denced by solution of part of the dibromide, the cooling bath was raised during the remainder of the addition. At the end, the ice-bath was removed and the mixture, which soon set to a heavy paste of white solid,[15] was stirred for 15 min. longer. Then 50 cc. of water was added to dissolve the zinc salt and the ethereal solution was decanted into a separatory funnel and washed with 400 cc. of water containing 25 cc. of 36% hydrochloric acid. After three more washings with 400 cc. of water, the solution was shaken with 300 cc. of water and 150 cc. of 25% sodium hydroxide solution and the ether layer tested to make sure it contained no trace of acetic acid (which readily acetylates the sterol during evaporation). The solution was then dried, evaporated to about 600 cc., 600 cc. of methanol was added, and the solution boiled down to the point of incipient crystallization (about 1 l.). After cooling (4°), the main crop of purified cholesterol was collected and dried: 108.4 g., m.p. 149.5–150°; a second crop of 8.4 g., m.p. 148–149°, was obtained after evaporation to a volume of 250 cc.; total yield 116.8 g. (93%). The residual mother liquor afforded about 4 g. of material containing bromine not removed by repetition of the treatment with zinc dust. If air-dried dibromide is used, 25 cc. of acetic acid should be added to the ethereal suspension before addition of zinc.

5α,6β-Dibromocholestane-3-one.—The moist dibromide from 150 g. of cholesterol was suspended in 2 l. of acetic acid in a 5-l. flask equipped with a stirrer and mounted over a bucket of ice and water that could later be raised, and a solution, preheated to 90°, of 80 g. of sodium dichromate dihydrate in 2 l. of acetic acid was poured into the stirred suspension (at 25°). The temperature of the mixture reached 55–58° during the oxidation and the solid all dissolved in 3–4 min. After another 2 min. the ice bucket was raised so that the flask was completely immersed and the stirrer was stopped for 10 min. to allow the dibromoketone to separate in easily filterable crystals. With stirring resumed, the temperature was brought to 25° and then, after addition of 400 cc. of water, to 15°. The product was collected, washed with methanol until the filtrate was colorless (500–600 cc.) and the white crystals, m.p. 73–75° dec., $[\alpha]^{25}D$ −46.8° Chf (*c* 2.11) were either used while still moist or dried in a dark cupboard at room temperature; yield 170.9 g. (96.5% in the oxidation, 81% from cholesterol). Butenandt[11] and Inhoffen[12] report m.p. (dec.) 80° and 68–69°.

Δ⁵-Cholestene-3-one.—The methanol-moist dibromocholestanone from 150 g. of cholesterol was covered with 2 l. of ether, 25 cc. of acetic acid was added, and the mixture stirred mechanically in an ice-bath and the temperature lowered to 15°. Then 40 g. of fresh zinc dust was added in portions in the course of 5 min. with maintenance of a temperature of 15–20° by cooling. When the exothermic reaction was over, the ice-bath was removed and stirring continued for 10 min. Then 70 cc. of pyridine was added and the resulting suspension of white complex stirred briefly; the solution was then filtered by suction and the filter cake washed well with ether. The colorless filtrate was washed three times with water and once with 600 cc. of 5% bicarbonate solution (to remove a trace of acetic acid), dried, and evaporated to a volume of 1 l. After addition of 500 cc. of methanol, evaporation was continued to a volume of 1.2 l. and the product let crystallize. It separated in large, pure white prisms, m.p. 126–129° (camphor-like), $[\alpha]D$ −2.5° Chf (*c* 2.03), no selective absorption at 242 mμ. The yield in the first crop was 87–94 g., and concentration of the mother liquor afforded 12–19 g. more of colorless material melting in the range 118–124° and suitable for conversion to the conjugated ketone; total yield 106 g. (88%, 71% from cholesterol).

Debromination with zinc and ethanol according to Butenandt and Schmidt-Thomé[11] when conducted on the same scale as above afforded crude Δ⁵-cholestene-3-one in 81–85% yield, but the material melted at 116–120°. Chromatography of the ethanolic mother liquor afforded Δ⁴-cholestene-3-one, Δ⁴-cholestene-3,6-dione and Δ⁴-cholestene-6β-ol-3-one; the last two products must be derived from cholesterol formed from the dibromide during the oxidation.

