Table XI—Determination of Extent of Hydrolysis of *N*-Chloroacetyl-L-isoleucine during Incubation

System	Primary Amino Nitrogen[a], mg
1. Test solution[b], refrigerated 19 hr	0.002
2. Test solution, incubated at 37°, 19 hr	0.002
3. 2.5 ml of uninoculated medium plus 2.5 ml of water, refrigerated 19 hr[c]	0.298
4. 2.5 ml of uninoculated medium plus 2.5 ml of water, incubated at 37°, 19 hr	0.294
5. 2.5 ml of uninoculated medium plus 2.5 ml of test solution, refrigerated 19 hr	0.302
6. 2.5 ml of uninoculated medium plus 2.5 ml of test solution, incubated at 37°, 19 hr	0.305

[a] Determined by Van Slyke nitrous acid method (5). The volumes of solution taken for the analysis were 1.5 ml for Systems 1 and 2 and 1.0 ml for Systems 3–6. For the volumes taken, Systems 1 and 2 should yield 0.201 mg and Systems 3–6 should yield 0.368 mg of primary amino nitrogen when the test compound is completely hydrolyzed. For comments on sensitivity to method, see text. [b] Test solution was chloroacetyl-L-isoleucine, 20.0 mg, plus 0.96 ml of 0.1 *N* NaOH with the volume made to 10.0 ml with water; pH of the test solution was 6. [c] The pH of the assay system (2.5 ml of medium plus 2.5 ml of test solution) was 6.52.

from that of the other chloroacetyl derivatives that showed inhibition in the 15–30% range. In the former, for example, the isomer of chloroacetyl-β-hydroxynorleucine showing the greatest activity was of the D-configuration. However, it is difficult to deduce the mechanism of inhibition in experiments of this type since the system is too complex.

In view of the differences in metabolic behavior between the free amino acids and the *N*-chloroacetylated derivatives, it might prove useful to test these derivatives in suitable mammalian tumor systems; this testing is now being done.

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Thermal and Photolytic Decomposition of Methotrexate in Aqueous Solutions

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Received March 10, 1977, from the *Pharmacy Department, The Clinical Center, National Institutes of Health, Bethesda, MD 20014.* Accepted for publication August 4, 1977.

Abstract □ The chemical kinetics of thermal and photolytic degradation of methotrexate in aqueous solutions were studied. At above pH 7 and 85°, methotrexate hydrolyzed to yield mainly N^{10}-methylpteroylglutamic acid. The hydrolysis rate followed first-order kinetics with respect to methotrexate concentration and increased rapidly above pH 9. When methotrexate solutions were kept under laboratory fluorescent light, the major degradation products were 2,4-diamino-6-pteridinecarbaldehyde, 2,4-diamino-6-pteridinecarboxylic acid, and *p*-aminobenzoylglutamic acid. The photolytic reaction followed zero-order kinetics with respect to methotrexate concentration and was catalyzed by bicarbonate ion. Evidence is presented for a proposed sequential cleavage of methotrexate by a free radical mechanism for the photolysis. Commercial parenteral methotrexate was found to be quite stable as marketed when stored in the original vial at room temperature.

Keyphrases □ Methotrexate—thermal and photolytic decomposition in aqueous solutions □ Thermal decomposition—methotrexate in aqueous solutions □ Photolytic decomposition—methotrexate in aqueous solutions □ Decomposition—thermal and photolytic, methotrexate in aqueous solutions □ Stability—methotrexate in aqueous solutions □ Antineoplastic agents—methotrexate, thermal and photolytic decomposition in aqueous solutions

Methotrexate (4-amino-N^{10}-methylpteroylglutamic acid), a widely used antileukemic agent, is often administered in the form of an aqueous parenteral solution. Although it is recognized that methotrexate decomposes in light and alkaline solutions, the chemical kinetics of methotrexate decomposition have not been reported.

Since commercially available methotrexate is only about 86% pure (1) and contains several impurities (2, 3), it is important to know whether methotrexate degradation accounts for some of the impurities. Furthermore, it is important to ascertain the stability of commercial methotrexate when it is diluted in large-volume intravenous solutions. The present study was undertaken to elucidate the decomposition rate of aqueous solutions of methotrexate under fluorescent light and at high temperatures and to identify the degradation products. Chemical kinetic

studies also were conducted to determine the order of the reactions and the influence of variables such as pH, ionic strength, and buffer species on the reaction rates.

EXPERIMENTAL

Reagents and Apparatus—Methotrexate USP reference substance[1], pure methotrexate prepared by purifying methotrexate USP by column chromatography on diethylaminoethylcellulose (1, 3), and commercial methotrexate preparations[2,3] were used. The four decomposition products from methotrexate were compared with authentic samples of N^{10}-methylpteroylglutamic acid[4], 2,4-diamino-6-pteridinecarbaldehyde[5], *p*-aminobenzoylglutamic acid[6], and 2-amino-4-hydroxy-6-pteridinecarboxylic acid[7].

The buffer generally employed was ammonium bicarbonate, although some studies were conducted with carbonate buffer, tris(hydroxymethyl)aminomethane buffer, or hydroxide ion provided by sodium hydroxide or ammonium hydroxide. The ionic strength of each buffered solution was adjusted with potassium chloride to 0.25.

