Biochemical Pharmacology, Vol. 29, pp. 2135–2141.

0006-2952/80/0801-2135 $02.00/0

INHIBITION OF BUTTERMILK XANTHINE OXIDASE BY FOLATE ANALOGUES AND DERIVATIVES*

HENRY G. KAPLAN†
Department of Medicine, Roger Williams General Hospital,
Brown University Program in Medicine, Providence, RI 02908, U.S.A.

(*Received* 23 *June* 1979; *accepted* 24 *January* 1980)

Abstract—Folic acid has been reported recently to be an effective agent for the treatment of hyperuricemia, although conflicting data exist. The relative inhibitory activities of this compound and its breakdown products, pterin aldehyde and 6-hydroxymethylpterin, for the enzyme xanthine oxidase have not been clear. In this study, folic acid purified from these two compounds competitively inhibited buttermilk xanthine oxidase under aerobic conditions by a mechanism kinetically distinct from that of pterin aldehyde, with an inhibition constant (K_i) of 0.12 μM. Methotrexate, leucovorin and N^5-methylH_4folate were competitive inhibitors of the enzyme with K_i values ranging from 12 to 53 μM. MethyleneH_4folate, H_2folate and H_4folate did not inhibit xanthine oxidase. N^5-MethylH_4folate could not be evaluated by the reduction of cytochrome *c* because of the nonenzymatic oxidation of this folate derivative by cytochrome *c* to a compound shown to be N^5-methylH_2folate. Unless high intracellular concentrations of unchanged folic acid, pterin aldehyde or hydroxymethylpterin can be achieved or folic acid proves to be a more effective inhibitor of reduced than of oxidized enzyme, it is unlikely that this compound will be an effective clinical agent for the inhibition of xanthine oxidase.

In 1948, Kalckar and Klenow [1] demonstrated that low concentrations of folic acid (pteroylglutamic acid, PGA) inhibited the enzyme xanthine oxidase (xanthine:oxygen oxido reductase, EC 1.2.3.2) which catalyzes the conversion of xanthine to uric acid. Subsequently, Lowry *et al.* [2, 3] showed that 2-NH_2-4-OH-pteridine-6-carboxaldehyde (pterin aldehyde), a breakdown product of folic acid produced by exposure of PGA to ultraviolet light, was a 100- to 1000-fold more potent inhibitor of this enzyme. Kalckar *et al.* [4, 5] confirmed this observation and suggested that the previously observed inhibition by folic acid was, in reality, due to contamination of the folate with pterin aldehyde. More recently, Stea *et al.* [6] have suggested that hydroxymethylpterin, another breakdown product of PGA, is also a potent inhibitor of xanthine oxidase.

Oster [7] has reported that clinical hyperuricemia can be effectively treated without toxicity by the oral administration of 40–80 mg of folic acid daily and has suggested that this may be due to inhibition of xanthine oxidase. Similar studies by Plouvier and Devulder [8], however, showed only a 10 per cent fall in urinary uric acid excretion in patients following initiation of folate therapy. This fall was transient.

For these reasons, a study was initiated to examine in depth the effect of folic acid on xanthine oxidase activity. In addition, other folates and folate analogues were evaluated because of both the frequent use of high doses of these agents in the treatment of neoplastic diseases [9] and the known *in vivo* conversion of folic acid to other folate species [10].

* Supported by NCI Grants: S07 RR 05765, P30 CA 13943 and P01 CA 20892.

† Present address: Department of Medicine, University of Washington, Health Sciences RG-20, Seattle, WA 98195, U.S.A.

METHODS

Chemicals. [3,5,9(n)-^{3}H]Folic acid (41 Ci/mmole) and [5-^{14}C]methylH_4folate (58 mCi/mmole) were purchased from Amersham-Searle, Arlington Heights, IL. Methotrexate (MTX) and 5-formyl-H_4folate (leucovorin, calcium salt) were provided by Dr. Ronald Stoller of our institution. Pterin aldehyde was provided by Dr. Robert Angier of Lederle Laboratories, Pearl River, NY. *Tert*-butyl alcohol and 2,5-dimethylfuran were purchased from Aldrich Chemicals, Milwaukee, WI. 2,4-Dinitrophenylhydrazine (DNPH) was obtained from Eastman Organic Chemicals, Rochester, NY. All other organic chemicals, including H_2folate and H_4folate, were purchased from the Sigma Chemical Co., St. Louis, MO. N^5N^{10}-MethyleneH_4folate was prepared as described previously [11]. Buttermilk xanthine oxidase (1.68 units/mg protein as defined by the manufacturer) was the highest purity grade available from Sigma. MN300F cellulose, DEAE cellulose and ECTEOLA cellulose thin-layer chromatography (t.l.c.) plates were from Brinkman Instruments, Westbury, N.Y.

