 bond or the C9–N10 bond occurs early in the oxidative reaction. Cleavage products containing the pterin ring formed more slowly. At pH 10, the formation of (*p*-aminobenzoyl)-glutamate was somewhat slower than the loss of tetrahydrofolate.

Pterin was the major pteridine product at pH 4, amounting to about 50% of the tetrahydrofolate oxidized. Much smaller amounts of pterin were produced at pH 7 and pH 10. From the intensity of a spot on a thin-layer plate, Blair and Pearson (1974) suggested that pterin was a major product of air oxidation of tetrahydrofolate at pH 3. Chippel and Scrimgeour (1970) identified pterin together with dihydroxanthopterin as products of oxidation of tetrahydrofolate under anaerobic conditions with ferricyanide as oxidizing agent at pH 5.6. They suggested a mechanism to account for the production of pterin in which electron abstraction takes place from the pteridine ring in tetrahydrofolate via two one-electron steps to produce a quinonoid dihydrofolate (Figure 4). Quinonoid dihydropterins have been well characterized as the kinetically favored products of the two-electron oxidation of simple tetrahydropterins (Archer and Scrimgeour, 1970; Archer et al., 1972). We were not able to observe the quinonoid dihydrofolate chromatographically, however. The thermodynamically unstable quinonoid dihydrofolate would then undergo acid-catalyzed breakdown to yield dihydropterin, (*p*-aminobenzoyl)glutamate, and formaldehyde (Figure 4). Further oxidation of the dihydropterin would yield pterin.

6-Formylpterin was the major pterin product at pH 7 and pH 10, while only a small amount of this compound

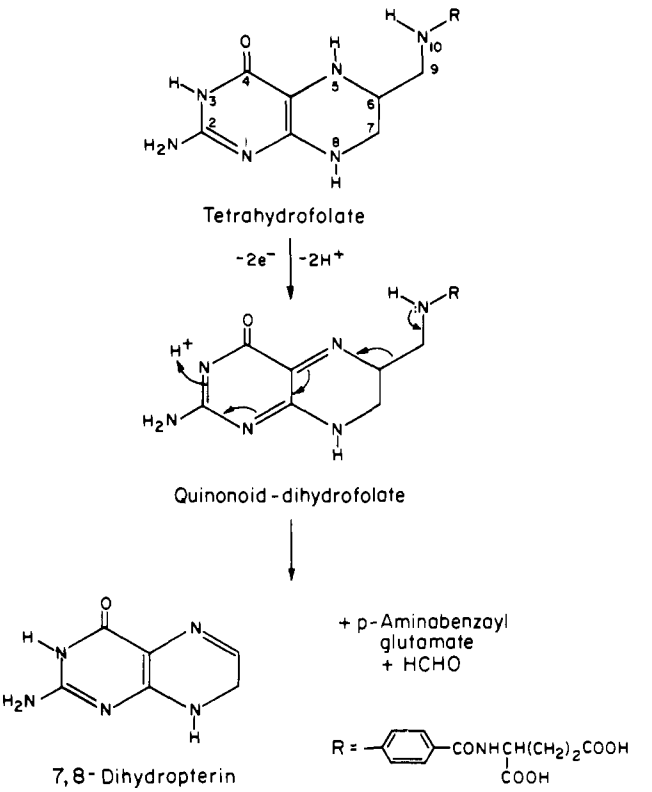

Figure 4. Mechanism of formation of dihydropterin from tetrahydrofolate.

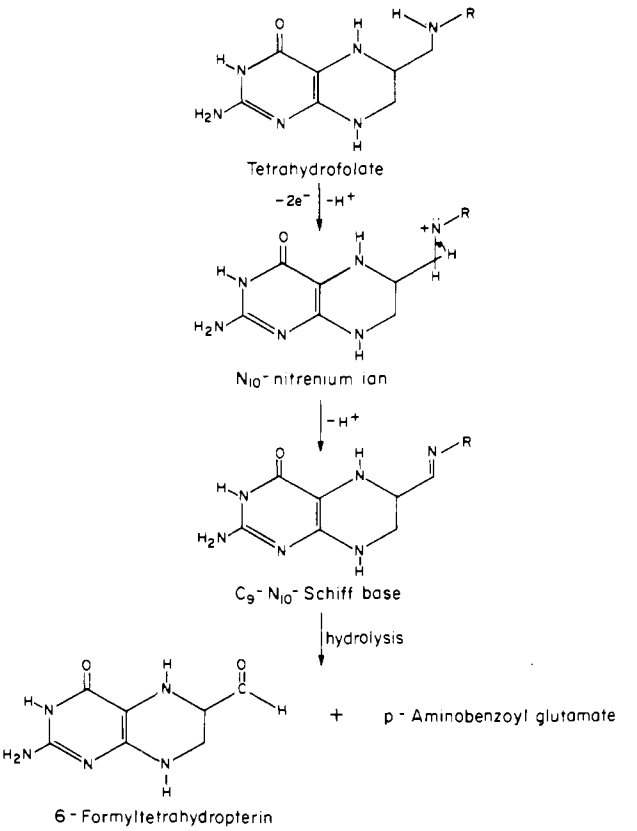

Figure 5. Mechanism of formation of 6-formyltetrahydropterin from tetrahydrofolate.