All other chemicals were reagent grade.

All spectrophotometric analyses were carried out on a recording spectrophotometer[8]. The apparatus for high-pressure liquid chromatography (HPLC) (4) and column chromatography on diethylaminoethylcellulose (1, 3) were reported previously. All pH measurements were taken at room temperature with a pH meter[9].

Thermal Degradation Studies—Methotrexate samples were diluted with appropriate buffers to a concentration of 0.1 mg of methotrexate/ml unless otherwise specified. Concentrations of up to 1 mg of methotrexate/ml were used only to confirm the reaction order. For studies between pH 8.5 and 9.5, the buffered methotrexate samples were sealed in clear glass ampuls, wrapped in aluminum foil, and immersed in a constant-temperature oil bath maintained at 85 ± 0.1°. For studies between pH 10 and 12, where reactions were considerably faster, buffered methotrexate samples were kept in amber glass volumetric flasks, tightly stoppered, and immersed in the constant-temperature oil bath. At appropriate time intervals, a sample (either an individual ampul or an aliquot from a volumetric flask) was taken out, cooled to room temperature, and analyzed for intact methotrexate and degradation products by procedures described under *Method of Analysis*.

Photolytic Degradation Studies—The buffered methotrexate samples, as described for thermal degradation studies, were transferred to 25-ml clear glass volumetric flasks and placed in a specially constructed light box. The box, 60.96 × 30.48 × 43.18 cm (height), was equipped with two fluorescent bulbs[10], 10.16 cm apart near the center of the top lid. The floor of the box was covered with white paper to give a uniformly illuminated background. The intensity of the light near the floor of the box was checked with a photometer and was uniform. At appropriate time intervals, 0.5-ml portions of the sample solutions were withdrawn and analyzed for intact methotrexate and photodecomposition products as described under *Method of Analysis*.

Methotrexate Dosage Forms—Diluted and undiluted solutions of commercial methotrexate injection were studied. In studies of diluted solution, methotrexate injection was diluted to a concentration of about 0.1 mg of methotrexate/ml with distilled water, adjusted to an initial pH of 8.3, and not readjusted thereafter during the runs. The diluted solution was then sealed in ampuls for thermal degradation studies and in 25-ml volumetric flasks for photolytic studies as previously described.

Photolytic degradation studies on the undiluted injection were made in the original vial. For thermal degradation studies, the contents of several undiluted injection vials were pooled and transferred to 2-ml ampuls as previously described. Methotrexate tablets were assayed initially, crushed, triturated into fine powder, spread on a petri dish, and then kept in the light box for 1 month. All samples were analyzed at appropriate time intervals for methotrexate and its decomposition products as described under *Method of Analysis*.

[1] Lot 1260x8105, Lederle Laboratories.
[2] Lot 467-164, 50-mg/2-ml vials, Lederle Laboratories.
[3] Lot 422-109, 5-mg tablets, Lederle Laboratories.
[4] Drug Research and Development Program, Division of Cancer Treatment, National Cancer Institute.
[5] Obtained from R. L. Dion, Laboratory of Medicinal Chemistry and Biology, Developmental Therapeutics Program, National Cancer Institute.
[6] Pfaltz and Bauer, Stamford, Conn.
[7] Sigma Chemical Co., St. Louis, Mo.
[8] Cary 17, Varian Instrument Division, Palo Alto, Calif.
[9] Beckman Expandometic SS-2.
[10] Sylvania F15T8-CW, 45.72 cm long.

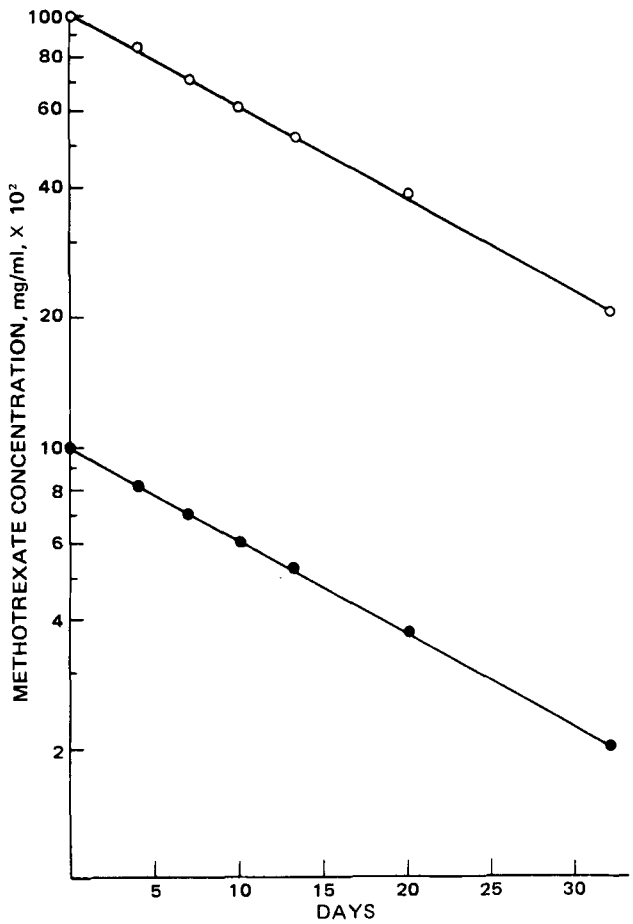


Figure 1—*Apparent first-order plots for the degradation of chromatographically pure methotrexate in the absence of light, pH 8.3, 0.1 M ammonium bicarbonate, 85°, and μ = 0.25. Key: ○, 1.0 mg of methotrexate/ml; and ●, 0.1 mg of methotrexate/ml.*