Gel electrophoresis. Polyacrylamide gel electrophoresis with lauryl sulfate was performed according to the method of Weber and Osborn [12]. Gel electrophoresis without lauryl sulfate was carried out by the same procedure, except that the gel buffer was 100 mM Tris containing 20 mM glycine and 1 g/100 ml Triton X-100 (New England Nuclear), pH 7.2.

Enzyme assay. Xanthine oxidase activity was assayed by two different spectrophotometric methods. Uric acid production was measured by an increase in optical density at 295 nm [13]. In the standard assay, xanthine (100 nmoles) was combined with 15 μg (25.3 munits) of enzyme preparation protein and inhibitor in the presence of 80 nmoles

EDTA-Na_2 and 40 μmoles potassium phosphate buffer, pH 7.4, in a volume of 1 ml. Enzyme was the final reactant added except in the preincubation studies.

Alternatively, activity was assayed by the cytochrome *c* reduction method [14]. The incubation conditions were identical to those in the uric acid production assay with the exception that cytochrome *c* (90 nmoles, Type VI, Sigma) was included in the reaction mixture, and the change of optical density was followed at 550 nm. This reaction was completely inhibited by the presence of 80 units of superoxide dismutase and both reactions gave similar blank rates (omitting either xanthine or xanthine oxidase) except in the presence of N^5-methylH_4folate (see below).

All spectrophotometric measurements were carried out in a Beckman DU spectrophotometer fitted with a constant temperature water bath, a Gilford automatic cuvette changer and continuous chart recorder, at 23° using 1 cm light path cuvettes. Radioactivity measurements were performed in a Packard Tri-Carb liquid scintillation counter with 10 ml Aquasol (New England Nuclear, Boston, MA) as scintillant. Counting efficiency for tritium was 47 per cent and for carbon-14 it was 90 per cent.

RESULTS

Enzyme purity. The purity of xanthine oxidase was assayed by gel electrophoresis. With polyacrylamide gels in Tris–glycine buffer a single protein band was found. When more than 10 μg of protein was loaded onto gels, two other faint bands were visible. In sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis, three bands were found, corresponding to molecular weights of 18,000, 37,000 and 74,000. Optical density measurements in potassium phosphate buffer (pH 7.4) revealed that $E_{280}/E_{450} = 6.0$ [15].

Because MTX is known to be a substrate for aldehyde oxidase but not xanthine oxidase [16], specific tests for the presence of aldehyde oxidase were carried out. Using the xanthine oxidase enzyme preparation, no oxidation of N^1-methylnicotinamide, a substrate of aldehyde oxidase but not xanthine oxidase, could be detected at concentrations up to ten times its reported K_m for aldehyde oxidase with either ferricyanide or cytochrome *c* as the electron acceptor [17]. Similarly, menadione, an inhibitor of aldehyde oxidase but not of xanthine oxidase [18], did not inhibit enzyme activity, whether measured by uric acid production from xanthine or by reduction of cytochrome *c* by xanthine oxidation at menadione concentrations up to 10 μM.

Folic acid purity. Folic acid (Sigma, Lot N.25C-0216) was assayed to be ≥99.6 per cent pure by Sigma. Stock solutions were prepared in 0.025 N KOH and dilutions were made in double distilled water. However, because of the question of contamination of folic acid with the potent xanthine oxidase inhibitors pterin aldehyde and hydroxymethylpterin, detailed analysis and purification of the folic acid were carried out.

The commercially obtained PGA was eluted from a 0.9 × 30 cm DEAE-Sephadex A-25 column at 4° utilizing a linear gradient of 0.2 to 2.0 M NH_4 acetate with 0.01 M 2-mercaptoethanol (ETSH) throughout [19]. Using [^{3}H]PGA alone, a number of rapidly migrating impurities were separated (Fig. 1) and not further characterized. Unlabeled PGA was then applied to the column and eluted similarly. A single peak of optical density at 285 nm was found which coincided with the major tritium peak. The fractions containing the PGA peak were then pooled, lyophilized and stored at −20° for future use. All procedures were carried out in the dark to prevent ultraviolet radiation-induced breakdown of the folic acid. PGA concentration was determined using the molar extinction coefficient at 282 nm (E^{1cm}_{max} = 27,600) [20]. Experiments using [^{3}H]PGA were carried out with labeled PGA prepared in this manner.

