Correction. In the article "Homoeosis in Drosophila: The Ultrabithorax larval syndrome" by Pliny H. Hayes, Takashi Sato, and Robin E. Denell, which appeared in number 2, January 1984, of Proc. Natl. Acad. Sci. USA (81, pp. 545-549), Fig. 1 was reproduced badly. A better reproduction is shown below.

Fig. 1. Superimposed camera lucida tracings of the spinule patterns of five larvae of the indicated genotypes. Note the consistency of the patterns within genotype and the differences between wild-type and mutant patterns. The magnification of the camera lucida was adjusted to compensate for size differences among larvae. To standardize the patterns, the dorsal sensory papillae (large circles) were superimposed; in the wild-type AB1 the lateral edges of the anterior spinules (arrow) were used for standardization. Hooked spinules in the posterior first abdominal segment of the five larvae are represented as small circles; their numbers ranged from zero to three in different larvae. The cross-hatched area is covered by spinules in all five larvae of each genotype.

Correction. In the article "Evaluation of ascorbic acid in protecting labile folic acid derivatives" by Susan D. Wilson and Donald W. Horne, which appeared in number 21, November 1983, of Proc. Natl. Acad. Sci. USA (80, 6500-6504), several editorial errors occurred in Table 1 on p. 6501. In the first column, the first, third, fifth, and sixth entries should read 10-HCO-H4PteGlu, 5-HCO-H4PteGlu, 5,10-CH2-H4PteGlu, and 5-CH3-H4PteGlu, respectively. Also, the fifth row in column 6 (headed 5,10-CH2-H4PteGlu) should have the entry 25.7 instead of a blank.
Evaluation of ascorbic acid in protecting labile folic acid derivatives

(folate metabolism/HPLC/microbiological assay/formaldehyde/Lactobacillus casei)

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ABSTRACT The use of ascorbic acid as a reducing agent to protect labile, reduced derivatives of folic acid has been evaluated by high-performance liquid chromatographic separations and Lactobacillus casei microbiological assay of eluate fractions. Upon heating for 10 min at 100°C, solutions of tetrahydropteroylglutamic acid (H$_4$PteGlu) in 2% sodium ascorbate gave rise to 5,10-methylene-H$_4$PteGlu and 5-methyl-H$_4$PteGlu. H$_4$PteGlu acid gave rise to 5-methyl-H$_4$PteGlu and PteGlu. 10-Formyl-H$_4$PteGlu gave rise to 5-formyl-H$_4$PteGlu and 10-formyl-PteGlu. 5-Formyl-H$_4$PteGlu gave rise to a small amount of 10-formyl-PteGlu. 5-Methyl-H$_4$PteGlu and PteGlu appeared stable to these conditions. These interconversions were not seen when solutions of these folate derivatives were kept at 0°C in 1% ascorbate. These observations indicate that elevated temperatures are necessary for the interconversions of folic acid in ascorbate solutions. Assays of ascorbic acid solutions indicated the presence of formaldehyde (=6 μM). This was confirmed by the identification of 3,5-diacetyl-1,4-dihydrolutidin dine by UV, visible, and fluorescence spectroscopy and by thin-layer chromatography of chloroform extracts of the reaction mixture of ascorbic acid solutions, acetylacetone, and ammonium acetate. These results indicate that solutions of sodium ascorbate used at elevated temperatures are not suitable for extracting tissue for the subsequent assay of the individual folic acid derivatives.

Reduced derivatives of folic acid (pteroylglutamic acid, PteGlu, and compounds with more glutamic acid residues), such as H$_4$PteGlu, 10-HCO-H$_4$PteGlu, 5,10-CH$_2$H$_4$PteGlu, and 5-CH$_3$H$_4$PteGlu, are very susceptible to oxidation (1, 2). O’Broin et al. (3) found that the $t_{1/2}$ for H$_4$PteGlu decomposition in the absence of antioxidants was about 10 min, for 10-HCO-H$_4$PteGlu the $t_{1/2}$ was 4 hr, and for 5-CH$_3$H$_4$PteGlu, 35 hr. 2-Mercaptoethanol (at 0.4 M or greater) or ascorbate was effective in protecting H$_4$PteGlu. However, ascorbate afforded better protection at lower concentrations; therefore, it seemed that ascorbate was the agent of choice for protecting the labile reduced folates during extraction, chromatography, and assay procedures. Much work has been done to determine the levels of folate derivatives in tissues and the effects that various metabolite states (e.g., vitamin B$_12$ or folate deficiency) and chemicals (e.g., ethanol, anticonvulsant drugs, and chemotherapeutic drugs) have on the distributions of these coenzymes in order to better understand how these conditions affect folate metabolism. Early studies employed the differential growth requirements of Lactobacillus casei, Streptococcus faecalis, and Pedicoccus cerevisiae (3–7). Subsequently these procedures were improved by prior separation of extracts by ion-exchange column chromatography (8–15). For the most part these studies employed ascorbate solutions at high temperatures to extract tissue folates prior to analysis.

