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The Degradation of Folic Acid in a Model Food System and in Beer

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ABSTRACT

The rate of degradation of folic acid in the light and in the dark is investigated in a model buffer solution and in beer, with and without the addition of sulphur dioxide.

In the model solution, held in the light, sulphur dioxide markedly accelerates the rate of degradation of folic acid whereas, in the dark, it exhibits a protective effect. In beer, the presence of sulphur dioxide has no effect on the degradation of folic acid, whether in the light or the dark. In the model solution, in the presence of sulphur dioxide, only the degradation product p-*aminobenzoyl glutamic acid was identified. In the absence of sulphur dioxide and in the beer samples, pterin-6-carboxylic acid was additionally identified as a degradation product.*

INTRODUCTION

Folates belong to the B vitamin group and are of nutritional importance in man. Among the most important sources are green leafy vegetables, peas, beans, certain fruits and beer. Folic acid is relatively unstable and can be destroyed during food processing and storage. Deficiency of folates in man leads to megaloblastic anaemia, often under conditions of pregnancy, infancy, old age, alcoholism or certain diseases.

Folic acid, a member of the folate group, has a pteridine nucleus linked via *p*-aminobenzoic acid to glutamic acid (Fig. 1). Reduction at the 5, 6, 7 and 8 positions and substitution on the 5 and 10 positions with methyl, methene or formyl groups, give the various folate forms that occur in nature. Also, in nature, folates are found in a 'bound' form, linked to a chain of glutamic acid residues, known as oligoglutamates.

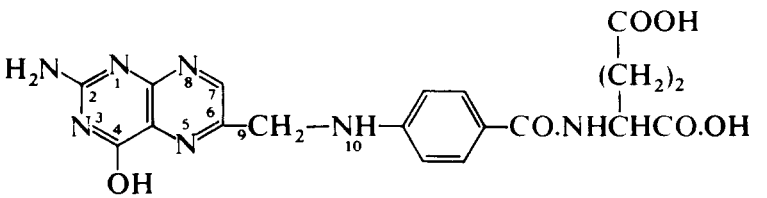

Fig. 1. Structure of folic acid.

The purpose of the present study is to determine the effect of sulphur dioxide on the stability of folic acid in aqueous solution and to compare the results with those in a beer, at the same pH.

Work published to date on the stability of folates in foods has been largely concerned with the effects of temperature, pH, oxygen and buffer ions. Folic acid is stable to heat in alkaline and neutral solution but unstable in acid solution. The rate of thermal destruction is dependent on a pH below 4. Treatment of folic acid with alkaline potassium permanganate cleaves the molecule at the C^9–N^{10} bond (Fig. 1), yielding *p*-aminobenzoylglutamic acid (*p*ABG) and pterin-6-carboxylic acid (Fig. 2). Aerobic hydrolysis of folic acid under acid conditions gives *p*ABG and 6-methylpterin (Fig. 2).

Chen & Cooper (1979) observed that the rate of thermal destruction of folacin in milk is partly dependent on the level of residual oxygen. They also noted, with other authors (Malin, 1977) that the natural antioxidant, ascorbic acid, retards destruction of folates in foods.

Sulphurous acid is also known to degrade folic acid (Blakly, 1969). Investigations into the degradation of folic acid consist essentially of rate studies. Unambiguous kinetic studies require quantitative determinations to be made quickly before secondary decomposition reactions can set in. Furthermore, rate studies consist of single determinations carried out over periods ranging from hours to weeks. For these reasons microbiological methods of analysis are unsuitable. Chemical analysis of folic acid has been performed by direct ultraviolet spectroscopy,