Δ⁴-Cholestene-3-one.—One hundred grams of Δ⁵-cholestene-3-one and 10 g. of anhydrous oxalic acid were dissolved in 800 cc. of 95% ethanol and the colorless solution was warmed for 10 min. on the steam-bath and then let cool to room temperature and seeded. The main crop of conjugated ketone (91.1 g.) separated as large, colorless prismatic needles, m.p. 81–82°, $[\alpha]D$ +92.0° Chf (*c* 2.01), λ^{EtOH} 242 mμ (17,000); constants reported[13] for material purified by chromatography are: m.p. 81–82°, λ^{EtOH} 240.5 mμ (18,000). Further crops were obtained first by concentration of the mother liquor and then by dilution with water, and these on recrystallization gave 6.8 g. of colorless product, m.p. 81–82°; total yield 97.9% (69% from cholesterol).

Isomerization of Δ⁵-cholestene-3-one in ethanol with either hydrochloric acid or sodium hydroxide (followed by neutralization of the yellow enolate solution with acetic acid) proved unsatisfactory on a large scale since a permanent yellow color developed and the first-crop material was yellowish and melted at 78–80°.

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(15) This solid, m.p. about 170° dec., contains zinc and affords cholesterol on crystallization from methanol or acetone; it was not obtained in a form suitable for analysis.

Oxidation of 10-Acyl- and 10-Alkylphenothiazines

BY HENRY GILMAN AND R. DAVID NELSON

RECEIVED MAY 9, 1953

A number of acid chlorides react readily with phenothiazine in pyridine to give 10-substituted phenothiazines.[1] Later reports[2] have shown that phenothiazine, and some of its nuclearly substituted derivatives, react with various haloacyl halides when heated in refluxing benzene or toluene to give the corresponding 10-haloacylphenothiazines in good yield. In this work, the 10-acylphenothiazines (Table I) were prepared by allowing the acid chloride to react with phenothiazine in the presence of dioxane and sodium carbonate.

A variety of oxidizing agents has been used to oxidize the sulfur of a number of phenothiazine derivatives to the sulfoxide or the sulfone. Those which oxidized the sulfur to the sulfoxide were potassium permanganate,[3,4] 30% hydrogen peroxide in ethanol,[5] sodium nitrite[6] and nitric acid.[6] In the latter case nitration also resulted. The sulfur has been oxidized to the sulfone by potassium permanganate,[4] 30% hydrogen peroxide in glacial acetic acid[7,8] and hypochlorous acid.[8] This note includes additional studies made on the oxidation of the sulfur of the phenothiazine nucleus.

The reaction of concentrated nitric acid with 10-chloroacetylphenothiazine in glacial acetic acid gave 3-nitrophenothiazine-5-oxide (I) and 10-chloroacetylphenothiazine-5-oxide but apparently no 3-nitro derivative of the latter compound. It appears that the acyl derivative was first oxidized by nitric acid to give the monoxide and that this reaction was then followed by hydrolysis and nitration resulting in the formation of I. Previous reactions using nitric acid and phenothiazine, or some of its derivatives, gave the corresponding

(1) S. E. Hazlet and C. E. Roderuck, THIS JOURNAL, **67**, 495 (1945).

(2) R. Dahlbom and T. Ekstrand, *Acta Chem. Scand.*, **5**, 102 (1951); T. Ekstrand, Swedish Patent 127,566 [*C. A.*, **45**, 1886 (1951)].

(3) E. DeB. Barnett and S. Smiles, *J. Chem. Soc.*, **97**, 188 (1910).

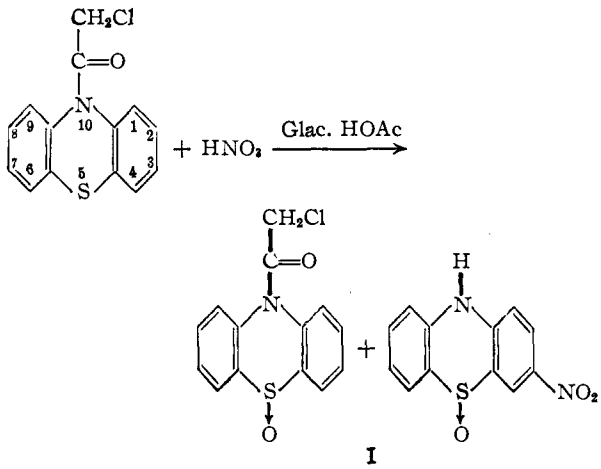
(4) H. I. Bernstein and L. R. Rothstein, THIS JOURNAL, **66**, 188 (1944).