We have been developing procedures to apply high-performance liquid chromatography (HPLC) to the task of quantitating the naturally occurring folate derivatives. In the course of this work we have discovered that extraction of folates by using ascorbate solutions at high temperatures leads to chemical interconversions of several of these derivatives. In particular, H$_4$PteGlu gave rise to significant amounts of 5,10-CH$_2$H$_4$PteGlu and 5,10-CH$_2$H$_4$PteGlu to 5-CH$_3$H$_4$PteGlu, and H$_4$PteGlu to 5-CH$_2$H$_3$PteGlu and PteGlu. Further studies, also reported here, have shown that these interconversions are due, in part, to generation of formaldehyde from ascorbate at high temperatures. Our findings clearly demonstrate that ascorbic acid solutions used at high temperatures are unsuitable for extracting tissue folates for the subsequent assay of the individual derivatives.

MATERIALS AND METHODS

Materials. Formaldehyde (35% wt/vol) was obtained from Baker. L-Ascorbic acid and sodium L-ascorbate were from Fisher and Sigma. Acetaldehyde and 2-furfuraldehyde were also from Fisher. The latter was distilled under reduced pressure prior to use. HPLC-grade water was from Burdick and Jackson (Muskegon, MI). Lyophilized cultures of $L.$ casei, subspecies rhamnosus (ATCC 7469) were obtained from the American Type Culture Collection. Folic acid casei medium was from Difco. Folic acid (PteGlu) and dl-5-formyltetrahydrofolate (5-HCO-H$_4$PteGlu) were from Sigma. dl-5-CH$_2$H$_4$PteGlu, H$_4$PteGlu, 10-HCO-H$_4$PteGlu, 10-HCO-PteGlu, and 5,10-CH$_2$H$_2$PteGlu were synthesized by published procedures as outlined by Horne et al. (16). The folate compounds were further purified by HPLC and quantitated by the $L.$ casei assay as described below. Purified standard folates were stored at −20°C in 1% (wt/vol) sodium ascorbate at reduced pressure under a nitrogen atmosphere.

HPLC–$L.$ casei assay showed the following purities: H$_4$PteGlu, 80%; 10-HCO-H$_4$PteGlu, 85%; H$_4$PteGlu, 94%; 5-HCO-H$_4$PteGlu, 5-CH$_2$H$_2$PteGlu, and PteGlu, 100%. The impurities in the 10-HCO-H$_4$PteGlu were accounted for by 5-HCO-H$_4$PteGlu and 10-HCO-PteGlu. Those in the H$_4$PteGlu standard eluted in a broad band after H$_4$PteGlu and are probably due to decomposition during chromatography.

HPLC. HPLC separations of PteGlu derivatives were carried out as described (16) with the following modifications. A Waters model 660 solvent programmer was used to construct

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Abbreviations: PteGlu, pteroyl-(aS)-glutamic acid; H$_4$PteGlu, 7,8-dihydropteroyl-(aS)-glutamic acid; H$_4$PteGlu, 6(1R,5R,7,8)-tetrahydropteroyl-(aS)-glutamic acid; Ac$_2$H$_4$Lut, 3,5-diacetyl-1,4-dihydrolutidin dine; Ac$_2$H$_4$Col, 3,5-diacetyl-1,4-dihydrocololidine.

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a concave gradient (setting 8 for 60-min duration) from 15% B to 30% B (solvent A was water; solvent B was a 1:1 mixture of water and 95% (vol/vol) ethanol; both solvents contained 5 mM tetrabutylammonium phosphate and 5 mM 2-mercaptoethanol) at a flow rate of 1.0 ml/min. A Beckman–Altx Ultrasphere-I.P. column (15 cm × 4.6 mm) was used and absorbance of column eluates was monitored at 280 nm. Fractions (1.0 ml) were collected into tubes containing 0.1 ml of 10% sodium ascorbate to protect labile, reduced folates. Injection efficiency was monitored by mixing about 1 μCi (1 Ci = 3.7 × 1010 Bq) of [3H]-H2O with the sample and subsequent scintillation counting of the first 10 eluate fractions.