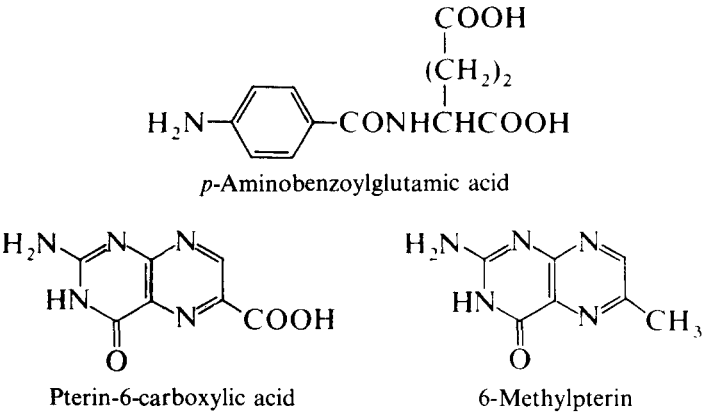

Fig. 2. Structures of degradation products of folic acid.

fluorimetry after oxidation to 2-amino-1, 4-dihydro-4-oxo-6-pteridine-carboxylic acid, polarography and colorimetry (Reif *et al.*, 1977). Chromatographic assays include thin layer chromatography on silica gel and high performance liquid chromatography on an octadecyl reversed-phase packing. The mobile phase used with the latter technique consisted of a phosphate buffer containing sodium perchlorate, methanol and sufficient potassium hydroxide to bring the pH to 7·2 (Reif *et al.*, 1977).

Bush *et al.* (1979) studied the effect of pH on the separation of oligoglutamates by reverse-phase HPLC. When the carboxylic groups of the solutes are largely undissociated at pH 2, the retention time of oligoglutamates increases with molecular weight. But, with a mobile phase of high pH, the carboxylic acid groups are dissociated and the elution order is reversed. At a pH of about 3·3, all the oligoglutamates are eluted together, but reproducibility may be difficult as the solutes are partially protonated. The oligoglutamates have also been separated on a Partisil-10 SAX column with a sodium chloride–phosphate buffer at pH 6·5 (Stout *et al.*, 1976).

Rouseff (1979) noted that the vitamin had been frequently reported as having fluorescent properties. However, when the eluate from a reversed-phase column was examined with fluorescence and UV detectors in series, the main UV absorbing peak did not coincide with any of the peaks from the fluorescence detector. By other techniques, it was concluded that the compounds exhibiting fluorescence were oxidative impurities. Day & Gregory (1981) separated six folates on coupled columns packed with

Ultrasphere IP and μBondapak phenyl with an acetonitrile–phosphate buffer mixture as eluant. The folates were detected by a post-column fluorescence derivatisation procedure or by UV absorbance at 280 nm. Reingold & Picciano (1982) described a more rapid ion-pairing high performance liquid chromatographic (HPLC) method for separating folates using a radially compressed C_8 cartridge which gave a separation of four folates in 16 min.

EXPERIMENTAL

Degradation experiments

Solutions (100 ml) of folic acid (50 mg litre^{-1}) in pH 5·0 acetate buffer, composed of 0·0296M acetic acid and 0·0704M sodium acetate, containing sulphur dioxide (130 mg litre^{-1}) were stored in capped 125 ml bottles at room temperature in a lighted cupboard (1 × 0·75 × 0·5 m) lined with white glossy paper and fitted with a 50 cm Atlas Double Life White 20W fluorescent lamp, or in the dark.

Reference solutions containing folic acid but not sulphur dioxide were similarly prepared and stored.

The headspace above the solutions was filled with nitrogen to prevent oxidation of the folic acid and the sulphur dioxide. Aliquots of the solution were removed periodically through the septum cap and analysed for folic acid and degradation products by HPLC and TLC.

Sulphur dioxide at levels of 50 and 140 mg litre^{-1} was added to beer prepared from a commercial light ale kit. Folic acid was added to these solutions and to untreated beer to give a final concentration of 50 mg litre^{-1}. Untreated beer contained 1·5 mg litre^{-1} of residual sulphur dioxide. The beer had a pH of 5. All beer samples were transferred to 25 ml capped bottles, which were filled to capacity and stored as described. Solutions were analysed periodically for folic acid by HPLC. Sufficient solutions were prepared so that each measurement in duplicate was made on a fresh sample.

Sulphur dioxide determinations on the solutions were made by the method of Tanner (1963). The sterility of the solutions was checked and shown to have an average count of less than one micro-organism per millilitre.