Investigational Methotrexate Injection—One gram of investigational methotrexate injection[11] was dissolved in 10 ml of distilled water; this solution was further diluted in an intravenous infusion bottle containing 500 ml of 0.05 *M* $NaHCO_3$ solution for injection and assayed. The infusion bottle was then kept on the laboratory counter and exposed to ordinary fluorescent light in the room for 24 hr. The intravenous solution was then analyzed for intact methotrexate and its decomposition products.

Method of Analysis—Intact methotrexate in the presence of its degradation products was assayed by HPLC using an anion-exchange resin column and eluting with a 0.6 *M* buffer (Mobile Phase 1) containing sodium phosphate and sodium perchlorate. The details of the column, solvent, sampling, and calculations were reported previously (4). Samples also were assayed on a diethylaminoethylcellulose column by gradient elution as reported in the literature (1, 4).

Determination and Identification of Degradation Products—The degradation products were initially separated by chromatography on a diethylaminoethylcellulose column (1, 3). The separated peaks were then identified by their UV spectra and retention times when compared with authentic samples. The degradation products were also monitored and measured by HPLC (4). For thermal decomposition products, the HPLC assay described under *Method of Analysis* was used. HPLC was also used for separation of photolytic products; however, a 0.005 *M* buffer[12] containing 3% acetonitrile (Mobile Phase 2) was used as the eluant.

RESULTS AND DISCUSSION

Methotrexate injection, as available commercially, is usually adjusted to pH 8.3. Therefore, most studies were conducted at pH 8.3 using am-

[11] Preservative-free methotrexate (Lot BC-76-221), 1-g vial manufactured for National Cancer Institute by BenVenue Laboratories, Bedford, Ohio.
[12] Buffer contained 0.0025 *M* sodium phosphates and 0.0025 *M* sodium perchlorate, pH 7.0.

Table I—Experimental Rate Constants for the Alkaline Hydrolysis of Methotrexate (0.1 mg/ml) at 85° in the Absence of Light and μ = 0.25

Sample	pH	Buffer	Half-Life, days	Rate, day^{-1}
Methotrexate USP	8.5	0.01 *M* NH_4HCO_3	13.0	0.0533
Methotrexate USP	8.5	0.1 *M* NH_4HCO_3	13.0	0.0533
Methotrexate USP	8.7	0.1 *M* NH_4HCO_3	13.8	0.0502
Methotrexate USP	9.0	0.1 *M* NH_4HCO_3	11.3	0.0613
Methotrexate USP	10.4	0.1 *M* NH_4HCO_3	0.416	1.666
Methotrexate USP	11.2	0.1 *M* NH_4HCO_3	0.158	4.378
Methotrexate USP	12.0	0.1 *M* NH_4HCO_3	0.038	18.14
Methotrexate USP	8.5	0.1 *M* tris(hydroxymethyl)aminomethane	9.1	0.076
Chromatographically pure methotrexate	8.5	0.1 *M* NH_4HCO_3	13.0	0.0533
Chromatographically pure methotrexate	8.5	0.1 *M* NH_4HCO_3	12.7	0.0546
Chromatographically pure methotrexate	8.5	0.1 *M* tris(hydroxymethyl)aminomethane	8.8	0.0787
Methotrexate injection[a] (undiluted)	8.3	—	17.0	0.0408

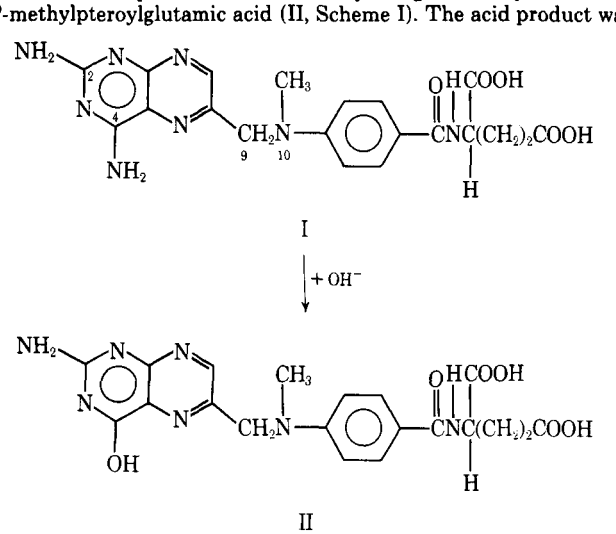
[a] Concentration of 25 mg/ml, initial pH 8.3, unbuffered.

monium bicarbonate buffer. The choice of the buffer was partially dictated by the purification method in which methotrexate in analytical quantities was purified by chromatographing on a diethylaminoethylcellulose column and eluting with a gradient of pH 8.3 ammonium bicarbonate buffer (1, 3).