Microbiological Assays. Assays were performed as described by Wilson and Horne (17), using glycerol-cryptoprotected L. casei as the inoculum. Either PteGlu or 5-HCO-H4PteGlu, both purified by HPLC as described above, were used as standards for the microbiological assay of folates. Assay results demonstrated no significant difference in the growth response of L. casei to these standards or to 5-CH3-H2PteGlu. In the case of racemic compounds (dl-5-HCO-H4PteGlu or dl-5-CH3-H2PteGlu) the amount of folate active for L. casei was taken to be one-half the amount determined by UV spectroscopy using molar extinction coefficients reported by Blakely (1). In assaying column eluate fractions, 250-μl aliquots were used. In such assays the standard curve received 250 μl of 25% (vol/vol) solvent B in solvent A.

Assay for Formaldehyde. Solutions of ascorbic acid and sodium ascorbate were assayed for formaldehyde by the chromotropic acid procedure (18) and by the Nash procedure (19) as described by Werringloer (20).

Synthesis of Hantzsch Reaction Product of Formaldehyde and Acetaldehyde. 3,5-Diacetyl-1,4-dihydrolutidine (Ac2H2Lut) and 3,5-diacetyl-1,4-dihydrolutidine (Ac2H2Col), the products of the Hantzsch reaction between ammonia, acetalacetone, and formaldehyde or acetaldehyde, respectively, were synthesized according to the procedures reported by Nash (19). The products were recrystallized twice from absolute ethanol prior to use.

RESULTS

The elution profile resulting from the HPLC separation and L. casei assay of eluate fractions of various standard synthetic folic acid derivatives is shown in Fig. 1. The individual folate derivatives were well separated under the standard elution conditions. This fact along with the sensitivity (<0.05 ng) and specificity of the microbiological assay led us to apply these procedures to estimate the level of the various folate derivatives in mammalian tissues. During our evaluation of these procedures we found that ascorbic acid solutions heated at 100°C can lead to chemical interconversion of several folate coenzymes.

Samples of each folic acid derivative (100 ng/ml) were heated separately in a boiling water bath for 10 min in a solution containing 2% (wt/vol) sodium ascorbate/3 mM Hepes buffer, pH 7.4. The solutions were cooled in an ice bath and assayed by the HPLC/L. casei procedure. Table 1 lists the results of these experiments. 10-HCO-H4PteGlu was seen to give rise to 5-HCO-H4PteGlu and 10-HCO-PteGlu. The presence of the 5-formyl derivative was not unexpected because it is known that 5- and 10-HCO-H4PteGlu are easily interconverted (21, 22). The presence of 10-HCO-PteGlu indicated that ascorbate did not completely prevent the oxidation of the reduced 10-formyl derivative. HPLC assay of unheated standard 10-HCO-H4PteGlu showed a lesser content of both 5-HCO-H4PteGlu (7%) and 10-HCO-PteGlu (8%). Heating H4PteGlu solutions with ascorbate resulted in considerable chemical interconversions. Of particular interest is the presence of 5,10-CH2-CH3-PteGlu and 5-CH3-H2PteGlu, neither of which was seen when unheated solutions of this compound (1% sodium ascorbate) were chromatographed. Because H4PteGlu reacts with formaldehyde to yield
5,10-CH$_2$H$_2$PteGlu (23), these results suggest that, upon heating, solutions of ascorbic acid generate formaldehyde. The presence of 5-CH$_3$H$_2$PteGlu also suggests that ascorbate solutions are capable of reducing 5,10-CH$_2$H$_2$PteGlu to 5-CH$_3$H$_2$PteGlu.

When ascorbate-containing solutions of 5-HCO$_2$H$_2$PteGlu were heated a small amount of 10-HCO$_2$PteGlu was seen. The latter eluted from the HPLC column between 5-CH$_2$H$_2$PteGlu and PteGlu. This conversion occurred, presumably, via conversion to 10-HCO$_2$H$_2$PteGlu, which then was oxidized to 10-HCO$_2$PteGlu. H$_2$PteGlu was partly converted to 5-CH$_3$H$_2$PteGlu and PteGlu. The overall recovery for H$_2$PteGlu (52%) indicated that, under these conditions, about 50% of this compound was converted to products not active for L. casei via rupture of the C$_9$-N$_10$ bond to give p-aminobenzoylglutamate and a pterin (ref. 1, pp. 78–82).