(5) H. Gilman and D. A. Shirley, *ibid.*, **66**, 888 (1944); D. F. Houston, E. B. Kester and F. DeEds, *ibid.*, **71**, 3819 (1949).

(6) F. Kehrmann and P. Zybs, *Ber.*, **52B**, 130 (1919).

(7) N. L. Smith, *J. Org. Chem.*, **16**, 415 (1951).

(8) J. G. Michels and E. D. Amstutz, THIS JOURNAL, **72**, 888 (1950).



I

nitromonoxides[5,6,9]; consequently, both oxidation and nitration had occurred. One exception involved a tetrachlorophenothiazine.[10] In that case only oxidation of the sulfur was observed, doubtless because the chlorine atoms occupied the positions normally affected by nitration reactions. The nitric acid oxidation was extended to other 10-acylphenothiazines.

In a second experiment, 10-chloroacetylphenothiazine gave an 86% yield of the monoxide and very little, if any, I (Table II). Under similar conditions, 10-phenacetylphenothiazine gave 50% of the oxide; 10-acetylphenothiazine gave a 19% yield of 10-acetylphenothiazine-5-oxide, as well as some I; and the 10-dichloroacetyl derivative was unaffected as evidenced by the almost quantitative recovery of starting material. Thus, it is evident that the group attached to the nitrogen of phenothiazine affects the success of the oxidation of the sulfur. This observation was made also by Bernstein and Rothstein.[4] They found that 10-ethylphenothiazine was converted to the sulfone and that 10-(*p*-toluenesulfonyl)-phenothiazine was oxidized to the monoxide by potassium permanganate in boiling water, whereas the *p*-acetamidobenzenesulfonyl derivative did not undergo reaction. Neither of the latter two derivatives was oxidized by hydrogen peroxide in acetone.

Pummerer and Gassner[11] reported the formation of phenothiazine-5-oxide in 75% yield by the oxidizing action of 30% hydrogen peroxide on phenothiazine dissolved in hot ethanol containing some potassium hydroxide. An attempt to repeat the reaction was unsuccessful. However, by carrying out the reaction in the absence of potassium hydroxide, phenothiazine-5-oxide was obtained in an almost quantitative yield.

A number of 10-acylphenothiazine-5-oxides was prepared by the action of excess 30% hydrogen peroxide on the acyl derivative in refluxing ethanol. There was no indication that any dioxide had been formed. That alkali could not have been used to catalyze the foregoing reactions was shown by the rapid hydrolysis of the acyl group upon addition of 10% sodium hydroxide to a hot ethanolic solution of the 10-acylphenothiazine-5-oxide.

(9) A. Bernthsen, *Ann.*, **230**, 73 (1885).
(10) O. Unger and K. A. Hofmann, *Ber.*, **29**, 1362 (1896).
(11) R. Pummerer and S. Gassner, *ibid.*, **46**, 2322 (1913).

An exception to the above reactions was the oxidation of 10-ethylphenothiazine by hydrogen peroxide in refluxing ethanol. Two products were isolated from the reaction mixture: the monoxide in 62% yield and the dioxide in 15.5% yield. The rather large excess of hydrogen peroxide used might account for the fact that the dioxide was formed. Early reports[12] stated that the oxidation of sulfides by excess 30% hydrogen peroxide in acetone or aqueous solutions at ordinary temperatures gave only the sulfoxides. These conditions are not strictly comparable to those used for the production of sulfoxides in this investigation. Thus, the observations of Smiles and Hinsberg do not preclude the formation of small amounts of the dioxide by carrying out the oxidation reaction in refluxing ethanol solution.

10-Methyl- and 10-ethylphenothiazine, as well as various 10-acyl derivatives, were oxidized to the corresponding dioxides (Table III), in fair to excellent yields, by means of excess 30% hydrogen peroxide in glacial acetic acid. In addition, a number of the monoxide derivatives was oxidized to the respective dioxides by the same procedure.