Thermal Degradation—Figure 1 shows methotrexate degradation in 0.1 *M* ammonium bicarbonate at an initial pH of 8.3[13] and 85°. The linearity of the semilogarithmic plots with the same slope at two different methotrexate concentrations indicated that the reaction was first order with respect to intact drug. Table I shows the experimental rate constants and half-lives for methotrexate degradation in the absence of light at various pH values and buffers, 85°, and μ = 0.25. The hydrolysis rate increased with hydroxide ion (pH) and was independent of ammonium bicarbonate concentration at constant ionic strength. However, in the presence of pH 8.3 tris(hydroxymethyl)aminomethane, samples degraded 50% faster than those in ammonium bicarbonate.

Furthermore, as shown in Table I, there were no significant differences between the hydrolysis rate of chromatographically pure methotrexate and methotrexate USP. The hydrolysis rate of methotrexate USP and commercial methotrexate is also the same because methotrexate USP is used in the preparation of commercial methotrexate. However, this fact is not evident from the data in Table I, because the undiluted commercial methotrexate sample was unbuffered and its pH had changed during the run. In all linear first-order plots from which the rate constant was calculated in Table I, the correlation coefficient was 0.998 or better.

Identification of Thermal Decomposition Product—In alkaline conditions above pH 8 and 85°, the major degradation product was N^{10}-methylpteroylglutamic acid (II, Scheme I). The acid product was confirmed by chromatographing a degraded solution of methotrexate on a cellulose column, collecting the major degradation peak, and identifying it by its UV spectrum and retention time in the system compared to an authentic sample. The acid product was further identified by comparing the UV spectra and retention times of an authentic sample of N^{10}-methylpteroylglutamic acid and the degradation peak in the HPLC method.



Scheme I

The product N^{10}-methylpteroylglutamic acid could be predicted because of the ease of alkaline hydrolysis of the 4-amino group as part of an amidine system. In comparison, the 2-amino group, as part of a quinidine system, is quite stable even under strongly alkaline conditions (5). The sequence of hydrolysis of methotrexate (I) at pH 8.3 and 85° is shown in Scheme I.

Photolytic Degradation of Methotrexate—Figure 2 shows methotrexate photolysis at room temperature in different concentrations of pH 8.3 ammonium bicarbonate. In all plots, the initial lag periods were followed by a zero-order loss of methotrexate with time. Identical samples, when stored in the absence of light at room temperature, did not undergo photolysis during the same period.

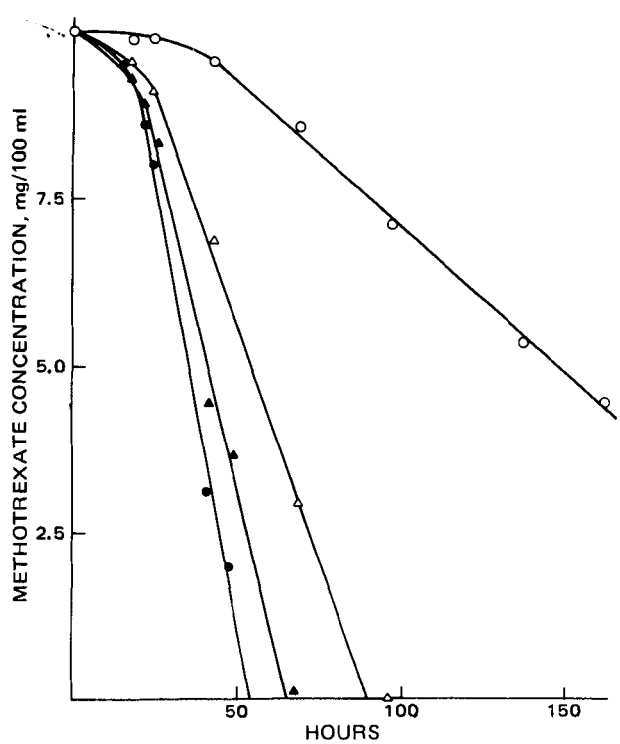


Figure 2—*Apparent zero-order plots for the photolytic degradation of chromatographically pure methotrexate at pH 8.3, 23 ± 2°, and μ = 0.25. Key (ammonium bicarbonate buffer): ○, 0.01 M; △, 0.05 M; ▲, 0.1 M; and ●, 0.15 M.*

[13] The pH value of 8.30 changes to 8.54 with time because of the volatility of the buffer (ammonia and carbon dioxide).

