ing with stirring was continued for 1.5 hours. A major portion of the solvent was then removed by distillation under reduced pressure. Four and three-tenths grams (80%) of white solid, m.p. 200–216° dec., crystallized from the cooled solution. Recrystallization of the product from ethanol gave 3.9 g. (68%) of small, flat, white crystals, m.p. 216–217°.

Anal. Calcd. for $C_{14}H_{11}NO_3S$: N, 5.13. Found: N, 5.18.

The dioxide was also prepared in 30% yield by hydrogen peroxide oxidation of 10-acetylphenothiazine-5-oxide in glacial acetic acid.

Hydrolysis of 10-Acetylphenothiazine-5-dioxide.—One and one-half milliliters of 10% sodium hydroxide was added to a hot solution of 0.50 g. (0.0018 mole) of the dioxide in 30 ml. of absolute ethanol. The color of the solution immediately became yellow. After a few minutes, part of the solvent was removed by distillation. The addition of water to the residual solution precipitated 0.42 g. (100%) of yellow solid, m.p. 255–257° dec. The mixed melting point with an authentic sample of phenothiazine-5-dioxide[19] was undepressed.

The other 10-acylphenothiazine-5-dioxides were hydrolyzed, in a similar fashion, to give phenothiazine-5-dioxide.

10-Methylphenothiazine.[4]—Fifteen milliliters of dimethyl sulfate was added with stirring to a mixture of 10 g. (0.05 mole) of phenothiazine, dissolved in 100 ml. of dioxane, and 50 g. of anhydrous potassium carbonate. The color of the mixture immediately turned brown and soon after heating to reflux, the color became yellow. After 3.5 hours of refluxing with stirring, another 10 ml. of dimethyl sulfate was added. The mixture was refluxed for a total of 24 hours. It was carefully poured into about 400 ml. of warm water, and after standing overnight, 10.5 g. of tan solid, m.p. 75–80°, was filtered off. After extracting this solid with hot ethanol, a tar remained. From the ethanol extract there crystallized 4.3 g. (40%) of light yellow needles, m.p. 91–94°. This solid was recrystallized from 95% ethanol giving 2.8 g. (26%) of yellow needles, m.p. 99–100°.

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Ion-exchange Chromatography of Pteroylglutamic Acid and Aminopterin[1]

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A separation of pteroylglutamic acid (PGA, folic acid) and 4-amino-PGA (Aminopterin) has been effected on columns of the anion exchanger Dowex-1. The procedure is of use in the analysis and purification of these compounds.

A "standard" PGA sample showed a single peak upon chromatography, and thus appeared to be pure (Fig. 1). The three samples of Aminopterin tested, however, contained approximately 20% of an impurity with the elution properties of PGA (Fig. 2). Total recoveries in the Aminopterin runs, based on optical density, were 80–90%, probably indicating further impurity which is not eluted.

In order to further characterize the impurity in the Aminopterin, some of the fractions from the second peak (Fig. 2) were combined, neutralized with sodium hydroxide, and evaporated to dryness in a vacuum desiccator. Paper chromatography of this material showed it to have the properties of PGA. Samples from both chromatographic peaks were also tested with *Tetrahymena pyriformis* (*geleii*). Material from the major (Aminopterin) peak gave neither inhibition nor stimulation of growth, contrary to the earlier report of 17% activity of (impure) Aminopterin for growth.[2] This confirms the complete separation of the two components, as shown in the ion-exchange chromatogram. Material from the minor (PGA) peak gave the growth stimulation with *Tetrahymena* expected from the PGA calculated to be present.

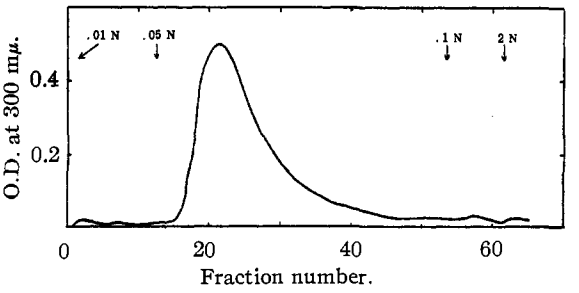

Fig. 1.—Pteroylglutamic acid (2.5 mg.) on column of Dowex-1-chloride 56 × 9 mm.: eluted at 0.6 ml./min. with hydrochloric acid of concentrations shown; 30-min. fractions, 93% recovery.