was formed at pH 4. Formation of 6-formylpterin can only be explained by a mechanism involving electron abstraction at N10 (Figure 5). Oxidation at N10 was first suggested by Whiteley et al. (1968) to account for formation of 6-formylpterin as a degradation product of dihydro-

folate. The N10 nitrenium ion formed by extraction of two electrons loses a proton from C9 to yield the Schiff base. The Schiff base would then hydrolyze to yield (*p*-aminobenzoyl)glutamate and 6-formyltetrahydropterin. Further oxidation would yield 6-formylpterin. As pointed out by Whiteley et al. (1968), support for this mechanism comes from the work of Sletzinger et al. (1955) and Waller et al. (1950) who showed that 6-formylpterin readily forms a Schiff base with hydroxylamine or aniline. The equilibrium for Schiff base formation is much less favorable with (*p*-aminobenzoyl)glutamate, and hence we may expect it to undergo facile hydrolysis.

The mechanism of oxidation via the N10 position is shown in Figure 5 with tetrahydrofolate. The evidence that tetrahydrofolate is indeed the species undergoing oxidation is firstly that the rate of loss of tetrahydrofolate and the rate of formation of (*p*-aminobenzoyl)glutamate are about the same. Thus, neither quinonoid dihydrofolate nor 7,8-dihydrofolate accumulates in the system. Secondly, we observed that although 7,8-dihydrofolate is oxidized to yield exclusively 6-formylpterin and (*p*-aminobenzoyl)-glutamate, the reaction time is very slow ($t_{1/2}$ = 4.5 h) and we would thus have expected to observe dihydrofolate as an intermediate in the oxidation of tetrahydrofolate. Thirdly, the dihydropterins only appear late in the reaction and there is a period between 1 and 3 h when no products appear on the liquid chromatograms. 6-Formyltetrahydropterin is not observable in our liquid chromatograms (it would have a long retention time and elute as a very broad peak, judging by the behavior of other tetrahydropterins). Thus, it seems likely that the major product at pH 7 in the period following the disappearance of tetrahydrofolate and before the appearance of dihydropterins is 6-formyltetrahydropterin.

The remaining problem, however, is to explain why there is a shift from pterin as the major product at pH 4 to 6-formylpterin at pH 7. The N10 position of tetrahydrofolate does not change its state of ionization over this pH range (pK_a = −1.25, Kellen and Jencks, 1966) and hence there is no obvious explanation for a shift in the position of electron abstraction from the ring to the side chain. One possibility, of course, is that oxidation of tetrahydrofolate at pH 4 does not take place in the ring, but takes place at N10 to form the N10-nitrenium ion which then loses the side chain to yield dihydropterin. We have no evidence to support this mechanism, however.

We only observed dihydrofolate as a product of the oxidation of tetrahydrofolate at pH 10 (Figure 3). Loss of a proton from the N3 position in the pterin ring (pK_a = 10.5, Kallen and Jencks, 1966) facilitates electron abstraction from the ring, and the resultant radical and cation are then stabilized by delocalization. Blair and Pearson (1974) in their kinetic study found that the increase in rate of oxidation of tetrahydrofolate with oxygen in air as the pH increases from 9 to 13 is proportional to the degree of ionization at N3. The abundance of base at pH 10 facilitates removal of the C6 proton rather than loss of the side chain of the quinonoid dihydrofolate observed at pH 4 (Figure 6). It is well known that rearrangement of quinonoid dihydropterin is subject to general base catalysis (Archer and Scrimgeour, 1970), and we may therefore expect the amount of 7,8-dihydrofolate formed from quinonoid dihydrofolate to depend on the concentration and species of buffer ions in solution. This would explain the variation in amount of dihydrofolate as an oxidation product of tetrahydrofolate by various observers. Figure 3 also indicates that 7,8-dihydrofolate, once formed, is oxidized but at a much slower rate than that of tetrahydrofolate, the final products being 6-formylpterin and (*p*-aminobenzoyl)glutamate. We did not detect folic acid as a reaction product under any conditions.