Table II—Photolytic Rate Constants and Lag Periods of Methotrexate Samples under Various Conditions in 25-ml Volumetric Flasks, pH 8.3, μ = 0.25, and Room Temperature

Run	Sample	Concentration, mg/ml	Buffer	Additives	Lag Period, hr	Rate, mg/ml × 10^4
1	Chromatographically pure methotrexate	0.1	None	None	72	1.6
2	Chromatographically pure methotrexate	0.1	0.01 *M* NH_4HCO_3	None	40	4.0
3	Chromatographically pure methotrexate	0.1	0.03 *M* NH_4HCO_3	None	30	10.0
4	Chromatographically pure methotrexate	0.1	0.05 *M* NH_4HCO_3	None	25	15.4
5	Chromatographically pure methotrexate	0.1	0.10 *M* NH_4HCO_3	None	23	20.0
6	Chromatographically pure methotrexate	0.1	0.10 *M* NH_4HCO_3	None	21	29.2
7	Chromatographically pure methotrexate	0.1	0.15 *M* NH_4HCO_3	None	20	26.0
8	Chromatographically pure methotrexate	0.1	0.25 *M* NH_4HCO_3	None	17	46.0
9	Chromatographically pure methotrexate	0.1	0.25 *M* NH_4HCO_3	0.1% edetate disodium	40	25.6
10	Chromatographically pure methotrexate	0.1	0.25 *M* NH_4HCO_3	2% 2-nitropropane	90	43.0
11	Chromatographically pure methotrexate	0.5	0.25 *M* NH_4HCO_3	None	17	52.0
12	Methotrexate USP	0.1	None	None	67	1.8
13	Methotrexate USP	0.1	0.10 *M* NH_4HCO_3	None	21	22.6
14	Methotrexate USP	0.1	0.25 *M* NH_4HCO_3	None	16	46.3
15	Methotrexate USP	0.1	0.1 *M* $NaHCO_3$	None	19	24.2
16	Methotrexate USP	0.1	0.1 *M* NH_4Cl	None	69	1.7
17	Methotrexate USP	0.1	0.1 *M* $NaHCO_3$ + 0.1 *M* NH_4Cl	None	22	21.6
18	Commercial injection	—	None	None	70	1.1

[a] Sample kept in 2-ml clear glass ampul.

Table II reports the photolytic rate constants and lag periods of methotrexate under various conditions. In Runs 8 and 11, different methotrexate concentrations degraded at approximately the same absolute rate (milligrams per hour) when other conditions such as light, vessel size, buffer concentration, and ionic strength were constant, and this result provides further support for a zero-order reaction.

Figure 2 and Table II show that photolyses of methotrexate solutions are strongly catalyzed by increasing ammonium bicarbonate concentration and that the same solution without ammonium bicarbonate is more stable to photodegradation. Additional studies in which either ammonium or bicarbonate was selectively excluded from the reaction mixture revealed that bicarbonate was the catalytic species (Table II, Runs 15 and 16).

Although no values are reported in the paper, the photodecomposition rate of methotrexate decreased when the light intensity was reduced. The rate also was influenced by the vessel size, which is predictable in photolytic reactions. As seen in Table II, Runs 5 and 6, a methotrexate solution sealed in a 2-ml clear glass ampul degraded about 1.5 times faster than the same sample kept in a 25-ml volumetric flask. This result can be explained by the larger surface area per milliliter of solution in the ampul. Finally, studies with the addition of edetate disodium slowed down the photolytic degradation rate, suggesting that trace metal-ion catalysis also may occur in the reaction.

Mechanism for Methotrexate Photolysis—Zero-order kinetics and the presence of a lag period (possibly explained by the buildup of the concentration of free radicals) strongly suggest a free radical mechanism for methotrexate photolysis. To support the proposed mechanism, a study was carried out in the presence of a free radical scavenger, 2-nitropropane. The lag period increased considerably in the presence of 2-nitropropane (Table II, Runs 8 and 10), which might have trapped the reactive free radical and thus delayed the onset of the reaction.

Identification of Photodegradation Products—Initial separation of degradation products was carried out in a cellulose column (1, 3). Figure 3 shows a chromatogram of a methotrexate solution approximately 60% photodegraded with three degradation peaks. The UV spectrum of peak 1 changed significantly with pH (Fig. 4) and provided a basis for its identification when compared with an authentic sample of 2,4-diamino-6-pteridinecarbaldehyde (6). Peak 2 exhibited a UV spectrum identical to that of 2,4-diamino-6-pteridinecarboxylic acid (7). Peak 3 was identified as *p*-aminobenzoylglutamic acid by its UV spectrum when compared to an authentic sample.

The HPLC method with a very dilute buffered eluant (Mobile Phase 2) was used for a more rapid identification of the photodegradation products. Figure 5*a* shows a chromatogram of methotrexate solution approximately 60% photodegraded; peak 1 (r_t = 7 min) was identified as 2,4-diamino-6-pteridinecarbaldehyde, peak 2 (r_t = 13 min) was

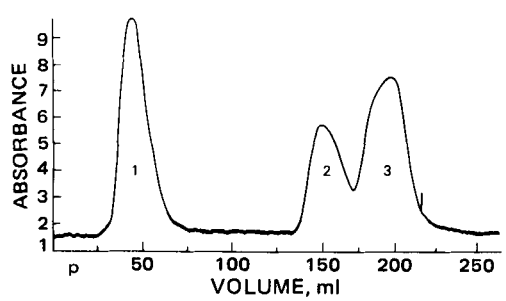


Figure 3—*Chromatogram of a partially photodegraded sample of methotrexate in 0.1* M *ammonium bicarbonate, pH 8.3, and 23 ± 2° from a cellulose ion-exchange column on linear gradient elution from 0.01 to 0.4* M *ammonium bicarbonate, pH 8.3. Key: peak 1, 2,4-diamino-6-pteridinecarbaldehyde; peak 2, 2,4-diamino-6-pteridinecarboxylic acid; and peak 3,* p*-aminobenzoylglutamic acid.*