High performance liquid chromatography

Analysis of folic acid in the model solutions was undertaken on a stainless steel column (250 × 5 mm inside diameter) packed with Zorbax-ODS, fitted with a guard column (located between pump and injector) packed with the same material. The mobile phase was prepared by dissolving 1·69 g of tetrabutylammonium hydrogen sulphate and 13·61 g potassium dihydrogen phosphate in about 700 ml distilled water. The solution was adjusted to pH 7·2 with sodium hydroxide solution and diluted to exactly 775 ml with distilled water. Methanol (225 ml) was added and the mixed eluant was degassed before use. The flow rate was 1 ml min^{-1}. Analysis of folic acid in beer was undertaken on a column (150 × 5 mm, inside diameter) packed with Ultrasphere 1P and fitted with a guard column packed with silica gel (40 μm). The mobile phase consisted of 5 mM aqueous tetrabutylammonium phosphate–methanol (7:3), degassed

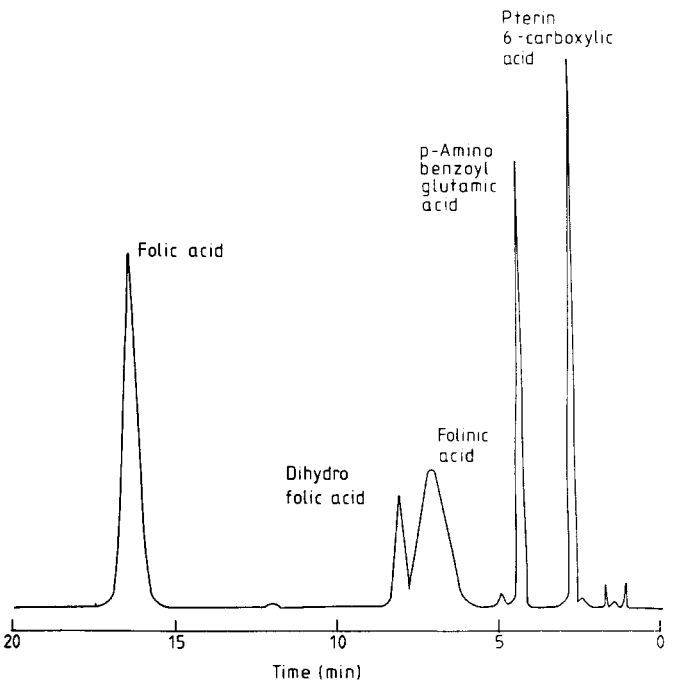

Fig. 3. Separation of folates and related compounds on an Ultrasphere 1P column (150 × 5 mm inside diameter) eluted with a mobile phase of 5 mM aqueous tetrabutylammonium methanol (7:3).

before use. The flow rate was 2 ml min^{-1}. Folic acid in all column eluants was detected spectrophotometrically at 280 nm. A typical separation is shown in Fig. 3.

Peak areas, determined on a computing integrator, were compared with standard solutions of folic acid. A calibration graph for folic acid showed a rectilinear relationship between peak area and weight of folic acid injected up to 1500 ng.

As folates are prone to oxidation in solution, standard solutions were prepared in a phosphate buffer containing ascorbic acid and a trace of mercaptoethanol (Reingold *et al.*, 1980).

Thin layer chromatography

Separations were made on precoated plates of cellulose (200 × 200 × 0·1 mm), which were cleaned by pre-development in 3 %(w/w) aqueous ammonium chloride solution. After appropriate spots of the sample or standard solution had been applied to the plate, it was developed in an equilibrated tank with 3 %(w/w) aqueous ammonium chloride solution to a height of 150 mm. Plates were dried at 40 °C and viewed under UV radiation (short and long wavelength). Plates were then sprayed with 0·05(w/w) aqueous potassium permanganate, dried at 40 °C and re-examined under UV radiation.