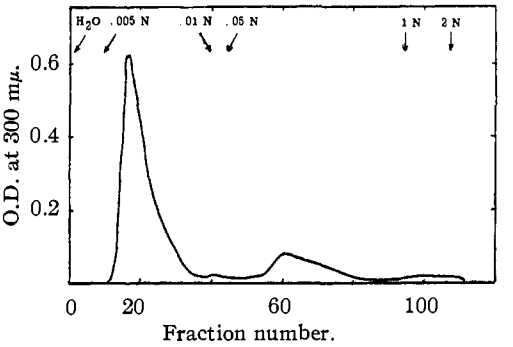

Fig. 2.—Aminopterin (2.5 mg.) on column of Dowex-1-chloride 50 × 9 mm.: eluted at 0.33 ml./min. with hydrochloric acid of concentrations shown; 45-min. fractions, the second peak is PGA.

PGA is not produced by deamination of Aminopterin during the separation, as shown by rechromatographing the pure Aminopterin fractions.

These studies confirm previous reports of the presence of PGA in Aminopterin,[3-5] and present a procedure for the purification of these compounds. Work is continuing on these and related materials.

The authors are grateful to the Lederle Laboratories for supplies of the compounds used.

Experimental

Columns of approximately 55 × 9 mm. were prepared from Dowex-1-chloride (200–400 mesh[6]) in the usual manner.[7] Solutions of PGA or analog were prepared in water, at a concentration of 0.5 mg./ml., neutralized to *p*H 7 with sodium hydroxide. Immediately before adsorption on the column, this solution was brought to *p*H 8–9 with ammonium hydroxide. Dilute hydrochloric acid was used for elution, 0.005 *N* for Aminopterin and 0.05 *N* for PGA in

(1) Supported in part by Contract No. AT(30-1)-1351 with the U. S. Atomic Energy Commission.

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(4) D. J. Hutchison and J. H. Burchenal, *Proc. Am. Assoc. Cancer Res.*, **1**, 26 (1953).

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(6) Kindly supplied by the Dow Chemical Company.

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these studies. The column and fraction collector were covered to exclude light; other operations were shielded insofar as possible. The optical density of samples at 300 mμ was determined in a Beckman DU spectrophotometer. Samples to be used later were neutralized to prevent decomposition. After each run the column was washed with about 200 ml. of 2 *N* hydrochloric acid, followed by water. The capacity of these columns for pterins has not been accurately determined, but it is much lower than their capacity for purines and pyrimidines.

Paper chromatography was carried out on strips of Whatman no. 1 paper in 1% aqueous dipotassium phosphate.[8]

To determine whether or not PGA was produced by deamination of Aminopterin during the ion-exchange procedure, combined fractions from the Aminopterin peak (285 ml.) were made alkaline with ammonium hydroxide and readsorbed by running through the column by gravity, in the dark. This required 28 hr. at room temperature. Elution in the same manner used previously gave only an Aminopterin peak, with a recovery of about 95% and no evidence of PGA.

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The Oxidation of Dialuric Acid by *o*-Iodosobenzoic Acid[1]

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The kinetics of oxidation of dialuric acid by oxygen has been studied by Hill[3] and by Richardson.[4] The dissociation constants and oxidation–reduction potentials of dialuric acid and ascorbic acid are of similar magnitude. Tautomeric formation of an enediol configuration is conceivable. In addition, the half-life of oxidized dialuric acid (alloxan)[5] is practically the same as the half-life of dehydroascorbic acid.[6] These similarities in properties suggested a study of the rate of oxidation of dialuric acid by *o*-iodosobenzoic acid to supplement work previously reported for ascorbic acid.[7]

Experimental

Dialuric acid was prepared by the method of Biltz and Damm.[8] General procedures for the preparation of reaction mixtures were the same as those previously described by us for the studies of oxidation of ascorbic acid. The rate of the reaction was followed by the spectrophotometric method using spirocyclohexylporphyrexide.[7,9] Solutions of dialuric acid were found to be too susceptible to oxidation by oxygen to permit use of the titrimetric procedure that utilizes porphyrindine.