When pterin aldehyde was co-chromatographed with PGA, three peaks were eluted (Fig. 2). Aliquots

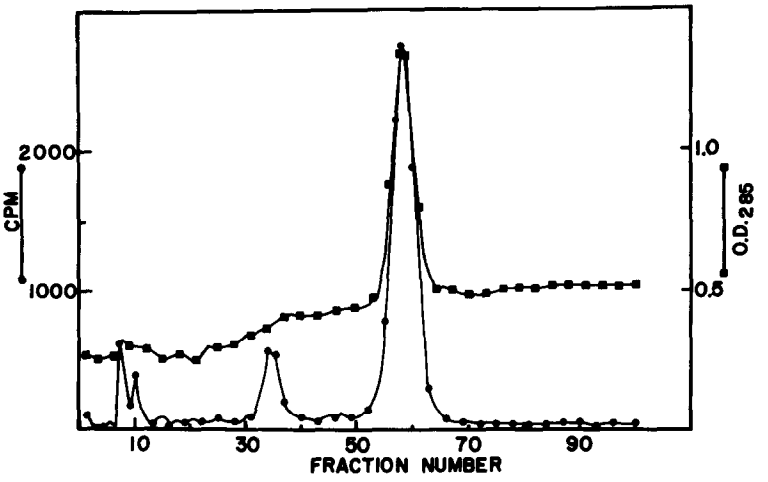

Fig. 1. Purification of folic acid by column chromatography. Either 2.5 pmoles (1 μCi) of [^{3}H]PGA (●) or 3.3 mg of unlabeled (■) PGA was applied to a 0.9 × 30 cm DEAE-Sephadex A-25 column equilibrated with 0.2 M NH_4 acetate containing 0.01 M ETSH and eluted with a linear gradient from 0.2 to 2.0 NH_4 acetate (0.01 M ETSH throughout). Each fraction contained 2.5 ml, and the flow rate was 14 ml/hr. Temperature was 4°, and gel filtration was carried out in the dark.

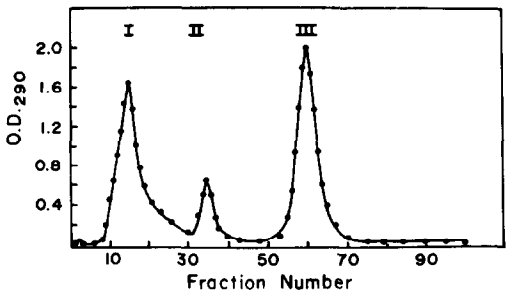

Fig. 2. Co-chromatography of pterin aldehyde, hydroxymethylpterin and folic acid. Three milligrams PGA and 2.4 mg pterin aldehyde were applied and eluted from a DEAE-Sephadex column under conditions shown in Fig. 1.

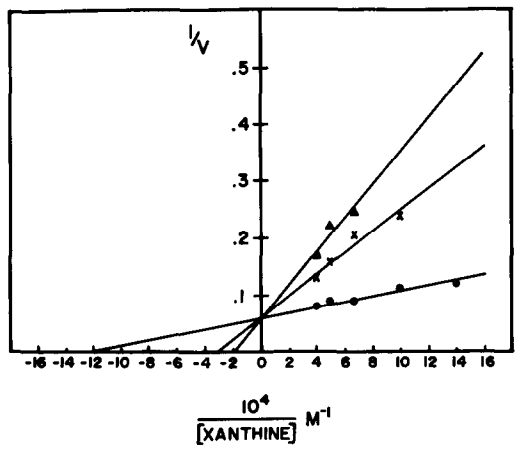

Fig. 3. Lineweaver–Burk plot for the inhibition of xanthine oxidase by folic acid. Xanthine oxidase, 15 μg (25.3 munits) of enzyme preparation protein, was combined with 100 nmoles xanthine, 80 μmoles EDTA-Na_2 and 40 μmoles potassium phosphate buffer (pH 7.4) in a volume of 1 ml with 0 (●), 0.9 (×) or 1.2 (▲) nmoles PGA, and reaction velocity was monitored at 23° at 295 nm using a 1 cm light path cuvette.

from each peak were analyzed by t.l.c. using MN300F cellulose plates with 3% (w/v) NH_4Cl solvent [21]. Material from peak I gave a single spot with $R_f = 0.32$, peak II a single spot with $R_f = 0.62$, and peak III a single spot with $R_f = 0.24$, consistent with pterin aldehyde, hydroxymethyl pterin and folic acid, respectively [6, 21]. The ultraviolet spectra of these compounds were determined and were consistent with data published previously [22].

The PGA was also chromatographed on MN300 ECTEOLA TLC plates [3% (v/v) NH_4OH] and DEAE-cellulose TLC plates [3% (v/v) NH_4OH]. In each instance, a single spot was observed with $R_f = 0.81$ and $R_f = 0.73$, respectively.