RESULTS AND DISCUSSION

Effect of sulphur dioxide on folic acid in a buffer solution

Folic acid (50 mg $litre^{-1}$) stored in the dark at pH 5·0, at room temperature, showed negligible degradation after 23 days. However, solutions of folic acid (50 mg $litre^{-1}$) containing sulphur dioxide (130 mg $litre^{-1}$) stored under identical conditions underwent a 20 % loss of folic acid during the same period. In the dark, therefore, sulphur dioxide had a destructive effect on folic acid (Fig. 4).

For solutions stored in the light cupboard, the reverse effect was observed. A solution of folic acid (50 mg $litre^{-1}$), at pH 5·0 and room temperature, was completely degraded in 23 days. Similar solutions containing sulphur dioxide (130 mg $litre^{-1}$) showed only 30 % loss of folic acid over the same period (Fig. 5). This protective action is probably due

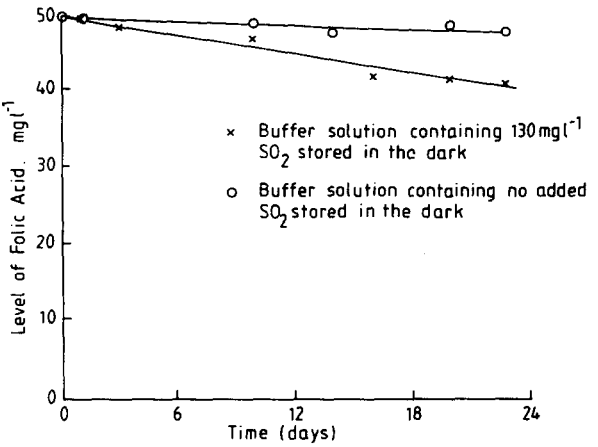

Fig. 4. Effect of sulphur dioxide (130 mg litre^{-1}) on the stability of folic acid in an acetate buffer stored in the dark.

to the antioxidant function of sulphur dioxide. It seems likely that the degradation of folic acid by light occurs by a mechanism which involves dissolved oxygen, the removal of which by sulphur dioxide reduces the rate of oxidation of folic acid. In the absence of light the action of sulphur dioxide significantly increases the rate of folic acid degradation.

Folic acid stored in the light without sulphur dioxide degraded to a mixture of three compounds, two of which were identified by the HPLC

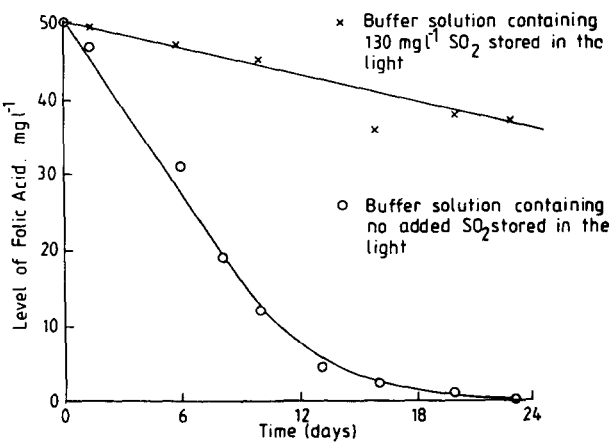

Fig. 5. Effect of sulphur dioxide (130 mg litre^{-1}) on the stability of folic acid in an acetate buffer stored in the light.

and TLC as the oxidative cleavage products, pterin-6-carboxylic acid and *p*-aminobenzoylglutamic acid (*p*ABG). The third compound was not identified but chromatographed close to the other two in both chromatographic systems, which suggested a similarity in chemical nature. It may have been 6-methylpterin, a product of aerobic hydrolysis of folic acid in acid conditions, or pterin-6-carboxaldehyde, a product of the photodecomposition of folic acid (Blakly, 1969).

It is worth noting that, although *p*ABG and the unknown compound appeared as soon as degradation of folic acid began, pterin-6-carboxylic acid only appeared in significant amounts after 8 days' storage in the light with no sulphur dioxide. Thereafter the proportion of pterin-6-carboxylic acid increased at the expense of the unknown compound. Blakly (1969) has suggested that folic acid is degraded by daylight to *p*ABG and pterin-6-carboxaldehyde and further irradiation converts pterin-6-carboxyaldehyde to pterin-6-carboxylic acid and then to pterin. Blakly's pathway of degradations suggests that, in the present work, the unknown compound is pterin-6-carboxaldehyde. Pterin and pterin-6-carboxylic acid elute together on HPLC, so that this method could not be used to detect pterin. None was detected by TLC, so it is likely pterin was either absent or present only in low concentrations.