The partial dissociation of 5,10-CH$_2$H$_2$PteGlu to H$_2$PteGlu upon heating in ascorbate solutions was anticipated because such dissociation at about pH 7 had been reported previously (23). Also shown in Table 1, about 12% of the 5,10-CH$_2$H$_2$PteGlu was apparently reduced to 5-CH$_3$H$_2$PteGlu by this treatment. This finding is consistent with the observation of 5-CH$_3$H$_2$PteGlu in heated ascorbate-containing solutions of H$_2$PteGlu (see above and Table 1) and further strengthens the conclusion that ascorbate solutions may reduce 5,10-CH$_2$H$_2$PteGlu to 5-CH$_3$H$_2$PteGlu. 5-CH$_3$H$_2$PteGlu and PteGlu were stable to this treatment.

Assay of Ascorbate Solutions for Formaldehyde. The finding that heated solutions of H$_2$PteGlu containing ascorbate gave rise to 5-10-CH$_2$H$_2$PteGlu suggested that such solutions generate formaldehyde. To examine this possibility solutions of ascorbate were assayed for formaldehyde content by the chromotropic acid method (18) and by Nash's procedure (19, 20). These assays indicated the presence of 1.1 mM and 6 mM formaldehyde, respectively, in 2% (wt/vol) sodium ascorbate solutions.

In separate experiments, equal aliquots (10 µl) of increasing sodium ascorbate concentration [0.1–10% (wt/vol)] were assayed via Nash's procedure. The results showed that the level of formaldehyde progressively increased (to about 6 mM) at ascorbate concentrations of 1% to 2% and plateaued thereafter.

Subsequent studies using the Nash assay showed similar amounts of formaldehyde in five different lots of ascorbate from two suppliers. Three samples were sodium ascorbate and two were the free acid; therefore, it seems unlikely that the formaldehyde was a contaminant present in the samples. This conjecture is supported by the fact that rechromatography of HPLC-purified samples of standard H$_2$PteGlu, stored at 0°C in 1% sodium ascorbate, showed no 5,10-CH$_2$H$_2$PteGlu. On the other hand, both the chromotropic acid assay and the Nash assay were positive for formaldehyde whether or not the ascorbate solutions were previously heated at 100°C for 10 min. However, both assays employed high temperatures (100°C for 30 min and 60°C for 10 min, respectively). Thus, the evidence favored the hypothesis that formaldehyde was generated during periods of heating, presumably via ascorbate decomposition.

It has been reported in a review article by Tolbert and Ward (24) that 2-furfuraldehyde is a decomposition product of ascorbic acid and dehydroascorbic acid. When assayed by the Nash procedure this compound gave only 0.9% of the absorbance of an equimolar amount of formaldehyde.

The products of the Hantzsch reaction (which is the basis of the Nash assay) with formaldehyde, Ac$_2$H$_2$Lut, and with acetaldehyde, Ac$_2$H$_2$Col, were synthesized as standards for spectroscopy and thin-layer chromatography. Subsequently the yellow products from the Nash assay with formaldehyde, sodium ascorbate, and 2-furfuraldehyde were extracted into CHCl$_3$. The CHCl$_3$ was dried over anhydrous Na$_2$SO$_4$. Authentic Ac$_2$H$_2$Lut and Ac$_2$H$_2$Col were dissolved in CHCl$_3$. All spectra were taken of samples in CHCl$_3$.

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**Fig. 2.** Absorption spectra of Nash assay extracts and standards. Tubes from the Nash assay of formaldehyde, sodium ascorbate, and 2-furfuraldehyde were extracted with 2 ml of CHCl$_3$. The CHCl$_3$ layer was dried over anhydrous Na$_2$SO$_4$. Authentic Ac$_2$H$_2$Lut and Ac$_2$H$_2$Col were dissolved in CHCl$_3$. All spectra were taken of samples in CHCl$_3$.