Inhibition of xanthine oxidase by folates. Xanthine oxidase was not inhibited significantly by H_2folate, H_4folate or N^5N^{10}-methyleneH_4folate at concentrations up to 100 μM in the presence or absence of 0.1 M ETSH. Folic acid (Figs. 3 and 4), as well as MTX, leucovorin and N^5-methylH_4folate, produced competitive inhibiton. The inhibition constants (K_i values) determined graphically from reciprocal plots are noted in Table 1. Methotrexate, leucovorin and N^5methylH_4folate were comparably weak inhibitors of the enzyme. PGA had a somewhat lower K_i of 0.12 μM. Determination of inhibition constants yielded comparable results using either xanthine oxidase assay method (Fig. 5) except in the case of N^5-methylH_4folate. Using this compound, nonlinear reaction rates were obtained with the cytochrome *c* reduction assay (see below). The pH curve of the inhibition of xanthine oxidase by folic acid demonstrated a broad plateau for degree of inhibition extending from pH 6.0 to 8.0. This is in contrast to uninhibited enzyme activity which rose steadily with increasing pH to pH 8.5 and then leveled off, a finding in agreement with previous reports [15].

The kinetic behavior of PGA-induced inhibition of xanthine oxidase was compared to that of pterin aldehyde. Preincubation of enzyme and PGA in the absence of xanthine for up to 60 min at 23° gave the same degree of inhibition as that obtained without preincubation with PGA concentrations from 0.05 to 0.4 μM. Preincubation of pterin aldehyde with enzyme resulted in an initial increase of inhibition

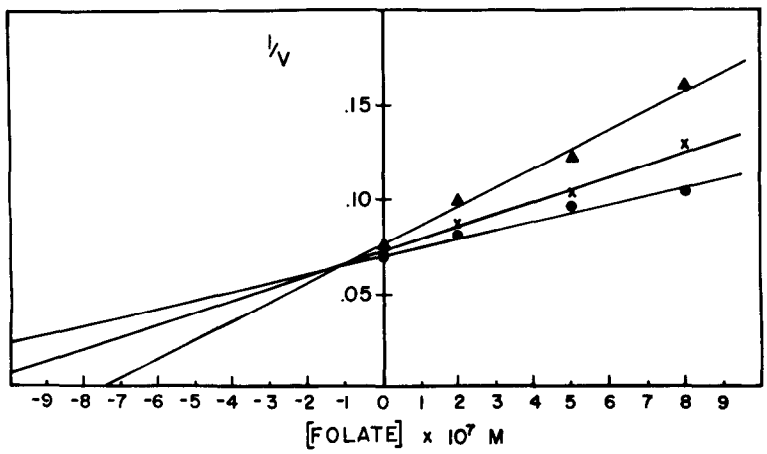

Fig. 4. Dixon plot for the inhibition of xanthine oxidase by folic acid. Amounts of xanthine included were 8 (▲), 16 (×) or 27 (●) nmoles. All other conditions were as in Fig. 3.

Table 1. Inhibition constants of folates for xanthine oxidase*

Folate derivative	K_i (μM)
Folic acid	0.12
5-FormylH_4folate	12
Methotrexate	25
5-MethylH_4folate	53
H_2folate	>100
H_4folate	>100
MethyleneH_4folate	>100

* K_i values were determined graphically from plots of $1/v$ vs [folate] as in Fig. 4. Enzyme activity was determined by measuring the change of optical density at 295 nm with time in the absence of cytochrome *c* with xanthine as substrate.

followed by a slower decrease as preincubation was prolonged beyond 10 min (Fig. 6).

Preincubation of PGA with DNPH was carried out exactly according to the methods of Kalckar *et al.* [5]. The PGA concentration was 8 μM and that of DNPH was 10 mM during preincubation. During enzyme assay the PGA concentration was 0.53 μM. No decrease of inhibition was observed when preincubation was carried out for 10–30 min at room temperature. In contrast, when pterin aldehyde (2 μM) was preincubated with the same amount of DNPH, inhibition of xanthine oxidase was blocked. This occurred whether or not the pterin aldehyde had been purified with ETSH-containing solutions.

Folates as substrates for xanthine oxidase. Folic acid, leucovorin, MTX and N^5-methylH_4folate were evaluated as substrates for xanthine oxidase using the cytochrome *c* reduction assay in the absence of xanthine. No activity was demonstrated at folate concentrations up to ten times the previously determined K_i values.

Since 2-amino-4-hydroxypteridine is known to undergo hydroxylation at the 7-position in the presence of xanthine oxidase [23, 24], new products were also sought using DEAE-Sephadex chromatography. Sixteen nmoles (10 μCi) of [^{3}H]PGA was incubated with 15 μg of enzyme protein preparation in 2.5 ml of 50 mM potassium phosphate buffer containing 0.1 mM EDTA (pH 7.4) for 30 min at 23°. An equal amount of additional [^{3}H]PGA was added and an aliquot was applied immediately to a DEAE-Sephadex column, according to the procedure outlined above. The only radioactive peak noted corresponded to that of unreacted folic acid. Similarly, incubation of enzyme with [^{3}H]PGA revealed no additional peaks of radiactivity by t.l.c. analysis.