Folic acid stored in the light or dark with sulphur dioxide was degraded to a mixture of *p*ABG and an unknown compound with an HPLC retention time identical to that of the unknown compound produced without sulphur dioxide. Blakly (1969) states that sulphurous acid cleaves the folic acid molecules to yield *p*ABG and a pterin-6-carboxaldehyde in which the pterin part of the molecule is in a reduced form. This compound is readily oxidised to the corresponding carboxaldehyde, thus giving degradation products similar to those produced by the action of light. It might be expected that pterin-6-carboxylic acid or pterin would be formed as the degradation continued but neither of these compounds was detected in any of the storage solutions which contained sulphur dioxide. It is possible that the pterin-6-carboxaldehyde forms a stable bisulphite addition compound and further degradation to the carboxylic acid and pterin is prevented.

The sulphur dioxide contents of the stored samples (where appropriate) were monitored throughout the test period of 23 days. The initial level (130 mg litre^{-1} SO_2) decreased within 1 day to 60–70 mg litre^{-1} SO_2 but then changed little throughout the remainder of the test. The test solutions were buffered at pH 5 and at this value the 'sulphur dioxide'

would be in an ionic form consisting of a nearly equimolar mixture of sulphite and bisulphite ions (Carswell, 1977). Accidental losses of gaseous sulphur dioxide during handling are therefore ruled out and the decrease must be attributed to combination with folic acid or oxidation to sulphuric acid.

The effect of sulphur dioxide on the degradation of folic acid in beer

A preliminary analysis of the prepared beer showed that it contained less than 1 mg litre^{-1} of free folic acid. For this reason, folic acid was added to the beer for the purposes of degradation experiments.

In beer stored in both the light and dark with no added sulphur dioxide, about one-third of the added folic acid was degraded after nearly

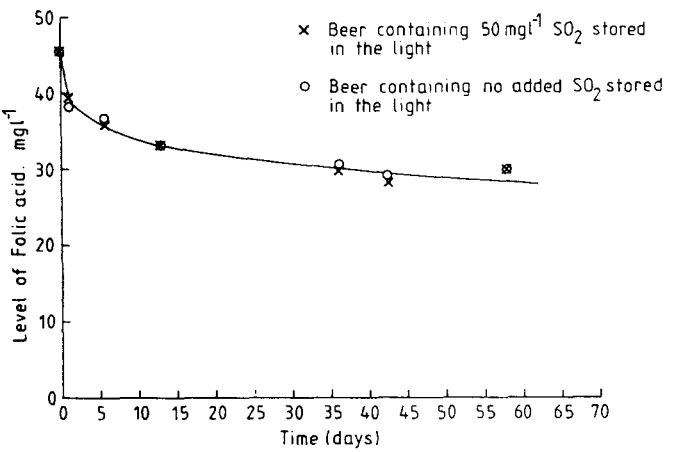

Fig. 6. Effect of sulphur dioxide (50 mg litre^{-1}) on the stability of folic acid in beer stored in the light.

2 months' storage (Figs 6 and 7). Folic acid was degraded rapidly during the first day, presumably due to reaction with dissolved oxygen which would be rapidly exhausted. The degradation thereafter, perhaps due to acid hydrolysis at pH 5·0, proceeded at a much lower rate and appeared to follow first order reaction kinetics. A rate constant of 5×10^{-7} day^{-1} was determined for the slower reaction. The known breakdown products of folic acid, pterin-6-carboxylic acid and *p*-aminobenzoylglutamic acid (Blakly, 1969) were identified in the beer by retention time. Although