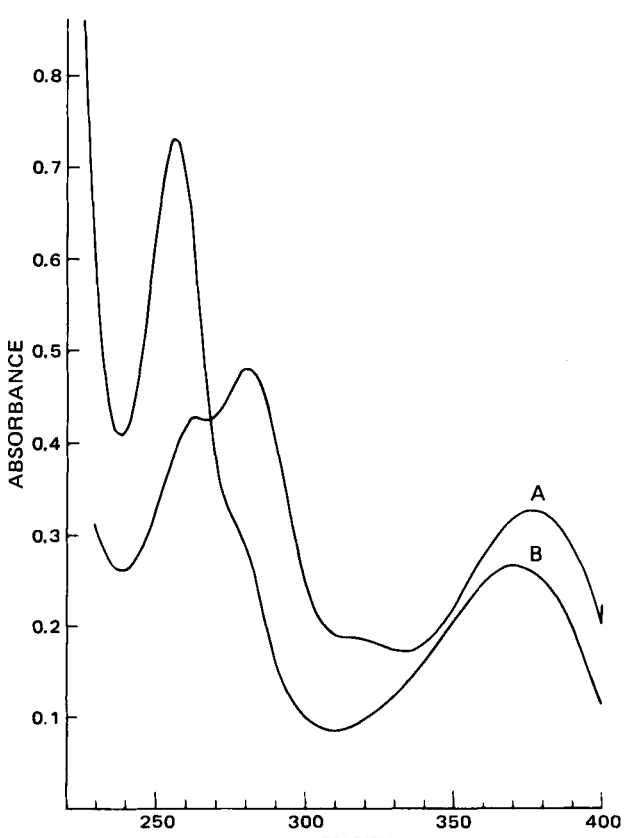


Figure 4—*UV spectra of peak 1 from Fig. 3 at two different pH values. Key: A, pH 8.3, 0.1* M *ammonium bicarbonate; and B, pH 13, 0.1* N *NaOH.*

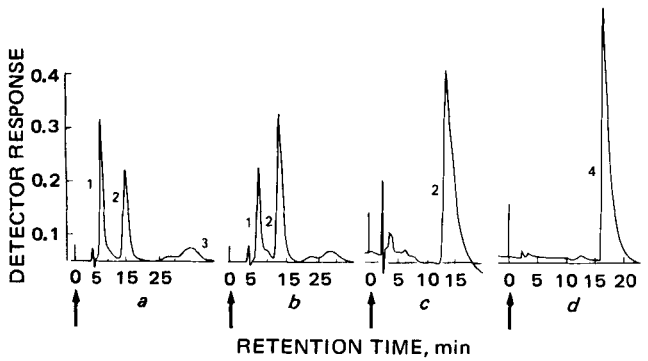


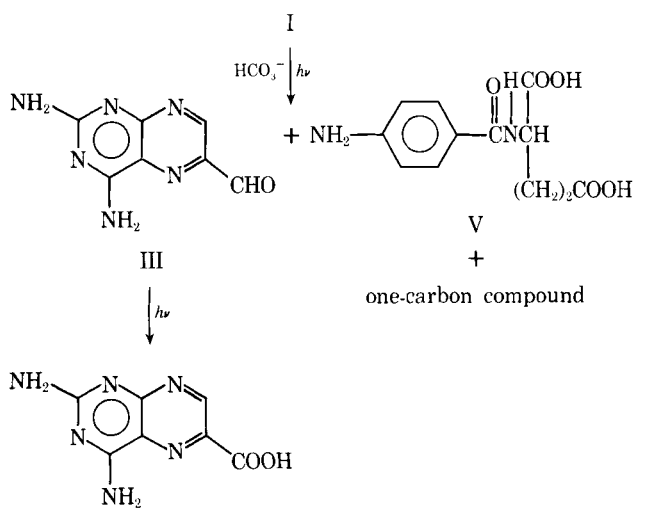
Figure 5—*HPLC changes for methotrexate photodegradation in 0.1 M ammonium bicarbonate (pH 8.3) at 23 ± 2° and μ = 0.25 with time. Key:* a, *2 days;* b, *6 days;* c, *20 days;* d, *fraction* c *boiled with 0.1* N *NaOH for 1 hr; peak 1, 2,4-diamino-6-pteridinecarbaldehyde; peak 2, 2,4-diamino-6-pteridinecarboxylic acid; peak 3,* p*-aminobenzoylglutamic acid; and peak 4, 4-amino-4-hydroxy-6-pteridinecarboxylic acid.*

identified as 2,4-diamino-6-pteridinecarboxylic acid, and peak 3 (r_t = 35 min) was identified as *p*-aminobenzoylglutamic acid when their UV spectra were compared to authentic samples. Figure 5*b* shows an increase in the acid peak 2 with a simultaneous decrease in the aldehyde peak 1 when the sample in Fig. 5*a* was exposed to additional light for 4 days.