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Ac$_2$H$_2$Lut standard; ---, formaldehyde extract; ---, Ac$_2$H$_2$Col standard; x---, 2-furfuraldehyde extract.
These samples were further characterized by thin-layer chromatography on silica gel G plates (EM Laboratories, Elmsford, NY). The solvent systems used were: solvent I, ethyl acetate/acetic acid/methanol (4:1:1, vol/vol); and solvent II, n-butyl alcohol/acetic acid/water (4:1:1, vol/vol). The Ac₂H₂Lut standard and the CHCl₃ extract of the Nash assay products of formaldehyde and ascorbate all gave a single yellow-green fluorescent spot of Rᵢ 0.69 in solvent I and 0.72 in solvent II. Upon standing these spots faded. The Rᵢ's for Ac₂H₂Col were 0.75 and 0.77 in solvents I and II, respectively. Furthermore, upon standing the fluorescent spots due to Ac₂H₂Col remained visible. The CHCl₃ extract of the Nash assay for 2-furfuraldehyde gave several spots—Rᵢ's of 0.92 and 0.83 in solvent I and 0.78 in II.

**DISCUSSION**

Our experiments document that chemical interconversion of several folate derivatives occurred when these compounds were heated for 10 min at 100°C in 2% (wt/vol) sodium ascorbate/5 mM Hepes, pH 7.4. In our hands such treatments led to the production of 5,10-CH₂-H₄PteGlu from H₃PteGlu. Also H₂PteGlu, H₃PteGlu, and 5,10-CH₂-H₃PteGlu gave rise to 5-CH₂-H₃PteGlu. These results suggested that heated ascorbate solutions are capable of reducing H₂PteGlu to H₃PteGlu, that these solutions generate formaldehyde that reacts chemically with H₃PteGlu to give 5,10-CH₂-H₃PteGlu (23), and that they are capable of reducing the latter to 5-CH₂-H₃PteGlu. None of these interconversions were seen when these standard folates were kept in 1% sodium ascorbate at -15°C and occasionally thawed and stored in an ice bath for periods of up to an hour. Thus, it appears that elevated temperatures are necessary for these interconversions to occur. Our observations are of particular interest in regard to procedures for estimating the content of individual folate derivatives in tissue extracts. Thus, it appears that the use of heated ascorbate solutions will result in overestimation of the proportion of 5-CH₃-H₄PteGlu. This might help explain some of the variability in reported values for hepatic 5-CH₃-H₄PteGlu. These have ranged from about 50% (9) to above 80% (14) of total folates. The conversion of H₂PteGlu to 5,10-CH₂-H₄PteGlu and 5-CH₂-H₃PteGlu would tend to lead to underestimation of H₃PteGlu. This conclusion would apply also to H₃PteGlu because it was converted to 5-CH₃-H₄PteGlu and PteGlu. The level of 5,10-CH₂-H₄PteGlu would similarly be underestimated due to its conversion to 5-CH₂-H₃PteGlu and dissociation to H₃PteGlu. The latter would result in the overestimation of H₃PteGlu and may explain why 5,10-CH₂-H₄PteGlu has not been found directly by chromatographic means in tissue extracts (8–15, 21, 22).

The presence of 5,10-CH₂-H₄PteGlu in heated ascorbate-containing solutions of H₃PteGlu suggested that formaldehyde was produced via decomposition of ascorbate. In a recent review (24) 2-furfuraldehyde was listed among the decomposition products of ascorbate and dehydroascorbate; however, formaldehyde was not. When 2% solutions of ascorbic acid or its sodium salt were assayed by two independent methods the presence of formaldehyde was indicated. Furthermore, it was observed that the chromophore (Ac₂H₂Lut) produced from authentic formaldehyde during the Nash assay was extractable into CHCl₃. Subsequently we showed that the absorption and fluorescence spectra of synthetic Ac₂H₂Lut were identical to the spectra of CHCl₃ extracts of tubes from the Nash assay of ascorbate solutions. Thin-layer chromatography of these extracts confirmed the spectral data and further showed that neither acetaldehyde nor 2-furfuraldehyde was present in detectable concentrations.

The present work emphasizes the fact that heating folate derivatives at 100°C in sodium ascorbate solutions, a routine practice for tissue extraction (1, 5–7, 9, 11–15), can lead to chemical interconversions of several of these compounds. This results in overestimation of some derivatives and underestimation of others and could explain the variability of reported tissue levels of individual folates. An extraction method that eliminates these problems could lead to lessened procedural variability and could allow a better understanding of nutritional and metabolic influences of folate biochemistry.

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