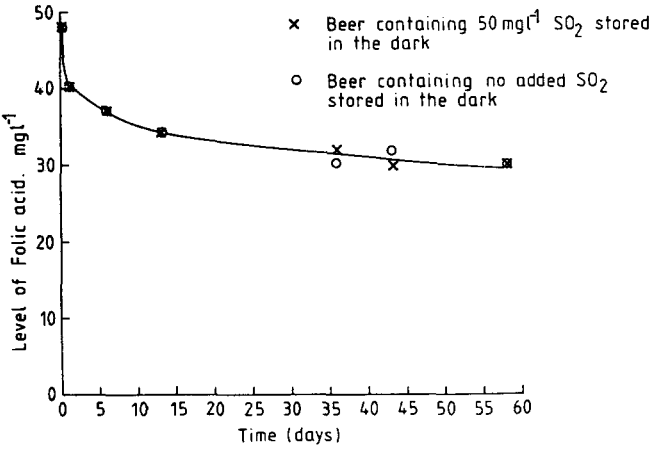

Fig. 7. Effect of sulphur dioxide (50 mg litre^{-1}) on the stability of folic acid in beer stored in the dark.

pterin-6-carboxylic acid was present naturally in the beer, an enhanced level was observed as the dedgradation study proceeded.

This is in contrast to the model solution containing sulphur dioxide, in which pterin-6-carboxylic acid was not detected.

The presence of sulphur dioxide at levels of either 50 or 140 mg litre^{-1} appeared to have little influence upon the rate of degradation (Figs 6 to 9). The measured sulphur dioxide contents of the stored samples, which were

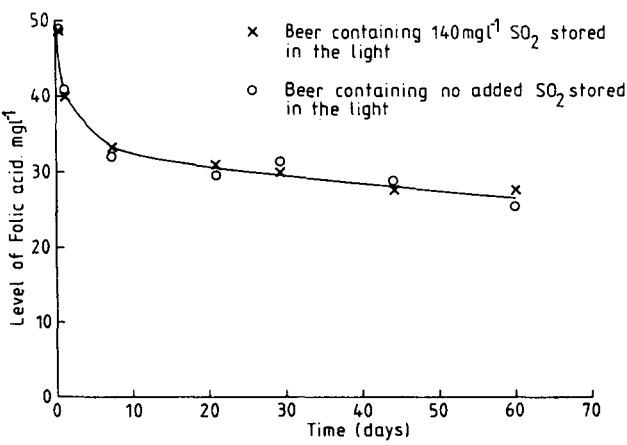

Fig. 8. Effect of sulphur dioxide (140 mg litre^{-1}) on the stability of folic acid in beer stored in the light.

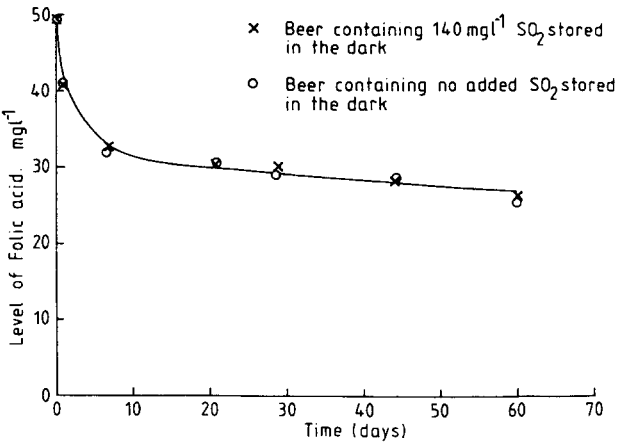

Fig. 9. Effect of sulphur dioxide (140 mg litre^{-1}) on the stability of folic acid in beer stored in the dark.

monitored throughout the test period, were initially 50 and 140 mg litre^{-1} but decreased to 40 and 120 mg litre^{-1}, respectively, after 1 day and then remained constant throughout the test period.

In sharp contrast to the model buffer solution at the same pH, sulphur dioxide in beer does not affect the rate of degradation of folic acid. It is presumed that this is due to components in the beer, which either have a protective effect on the folic acid or form loose complexes with the sulphur dioxide, which prevent it from reacting with the folic acid.

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