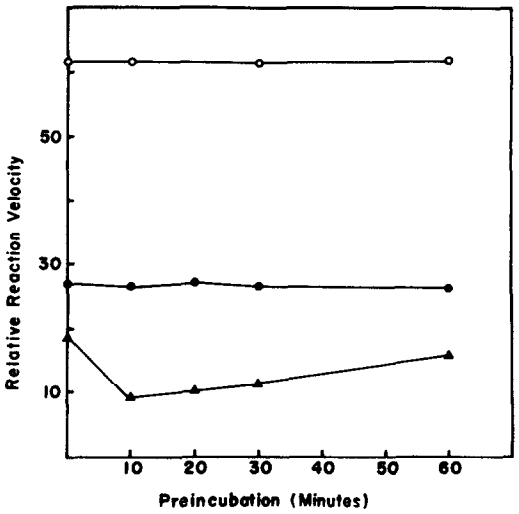

Fig. 6. Preincubation of xanthine oxidase with folic acid or pterin aldehyde. Xanthine oxidase, 15 μg (25.3 munits) of enzyme preparation protein, was preincubated at 23° with 0.8 nmoles PGA (●), 0.2 nmole pterin aldehyde (▲) or water(○) for varying periods of time with 40 μmoles potassium phosphate buffer (pH 7.4) and 80 nmoles EDTA-Na_2 in a volume of 825 μl. After the preincubation period, 100 nmoles xanthine and 90 nmoles cytochrome *c* were added in a volume of 175 μl, and reaction velocity was followed at 550 nm.

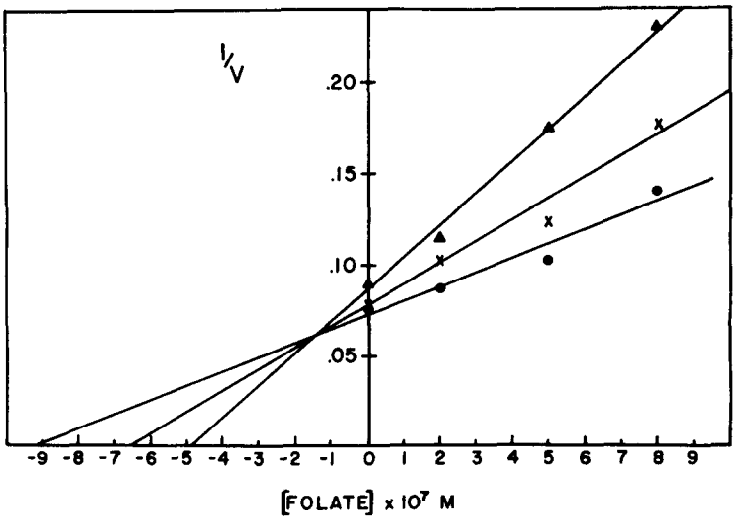

Fig. 5. Dixon plot for the inhibition of xanthine oxidase by folic acid. Xanthine oxidase, 15 μg (25.3 munits) of enzyme protein, was combined with folic acid and 10 (▲), 18 (×) or 25 (●) nmoles xanthine. All other conditions were as in Fig. 3 except that 90 nmoles of cytochrome *c* was included in the reaction mixture, and reaction velocity was measured by the change in optical density at 550 nm.

N^5-MethylH_4folate and cytochrome c. When N^5-methylH_4folate was evaluated in this study, high nonlinear blank rates were observed in its presence in the cytochrome *c* assay, but not in the uric acid production assay. This change in optical density at 550 nm occurred when N^5-methylH_4folate and cytochrome *c* were combined in the absence of either xanthine or xanthine oxidase, suggesting a nonenzymatic reaction. This reaction was not inhibited by 100 mM mannitol, 80 units/ml superoxide dismutase, 1 mM 2,5-dimethylfuran, 100 mM *tert*-butyl alcohol or 8.25 units/ml catalase [25]. The cytochrome *c* preparation (horse heart type VI) that was used migrated as a single band in Tris–glycine polyacrylamide gel electrophoresis [12].

Three milligrams (6.5 μmoles) N^5-methylH_4folate was combined with 1.33 μCi [5-^{14}C]-N^5-methylH_4folate and purified as above on a DEAE-Sephadex column [19]. The fractions containing the compound were pooled, lyophilized and redissolved in water. This material had an R_f of 0.80 in t.l.c., in agreement with literature values for N^5-methylH_4folate [23]. A solution of 0.54 μmole of purified N^5-methylH_4folate (0.2 mCi/mmole) and 0.54 μmole of cytochrome *c* in a total volume of 1 ml was incubated for 30 min at 23° and then applied to the DEAE-Sephadex column. The cytochrome *c* eluted immediately after the void volume in fractions 2–9, as monitored by optical density at 410 nm. The radioactivity eluted primarily in fractions 27–34 (Fig. 7, peak I). Radioactivity from an aliquot of fraction 30 migrated with an R_f of 0.88 in t.l.c., in close agreement with the value of 0.87 reported previously for N^5-methylH_2folate [23]. Radioactivity from an aliquot from fraction 44 (peak II) migrated with the same R_f as N^5-methylH_4folate. These experiments suggest that oxidation of N^5-methylH_4folate to N^5-methylH_2folate is stimulated by cytochrome *c*.