One method of forming peracetic acid[13] is by heating a mixture of 30% hydrogen peroxide and glacial acetic acid. Since hydrogen peroxide in ethanol oxidized the sulfur of the phenothiazine derivatives to the sulfoxide, and the use of hydrogen peroxide in acetic acid resulted in the oxidation of the sulfur to the sulfone, it seems possible that in the latter reaction peracetic acid would be formed *in situ* and thus behave as the oxidizing agent. This very likely might account for the difference in degree of oxidation upon using ethanol or glacial acetic acid as the solvent.

Potassium amide reacts readily with aryl halides in liquid ammonia at −33°.[14] In view of this fact and also of the fact that 10-sodiophenothiazine reacts readily with ethyl bromide in liquid ammonia,[15] the reaction of 10-sodiophenothiazine with iodobenzene was tried. Under corresponding conditions the reaction was found to be unsuccessful. Thus, it seems that the amide ion is a stronger attacking agent than the 10-phenothiazyl ion in the nucleophilic displacement reaction on carbon.

Methylation of 3-methylphenothiazine with dimethyl sulfate in acetone containing sodium hydroxide was successful.[16] It was found that phenothiazine could also be methylated by dimethyl sulfate.

The authors are grateful to Parke, Davis and Company for arranging for the testing of some of the compounds. The results of these tests will be reported elsewhere.

Experimental

10-Acetylphenothiazine.[17]—This example is typical of procedures used to prepare three other acyl derivatives which are described in Table I.

(12) M. Gazdar and S. Smiles, *J. Chem. Soc.*, **93**, 1833 (1908); O. Hinsberg, *Ber.*, **41**, 2836 (1908).
(13) D. Swern, *Chem. Revs.*, **45**, 1 (1949).
(14) F. W. Bergstrom and W. C. Fernelius, *ibid.*, **12**, 98 (1933).
(15) H. Gilman, R. D. Nelson and J. F. Champaigne, Jr., THIS JOURNAL, **74**, 4205 (1952).
(16) Ng. Ph. Buu-Hoï and Ng. Hoán, *J. Chem. Soc.*, 1834 (1951).
(17) The compound was also prepared in 99% yield by refluxing a solution of phenothiazine in acetic anhydride. See reference 9.

TABLE I

10-ACYLPHENOTHIAZINES

Acyl group	Recrystallizing solvent	M.p., °C.	Yield, %	Formula	Nitrogen, % Calcd.	Found
$COCH_2Cl$[a]	Benzene–pet. ether (b. 60–70°)	113.5–114.5	45	$C_{14}H_{10}ClNOS$	...	.....
$COCHCl_2$[b]	Ethanol	149–151	23	$C_{14}H_9Cl_2NOS$	4.52[c]	4.87, 4.66
$COCH_2C_6H_5$	Benzene	152–153	66	$C_{20}H_{15}NOS$	4.46	4.43

[a] Reference 2. [b] On using dichloroacetic acid anhydride a 32% yield of the product, m.p. 154–155°, was obtained. It was purified by chromatographic adsorption on a column of alumina. The reaction of the acid chloride in pyridine or the reaction of ethyl dichloroacetate with phenothiazine so far has not given the desired derivative. [c] Chlorine analysis. Calcd.: Cl, 22.86. Found: Cl, 22.32, 22.42, 22.42.

Two and four-tenths grams (0.03 mole) of acetyl chloride in 20 ml. of dioxane was slowly added with stirring to a solution of 2 g. (0.01 mole) of phenothiazine in 40 ml. of dioxane in which there was suspended 4 g. of anhydrous sodium carbonate. The mixture was stirred at room temperature for a few minutes and then slowly heated to a gentle reflux. The heating was continued for 40 minutes. The mixture was poured into very dilute hydrochloric acid to precipitate 2.2 g. (91%) of yellow solid, m.p. 184–188°. The crude product was recrystallized from ethanol giving 1.9 g. (79%) of light yellow crystals, m.p. 197–198°.

10-Acetylphenothiazine-5-oxide.—The following procedures are typical of the methods by which the oxides listed in Table II were prepared.