Results and Discussion

Dialuric acid (DA) was found to be oxidized by *o*-iodosobenzoic acid (RIO) in a second-order process expressed by the differential equation

$$-\mathrm{d(DA)/dt} = k''\mathrm{(DA)(RIO)} \qquad (1)$$

The integrated form of equation 1 was used to evaluate k'' from the experimental data. Typical results are shown in Table I.

TABLE I

OXIDATION OF DIALURIC ACID BY *o*-IODOSOBENZOIC ACID

Temperature 15.0°

No.	Buffer	*p*H	(DA) × 10⁴, moles per l.	(RIO) × 10⁴, moles per l.	k'', l. mole^{-1} min.$^{-1}$
1	Phosphate, 0.10 *M*	7.05	6.91	9.43	47
2	Phosphate, .10 *M*	7.05	6.99	5.66	49
3	Phosphate, .10 *M*	7.05	13.69	9.43	46
4	Phosphate, .10 *M*	7.05	7.03	9.43	48
5[a]	Phosphate, .60 *M*	6.97	6.97	9.43	104
6[b]	Phosphate, .10 *M*	6.69	6.84	9.43	53
7	Phosphate, .10 *M*	6.04	6.91	9.43	47
8	Phosphate, .10 *M*	7.68	6.83	9.43	24
9[c]	Phosphate, .10 *M*	7.05	6.91	9.43	96
10[d]	Phosphate, .10 *M*	7.05	6.96	9.43	61
11	Veronal, 0.05 *M*	6.96	7.02	9.43	19
12[e]	Phosphate, 0.10 *M*	7.05	6.99	13.58	116

[a] Ionic strength, 1.44. [b] Ionic strength, 1.42 by addition of KCl. [c] $FeSO_4$, 1×10^{-6} *M*. [d] $CuSO_4$, 1×10^{-6} *M*. [e] Temperature, 25.0°.

In expt. 1–3, the concentrations of dialuric acid and *o*-iodosobenzoic acid were varied independently over a limited range owing to the low solubility of *o*-iodosobenzoic acid. The agreement of the values for k'' indicates that the reaction is first order with respect to each of the reactants. The value of k'' increased from 48 to 104 as the concentration of phosphate buffer was increased from 0.10 to 0.60 *M* (expt. 4 and 5). This increase in rate is not associated primarily with an increase in ionic strength since addition of potassium chloride to produce an equivalent ionic strength had no significant effect on the rate (expt. 6). In phosphate buffer, the rates were similar at *p*H 6 and 7 but decreased at *p*H 7.7 (expt. 7 and 8). In veronal buffer at *p*H 7 (expt. 11) the rate was much less than in phosphate buffer. No experiments were conducted in nonbuffered solutions. Iron was a more effective catalyst than copper (expt. 9 and 10). In 0.10 *M* phosphate buffer at *p*H 7.05, k'' increased from 48 to 116 liters mole^{-1} min.$^{-1}$ as the temperature was increased from 15 to 25° (expt. 12).

Analyses of solutions after reactions were completed indicated that, under all conditions studied, one mole of *o*-iodosobenzoic acid had been reduced for each mole of dialuric acid oxidized. There was no evidence of formation of any products from alloxan capable of reducing either iodine or *o*-iodosobenzoic acid.

These preliminary results suggest that the oxidation of dialuric acid by *o*-iodosobenzoic acid is similar in some respects to the oxidation of ascorbic acid. The principal reactions are second order; the rate is proportional to the concentration of buffer but is independent of the ionic strength; both reactions exhibit catalysis by copper and iron.

Points of difference also may be noted. Under the same conditions, dialuric acid is oxidized at a rate fifteen times that of ascorbic acid. No reducing substance is formed from oxidized dialuric acid. The catalytic effect of copper is much greater than

(1) One of several investigations supported in part by a research grant from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

(2) Predoctorate Research Fellow of the National Institutes of Health, 1949–1950.

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