DISCUSSION

The discovery of allopurinol as a potent inhibitor of xanthine oxidase has made a dramatic impact on the clinical treatment of hyperuricemia. However, its usefulness has been limited by the development of allergic reactions, the lack of general availability of a parenteral form of the drug, and its reported possible myelosuppressive effects which appear to be independent of its capacity to inhibit xanthine oxidase [26, 27].

Allopurinol bears a close structural resemblance to the pteridine ring of folic acid. Indeed, 2-NH_2-4-OH-pteridine-6-aldehyde is one of the most potent known inhibitors of xanthine oxidase [2–5] and hydroxymethylpterin has been reported to be almost as potent an inhibitor [6]. When Kalckar *et al.* [5] proposed that PGA-induced inhibition of xanthine oxidase was, in fact, due to contamination with pterin aldehyde, they pointed out that: (1) the degree of inhibition varied with the purity of the preparation; (2) incubation of folic acid with 2,4-dinitrophenylhydrazine decreased (but did not abolish) inhibition; (3) preincubation of PGA and enzyme altered the amount of inhibition observed; and (4) butanol extraction of folate also decreased inhibition. However, the inhibitory activity of pure folic acid was not reported. Since folic acid is available in both oral and parenteral forms and appears to be relatively nontoxic, we explored the inhibitory potential of this compound for xanthine oxidase.

Buttermilk xanthine oxidase was used in these studies. It is available in relatively pure form and Bergmann and Dikstein [28] have demonstrated a close correlation of substrate specificities between milk enzyme and that of human liver. Boss and Ragsdale [29] have recently published data comparing the effects of PGA on bovine milk and human liver xanthine oxidase. Using xanthine as the sub-

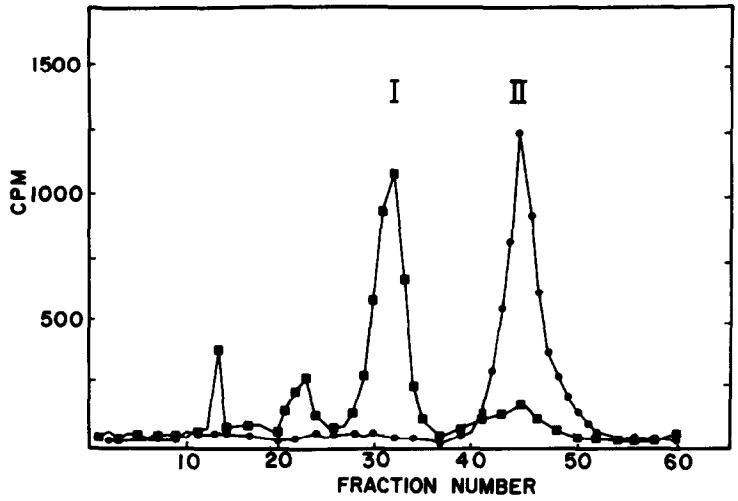

Fig. 7. Elution of N^5-methylH_4folate and its products of reaction with cytochrome *c*. N^5-MethylH_4folate, 3 mg (6.5 μmoles), was combined with 2 μCi [5-^{14}C]-N^5-methylH_4folate and eluted from a DEAE-Sephadex column as in Fig. 1 yielding a major peak in fractions 40–50 (●) (peak II). This material was pooled and lyophilized. Then 0.54 μmole [5-^{14}C]-N^5-methylH_4folate (0.3 mCi/mmole) was incubated with 0.54 μmole cytochrome *c* at 23° for 30 min in a volume of 1 ml containing 50 μmoles potassium phosphate buffer (pH 7.4) and 1 μmole EDTA-Na_2, after which an aliquot was reapplied to the column and eluted as previously, yielding a major peak in fractions 27–34 (■). Cytochrome *c* eluted in fractions 2–9, as monitored by optical density at 410 nm. Fractions contained 2.5 ml. Flow rate = 15 ml/hr.

strate, PGA yielded a K_i of 0.9 μM for the bovine milk enzyme and 0.8 μM for the human liver enzyme, suggesting that the two enzymes behave similarly toward PGA. However, they did not provide data on the source of PGA used or its purity. Studies similar to those presented in this paper, using human enzyme and highly purified PGA, will be necessary to confirm the similarity in behaviors of the human liver and buttermilk enzymes toward PGA.