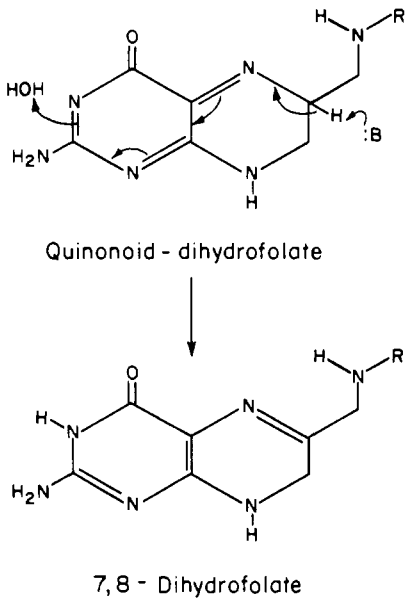

Figure 6. Mechanism of base-catalyzed rearrangement of quinonoid dihydrofolate to 7,8-dihydrofolate.

A minor product of oxidation of tetrahydrofolate in our experiments at pH 7 and pH 10 was xanthopterin. This product did not form at pH 4. In their experiments on the oxidation of tetrahydrofolate by 4 equiv of ferricyanide in MES buffer at pH 5.6, Chippel and Scrimgeour (1970) found that dihydroxanthopterin was the major product with smaller amounts of pterin. They found that dihydrofolate oxidation by 2 equiv of ferricyanide gave rise to both dihydroxanthopterin and 6-formylpterin. The ratio of the two products depended strongly on the buffer species. Amine buffers gave high levels of dihydroxanthopterin, whereas carboxylic and phosphate buffers enhanced formation of 6-formylpterin through a mechanism in which the buffers probably act as bridging groups in the transfer of electrons to ferricyanide from the dihydrofolate. Others have noted that phosphate promotes degradation of dihydrofolate to 6-formylpterin (Hillcoat et al., 1967). Thus the rather high 6-formylpterin/xanthopterin ratios in our experiment are explained by our use of borate–citrate–phosphate buffers.

Chippel and Scrimgeour (1970) showed that 6-formyldihydropterin is not likely to be the intermediate in the formation of xanthopterin. They showed that 6-formyldihydropterin was converted to xanthopterin when aerated at room temperature in the dark at pH 13, but the reaction took more than 24 h to reach completion. They also showed that neutral solutions of 6-formyldihydropterin were only slowly changed under aerobic conditions to dihydroxanthopterin. Chippel and Scrimgeour suggest that dihydrofolate is the precursor of dihydroxanthopterin in their experiments via formation of a covalent hydrate across the N5–C6 double bond. In our experiments, however, we saw no xanthopterin, following oxidation of dihydrofolate.

Although our experiments do not allow us to specify definitely the precursor of xanthopterin, a possibility is 7,8-dihydropterin which would undergo base-catalyzed covalent hydration of the N5–C6 double bond to yield 6-hydroxytetrahydropterin, followed by oxidation to xanthopterin (Figure 7). The N5–C6 double bond of 7,8-dihydropterin is known to be readily susceptible to nu-

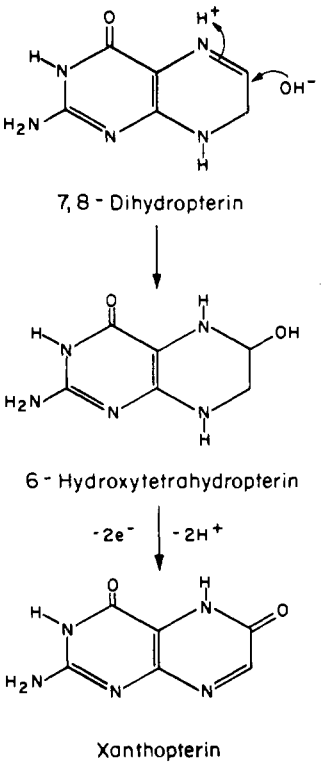

Figure 7. Mechanism of formation of xanthopterin from 7,8-dihydropterin.

cleophilic attack (Stuart et al., 1966).

In conclusion, we have demonstrated that tetrahydrofolate undergoes a variety of degradation pathways in air. Our results have suggested certain precursor–product relationships. Both the rate of reaction and nature of the products depend markedly on pH. At pH 7 and below, tetrahydrofolate was degraded exclusively to (*p*-aminobenzoyl)glutamate and pterin products. Hence under these conditions, vitamin activity will be completely lost. At higher pH's, dihydrofolate is a product, but this too oxidizes slowly in air to destroy the integrity of the folic acid molecule. In food systems there may be catalytic or inhibitory factors that change the distribution of products. With an understanding of the degradative chemistry of tetrahydrofolate in aqueous solution, it is now important to investigate the behavior of naturally occurring reduced folates in real food systems.

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