When the sample in Fig. 5*b* was further exposed to light for 2 weeks, loss of peak 1 resulted in an increase in the formation of peak 2 (r_t = 13.5 min) (Fig. 5*c*). When the sample seen in Fig. 5*c* was boiled with 0.1 *N* NaOH for 1 hr, peak 2 shifted upfield and became peak 4 with a retention time (r_t = 18 min) (Fig. 5*d*) identical to that of an authentic sample of 2-amino-4-hydroxy-6-pteridinecarboxylic acid. Formation of peak 4 could be explained by the ease of hydrolysis of the 4-amino group of 2,4-diamino-6-pteridinecarboxylic acid in alkaline solution.

Identification of peak 3 warrants further discussion. To determine whether N–CH_3 cleavage occurs under the experimental conditions described for methotrexate photolysis, *p-N*-methylaminobenzoic acid (0.10 mg/ml) was dissolved in 0.25 *M* $NaHCO_3$ (pH 8.3) and kept under light. After about 10 days, 60% of the initial reactant, *p-N*-methylaminobenzoic acid, was converted to *p*-aminobenzoic acid. In a similar study in which sodium bicarbonate was omitted but the pH was maintained at 8.3, about 10% degradation occurred. It appears, therefore, that the bicarbonate ion has a photocatalytic effect in breaking the N–CH_3 bond when nitrogen is attached to an aromatic group. Considering the abundance of such groups in medicinal agents and the common use of bicarbonate buffers, this observation warrants further systematic investigation.

Scheme II presents the proposed sequence for photolytic reaction of methotrexate in the presence of bicarbonate buffer. As described earlier, 2-nitropropane trapping suggested the presence of bicarbonate free radicals. These radicals probably act as the catalyst in the initial breaking of N^{10}-alkyl linkages. In Scheme II, both alkyl linkages, CH_2–N and the N^{10}–CH_3, are shown to be cleaved in one step. However, this may not necessarily be true, and either of the alkyl groups could be cleaved first.



Scheme II

The relatively slow rate of cleavage of the methyl group from *p-N*-methylaminobenzoic acid compared to the rate of appearance of *p*-aminobenzoylglutamic acid suggests that *p-N*-methylaminobenzoylglutamic acid is not the precursor of *p*-aminobenzoylglutamic acid during methotrexate photolysis. Therefore, the methyl group must cleave away before or simultaneously with the CH_2–N group. Formation of 4-aminopteroylglutamic acid (aminopterin) as an intermediate is a possibility, but it has not been identified in the reaction mixture.

The formation of the aldehyde that oxidizes to the corresponding acid in alkaline pH also occurred in folic acid photolysis (8). In Scheme II, the conversion of 2,4-diamino-6-pteridinecarbaldehyde to 2,4-diamino-6-pteridinecarboxylic acid also was photocatalyzed; when the sample in Fig. 5*a* was kept in the dark for 4 days, there was no significant increase in the acid peak even though it was kept in moderate alkaline pH. The influence of the buffer species on the degradation products in the reaction has not been investigated, nor has the fate of the one-carbon compound coming from the methyl group attached to the N^{10}-portion of methotrexate. However, formaldehyde and formic acid may be the products formed that are similar to formation of 2,4-diamino-6-pteridinecarbaldehyde and 2,4-diamino-6-pteridinecarboxylic acid from the other substituent (2,4-diaminopteridylmethyl) on N^{10}.

Studies with Commercial Preparations—The results of a thermal study with commercial methotrexate injection at 85° are presented in Table I. The degradation rate of the injection was similar to that of methotrexate USP in 0.1 *M* ammonium bicarbonate at an initial pH of 8.3. Since methotrexate USP in 0.1 *M* ammonium bicarbonate kept in the dark at room temperature for 1 year showed about 3% degradation, one can assume that commercial methotrexate injection is at least as stable. Therefore, it was concluded that the injection should be stable (less than 10% degradation) for about 3 years at room temperature.

No photodegradation products previously described were found in recently manufactured commercial methotrexate injections stored in the original package for about 1 year. When methotrexate injection was diluted to 0.1 mg of methotrexate/ml with water and kept under light, there was only 5% degradation after 10 days and 11% degradation after 20 days. However, solutions of methotrexate USP without benzyl alcohol but otherwise identical to commercial methotrexate degraded 8% in 10 days and 17% in 20 days. Benzyl alcohol in the commercial injection apparently has some stabilizing effect against methotrexate photodegradation. However, methotrexate stability in undiluted concentrated solution is much different. Undiluted vials of 25 mg of methotrexate/ml in the original container, when kept under light for more than 1 month, showed negligible photodegradation. This result may be explained by the fact that the more concentrated the solution undergoing zero-order degradation, the smaller the percent degradation.

In high dose (10–25 g) methotrexate therapy, bicarbonate solutions are often administered along with methotrexate to alkalinize the urine and increase methotrexate excretion. Methotrexate mixed with bicarbonate in the infusion bottle may often be exposed to room light over 12–24 hr. Considering the catalytic effect of the bicarbonate ion on methotrexate photolysis, a study was carried out in light using a low concentration of methotrexate, 2 mg/ml (for a maximum percent degradation), and a high concentration of the bicarbonate ion (0.05 *M* $NaHCO_3$) in an infusion bottle. No photodegradation products were observed in 12 hr. This result may be explained by the fact that the solution was more concentrated (than solutions in the study) and that the lag period must be considerably longer in room light than in the light box used in the photolytic studies. Thus, mixing methotrexate in bicarbonate solution was acceptable if the solution was used within 12 hr. However, the bicarbonate ion catalyzes the photolysis of methotrexate and should be avoided if possible.