Because the purity of the PGA that was used was critical to these studies, detailed analyses of the purity of the PGA were carried out. No pterin-6-aldehyde could be detected by t.l.c. or ultraviolet spectral analysis. The folic acid preparation was purified on a DEAE-Sephadex column by a method demonstrated to separate PGA from pterin aldehyde and hydroxymethylpterin (Figs. 1 and 2). After incubation of [^{3}H]PGA with enzyme, no new peaks of radioactivity were noted, suggesting that neither the aldehyde nor hydroxymethylpterin was produced during incubation of [^{3}H]PGA with enzyme. The kinetic behavior of PGA was that of a freely reversible competitive inhibitor of xanthine oxidase (Figs. 3–5), whereas Lowry *et al.* [2] have shown that pterin aldehyde is such a tight-binding inhibitor of xanthine oxidase that its inhibition does not conform to simple Michaelis–Menten kinetics. In addition, we have demonstrated that preincubation of folic acid with xanthine oxidase in the absence of substrate does not affect the degree of inhibition, whereas preincubation of pterin aldehyde with enzyme results in an initial increase of inhibition followed by a slow decline (Fig. 6). The early increase of inhibition is consistent with the analyses of Cha *et al.* [30, 31] demonstrating that tight-binding inhibitors often have relatively slow association rates. The gradual decrease of inhibition was attributed previously to oxidation of pterin aldehyde to less active inhibitors by xanthine oxidase [5].

Finally, inhibition of xanthine oxidase by folic acid was not affected by preincubation with 2,4-dinitrophenylhydrazine, while pterin aldehyde-induced inhibition was blocked by identical conditions. Therefore, by chemical analysis, purification, and kinetic behavior, the inhibition of xanthine oxidase by folic acid was shown to be distinct from that by pterin aldehyde and hydroxymethylpterin.

In man, the major form of folate both in serum and intracellularly in liver and red blood cells is N^5-methylH_4folate [32]. Previous investigators have estimated total liver folate content to be approximately 20 μmoles/kg [33], with much of the folate existing as polyglutamates. However, Stea *et al.* [6] have shown that a variety of malignant cells in tissue culture metabolize folic acid to 6-hydroxymethyl pterin, possibly via production of pterin aldehyde [2, 3]. It would be of interest, therefore, to examine human liver for the possible production of these metabolites after the administration of pharmacologic doses of folic acid.

The K_i of MTX for xanthine oxidase is 25 μM. Many investigators have shown that levels in excess of this are reached in humans during 'high-dose MTX' therapy with leucovorin [9] and thymidine [34] 'rescue'. However, this is a transient state and unlikely to be of clinical significance.

Despite earlier studies demonstrating the 7-hydroxylation of 2-NH_2-4-OH-pteridine by xanthine oxidase [23, 24], we were unable to show enzymatic oxidation of any of the folates tested. However, cytochrome *c* is reduced in the presence of N^5-methylH_4folate, extending the observation of Donaldson and Keresztesy [35], who hypothesized the oxidation of N^5-methylH_4folate to N^5-methylH_2folate to explain the alteration of the capacity of N^5-methylH_4folate to support methyleneH_4folate reductase activity after exposure to a variety of oxidizing agents including cytochrome *c*. The behavior of the product of N^5-methylH_4folate and cytochrome *c* in t.l.c. confirms its identity as N^5-methylH_2folate [21]. The failure of scavengers of superoxide anion, hydrogen peroxide, singlet oxygen and hydroxyl radicals to inhibit this reaction makes their participation in the oxidation of N^5-methylH_4folate unlikely [25].

Further evaluation of folic acid and its analogues as clinically useful inhibitors of xanthine oxidase is needed. Of special interest would be the behavior of folates with reduced xanthine oxidase. Many of the folates evaluated in the present study, as well as a variety of other compounds already reported, exhibit K_i values in the range of 10–1000 nM under the conditions used here [15, 36]. However, allopurinol, the clinically used xanthine oxidase inhibitor, is converted *in vivo* to oxypurinol which has an extremely high affinity for reduced xanthine oxidase, producing 50 per cent inhibition of enzyme activity at drug concentrations of approximately 0.5 nM [37, 38]. Agents which would maintain this enzyme preparation in the reduced state under aerobic conditions, such as dithionite, however, would also reduce folic acid. While Boss and Ragsdale [29] have reported that H_2folate also inhibits oxidized xanthine oxidase, we have been unable to confirm this observation. Therefore, we have not yet examined the inhibition of reduced xanthine oxidase by PGA.

Unless extremely high intracellular concentrations of unchanged folic acid or pterin aldehydes can be achieved, or folates prove to be more effective as inhibitors of reduced rather than oxidized xanthine oxidase, it seems unlikely that folic acid will be an effective clinical agent for inhibiting xanthine oxidase.