TABLE II

10-ACYLPHENOTHIAZINE-5-OXIDES

Acyl group	Method	M.p., °C.	Yield, %	Formula	Nitrogen, % Calcd.	Found
$COCH_2Cl$[a]	A	186–187	86	$C_{14}H_{10}ClNO_2S$	4.80[b]	4.72
$COCH_2C_6H_5$	A	141–141.5[c]	50	$C_{20}H_{15}NO_2S$	4.20	4.39
$COCH_2C_6H_5$	B	138–140[d]	70			

[a] In the first experiment, 3-nitrophenothiazine-5-oxide and a small amount of 10-chloroacetylphenothiazine-5-oxide were isolated. [b] Chlorine analysis. Calcd.: Cl, 12.15. Found: Cl, 12.36, 12.08. [c] The compound was purified by chromatographic adsorption on a column of alumina. [d] The product was recrystallized from ethanol only.

Method A.—Five milliliters of concentrated nitric acid (sp. gr. 1.42) was added slowly to a solution of 5 g. (0.021 mole) of 10-acetylphenothiazine in 105 ml. of glacial acetic acid cooled in an ice-bath. After 20 minutes an additional milliliter of nitric acid was added. The reaction mixture was stirred occasionally while standing in the ice-bath for 30 minutes and then poured into water. After a few minutes a dark red solid began to separate. This was filtered off and shown to be 3-nitrophenothiazine-5-oxide[9] (mixed m.p.). The filtrate was poured over ice and 2.5 g. (46%) of light brown powder, m.p. 156–159°, separated. This product was purified by five recrystallizations from ethanol to give 1 g. (19%) of flat, white needles, m.p. 169.5–170°.

Anal. Calcd. for $C_{14}H_{11}NO_2S$: N, 5.45. Found: N, 5.47.

Method B.—A solution of 5 g. (0.021 mole) of 10-acetylphenothiazine, 500 ml. of ethanol and 60 ml. of 30% hydrogen peroxide was refluxed for 5 hours. Another 20 ml. of hydrogen peroxide was added and the refluxing continued for 30 minutes. After the solution had stood at room temperature for a few hours, approximately 450 ml. of solvent was removed by distillation. Water was added to the residual solution and 4.7 g. (88%) of white solid, m.p. 169–170°, separated. The mixed melting point with the 10-acetylphenothiazine-5-oxide prepared by the former method was undepressed. There was no evidence that any of the dioxide had been formed in the reaction.

Hydrolysis of 10-Acetylphenothiazine-5-oxide.—A solution of 1.0 g. (0.0039 mole) of the oxide in 15 ml. of ethanol and 2 ml. of 10% sodium hydroxide was refluxed for a few minutes. The color of the solution immediately became brown. After a short time colorless platelets crystallized. The solution was cooled and 0.6 g. (72%) of solid, m.p. 250–251° dec., filtered off. The mixed melting point with an authentic specimen of phenothiazine-5-oxide was undepressed.

The other 10-acylphenothiazine-5-oxides were hydrolyzed, by a similar procedure, to give phenothiazine-5-oxide.

Phenothiazine-5-oxide.[11]—A solution of 24.5 g. (0.123 mole) of phenothiazine, 800 ml. of ethanol, 8 ml. of 10% ethanolic potassium hydroxide and 24 ml. of 30% hydrogen peroxide was heated with stirring on a steam-bath for 3 hours.[11] A small amount of solid was filtered off. Since very little more solid separated after 10 days at room temperature, the solution was poured into 3 l. of water to give 24.1 g. of solid, m.p. 160–163° dec. Apparently the reaction did not go to completion since phenothiazine-5-oxide melts at 250° dec.[18] Thus, the solid, m.p. 160–163° dec., was redissolved in 750 ml. of ethanol, and 35 ml. of 30% hydrogen peroxide was added to the refluxing solution. The refluxing was continued for 4 hours. Most of the solvent was removed by distillation and the remaining solution was poured into water; 25.4 g. (96%) of yellow solid, m.p. 242–242.5° dec., separated. Recrystallization of the solid did not change its melting point. The mixed melting point with an authentic sample of the oxide was undepressed.

Hydrogen Peroxide Oxidation of 10-Ethylphenothiazine.—Three hundred milliliters of 30% hydrogen peroxide was added to a hot solution of 29.5 g. (0.13 mole) of 10-ethylphenothiazine[15] in 1200 ml. of absolute ethanol. The mixture was refluxed for a total of 8 hours. After 5 hours, more hydrogen peroxide (100 ml.) was added. The solution stood for 15 hours and then was concentrated by distilling off 700 ml. of solvent. The remaining solution was poured into 2 l. of ice-water to precipitate 25.5 g. of white solid, m.p. 146–149°. Recrystallization of the product from 125 ml. of absolute ethanol did not change the melting point. Since this one recrystallization from ethanol did not seem to purify the product, the total amount of crude material was dissolved in 800 ml. of benzene and the solution chromatographed on a 38 × 196 mm. column of alumina (Alcoa Activated Alumina, F-20). The column was eluted with benzene and finally absolute ethanol, the eluate being collected in 125-ml. portions. This procedure resulted in the isolation of 5.2 g. (15.5%) of 10-ethylphenothiazine-5-dioxide,[4] m.p. 161–163°, and 19.7 g. (62%) of 10-ethylphenothiazine-5-oxide, m.p. 162–163°. The dioxide passed through the column in the first portions of the eluate. The mixed melting point of the two products was depressed to 138–141°.

Anal. Calcd. for $C_{14}H_{13}NOS$: N, 5.76. Found: N, 5.85.

10-Acetylphenothiazine-5-dioxide.—The dioxides listed in Table III were prepared by the following procedure.

TABLE III

10-SUBSTITUTED PHENOTHIAZINE-5-DIOXIDES

R	M.p., °C.	Yield, %	Formula	Nitrogen, % Calcd.	Found
CH_3[a]	220–221[b]	44	$C_{13}H_{11}NO_2S$	..	..
CH_2CH_3[c]	162–164	95	$C_{14}H_{13}NO_2S$	..	..
$COCH_2Cl$	211[b]	51	$C_{14}H_{10}ClNO_3S$	4.56	4.60
$COCHCl_2$	211–212[b]	80	$C_{14}H_9Cl_2NO_3S$	4.09	4.12
$COCH_2C_6H_5$[d]	215–216[b]	70	$C_{20}H_{15}NO_3S$	4.01	4.22

[a] Reference 9. [b] Decomp. [c] Reference 4. [d] The product was purified by recrystallization from xylene. It was also prepared in a 77% yield by the hydrogen peroxide oxidation of the monoxide in glacial acetic acid.

Five milliliters of 30% hydrogen peroxide was added with stirring to a solution of 5 g. (0.021 mole) of 10-acetylphenothiazine in 150 ml. of glacial acetic acid at room temperature. After heating the solution at 60–70° for 15 minutes another 3 ml. of hydrogen peroxide was added. The heat-

(18) E. DeB. Barnett and S. Smiles, *J. Chem. Soc.*, **95**, 1253 (1909).

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°.

(19) A. Bernthsen, *Ber.*, **39**, 1807 (1906).

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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 (gelei)*. 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.

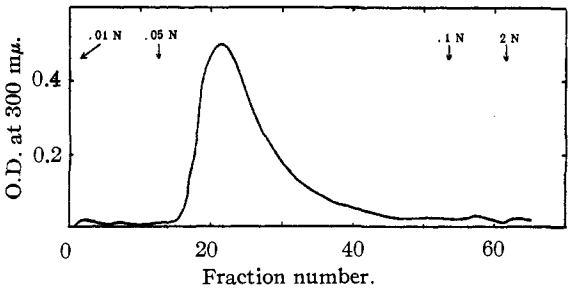

Fig. 1.—Pteroylglutamic acid (2.5 mg.) on column of Dowex-1-chloride 56 × 9 mm.: eluted at 0.6 ml./min. with hydrochloric acid of concentrations shown; 30-min. fractions, 93% recovery.

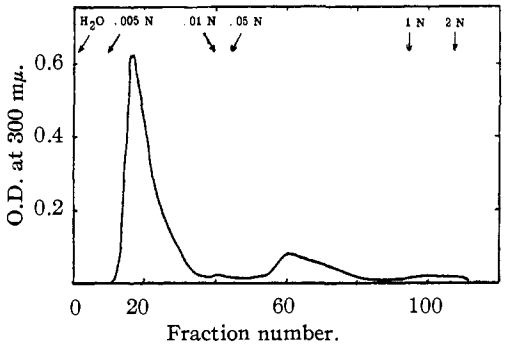

Fig. 2.—Aminopterin (2.5 mg.) on column of Dowex-1-chloride 50 × 9 mm.: eluted at 0.33 ml./min. with hydrochloric acid of concentrations shown; 45-min. fractions, the second peak is PGA.

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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