Acknowledgement—The author wishes to acknowledge the expert technical assistance of Ms. Eileen Jurista.

REFERENCES

1. H. M. Kalckar and H. Klenow, *J. biol. Chem.* **172**, 349 (1948).
2. O. H. Lowry, O. A. Bessey and E. J. Crawford, *J. biol. Chem.* **180**, 389 (1949).
3. O. H. Lowry, O. A. Bessey and E. J. Crawford, *J. biol. Chem.* **180**, 399 (1949).
4. H. M. Kalckar, N. O. Kjeldgaard and H. Klenow, *J. biol. Chem.* **174**, 771 (1948).
5. H. M. Kalckar, N. O. Kjeldgaard and H. Klenow, *Biochim. biophys. Acta* **5**, 586 (1950).
6. B. Stea, P. S. Baklund, P. B. Berkey, A. K. Cho, B. C. Halpern, R. M. Halpern and R. A. Smith, *Cancer Res.* **38**, 2378 (1978).
7. K. A. Oster, *Ann. intern. Med.* **86**, 367 (1977).

8. B. Plouvier and B. Devulder, *Ann. intern. Med.* **88**, 269 (1978).
9. R. G. Stoller, S. A. Jacobs, J. C. Drake, R. J. Lutz and B. A. Chabner, *Cancer Chemother. Rep.* **6** (part 3), 19 (1975).
10. E. L. R. Stokstad and J. Koch, *Physiol. Rev.* **47**, 83 (1967).
11. H. G. Kaplan and C. E. Myers, *J. Pharmac. exp. Ther.* **201**, 554 (1977).
12. K. Weber and M. Osborn, *J. biol. Chem.* **224**, 4406 (1969).
13. I. Fridovich, *J. biol. Chem.* **237**, 584 (1962).
14. J. M. McCord and I. Fridovich, *J. biol. Chem.* **234**, 5753 (1968).
15. R. C. Bray, in *The Enzymes* (Ed. P. D. Boyer), Vol. 12, pp. 299–419. Academic Press, New York (1975).
16. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Boothe and J. R. Bertino, *Biochim. biophys. Acta* **105**, 380 (1965).
17. T. A. Krenitsky, S. M. Neil, G. B. Elion and G. H. Hitchings, *Archs Biochem. Biophys.* **150**, 585 (1972).
18. K. V. Rajagoplan and P. Handler, *J. biol. Chem.* **239**, 2022 (1964).
19. P. F. Nixon and J. R. Bertino, *Analyt. Biochem.* **43**, 162 (1971).
20. R. L. Blakley, *The Biochemistry of Folic Acid and Related Pteridines*, p. 92. North-Holland, Amsterdam (1969).
21. J. P. Brown, G. E. Davidson and J. M. Scott, *J. Chromat.* **79**, 195 (1973).
22. C. W. Waller, A. A. Goldman, R. B. Angier, J. H. Boothe, B. L. Hutchings, J. H. Mowat and J. Semb, *J. Am. chem. Soc.* **72**, 4630 (1950).
23. C. N. Hodnett, J. J. McCormack and J. A. Sabean, *J. pharm. Sci.* **65**, 1150 (1976).
24. D. M. Valerino and J. J. McCormack, *Biochim. biophys. Acta* **184**, 154 (1969).
25. E. W. Kellogg and I. Fridovich, *J. biol. Chem.* **250**, 8812 (1975).
26. Boston Collaborative Drug Surveillance Program, *J. Am. med. Ass.* **227**, 1036 (1974).
27. M. S. Greenberg and S. S. Zambrano, *Arthritis Rheum.* **15**, 413 (1972).
28. F. Bergmann and S. Dikstein, *J. biol. Chem.* **223**, 765 (1956).
29. G. R. Boss and R. A. Ragsdale, *Fedn Proc.* **37**, 1541 (1978).
30. S. Cha, *Biochem. Pharmac.* **24**, 2177 (1975).
31. S. Cha, R. P. Agarwal and R. E. Parks, *Biochem. Pharmac.* **24**, 2186 (1975).
32. R. Y. Pratt and B. A. Cooper, *J. clin. Invest.* **50**, 455 (1971).
33. I. Chanarin, M. Hutchinson, A. McLean and M. Moule, *Br. med. J.* **1**, 396 (1966).
34. W. D. Ensminger and E. Frei, *Cancer Res.* **37**, 1857 (1977).
35. K. O. Donaldson and J. C. Keresztesy, *J. biol. Chem.* **237**, 3815 (1962).
36. K. Hande, E. Reed and B. Chabner, *Clin. Pharmac. Ther.* **23**, 598 (1978).
37. T. Spector and D. G. Johns, *J. biol. Chem.* **245**, 5079 (1970).
38. T. Spector, *Biochem. Pharmac.* **26**, 355 (1977).