Methotrexate tablets, when powdered, spread over a large surface area, and kept under light, showed no photodegradation products over 2 months.

It can be concluded from the results of this study that, except for N^{10}-methylpteroylglutamic acid, the impurities in commercial methotrexate injection (2, 3, 9) did not result from the degradation of methotrexate but appear to have resulted during the synthesis of the drug.

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ACKNOWLEDGMENTS

The authors thank Mrs. Altice G. Frazier for technical assistance.

Pharmacological Studies on *N*-Demethylated Carbachol

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Received March 17, 1977, from the *Department of Pharmacology and Therapeutics, College of Medicine, University of Florida, Gainesville, FL 32610.* Accepted for publication August 2, 1977.

Abstract □ In attempts to find a drug more active than pilocarpine, the tertiary nitrogen derivative of carbachol, *N*-demethylated carbachol, was synthesized and tested on several autonomic nervous system preparations. *N*-Demethylated carbachol was active at muscarinic and nicotinic sites *in vivo* and *in vitro*. In superfusion studies, *N*-demethylated carbachol contracted the smooth muscle of the guinea pig ileum as well as skeletal muscles of frog rectus abdominis and chick biventer cervicis. *N*-Demethylated carbachol decreased blood pressure in the rat, with an ED_{50} (±*SEM*) of 4.82 ± 0.78 mg/kg. After close arterial injection to the cat superior cervical ganglion, *N*-demethylated carbachol elicited contractions of the nictitating membrane (ED_{50} of 1.68 ± 0.24 mg/kg) that were not significantly affected by atropine. *N*-Demethylated carbachol stimulated salivation in dog Wharton duct preparations with an ED_{50} of 2.55 ± 0.81 mg/kg. In contrast, pilocarpine had no effects on skeletal muscles *in vitro*, produced ganglionic effects blocked by atropine, had a prominent effect on salivation, and tended to elevate blood pressure.

Keyphrases □ *N*-Demethylated carbachol—synthesized, cholinergic activity evaluated *in vivo* and *in vitro* □ Carbachol, *N*-demethylated—synthesized, cholinergic activity evaluated *in vivo* and *in vitro* □ Cholinergic activity—*N*-demethylated carbachol evaluated *in vivo* and *in vitro*

It is desirable at times to produce effects that mimic the stimulation of autonomic pathways. One of the simplest ways of achieving this condition, at least in theory, would be administration of the appropriate neurotransmitter to the target organ. Acetylcholine, the transmitter at parasympathetic neuroeffector sites, is virtually useless as a therapeutic agent, largely because of its rapid hydrolysis by both acetylcholinesterase and butyrylcholinesterase. Studies on carbachol, the carbamic acid ester of choline, were reported in 1932 (1). Substitution of the carbamyl moiety in place of the acetyl group of acetylcholine increased resistance to hydrolysis by cholinesterases. At the same time, the essential pharmacological properties of acetylcholine were retained in the new ester.

Clinical use of carbachol has been hindered by the inability of the permanently charged molecule to penetrate biological membranes. Its effectiveness, *e.g.*, as an ophthalmic agent, was maintained only with high concentrations and the addition of wetting agents such as benzalkonium chloride to increase corneal penetration (2). Side effects and toxicity resulting from these procedures have reduced carbachol use in ophthalmology.

Drugs containing a tertiary nitrogen rather than a quaternary nitrogen moiety in their structures have the advantage of higher lipid solubility and can thus penetrate membranes. This ability accounts for the extensive topical use of pilocarpine in glaucoma treatment. This naturally occurring cholinomimetic alkaloid bears little structural resemblance to acetylcholine, however, and certain aspects of its action, mechanism, and inactivation are ambiguous (3).

Considerations of stability, penetrability, and structure suggest that a tertiary nitrogen derivative of carbachol might be an alternative to pilocarpine in ocular therapy.

At least two groups in the past have reported the synthesis of this tertiary nitrogen compound, 2-(dimethylamino)ethyl carbamate (*N*-demethylated carbachol) (I) (4, 5). Despite the therapeutic potential of such a derivative, little work has been done on its pharmacology. Therefore, studies were initiated to investigate its properties.

EXPERIMENTAL

Drugs—Compound I hydrochloride was prepared using a procedure described by Hazard *et al.* (5). Its identity was confirmed by melting point, elemental analysis, and mass and NMR spectral analysis. Acetylcholine iodide[1], atropine sulfate[1], pilocarpine nitrate[1], tubocurarine chloride[1], and carbachol[2] were used as received.

Superfused Guinea Pig Ileum Preparation—Guinea pigs, 300–500 g, were stunned; the terminal portion of the ileum was removed, cleaned, cut into 2-cm long segments, and suspended on a superfusion assembly with an isometric force transducer[3] under an initial tension of 1 g. Tyrode solution (137 m*M* NaCl, 2.7 m*M* KCl, 1.35 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 11.9 m*M* $NaHCO_3$, 0.36 m*M* NaH_2PO_4, and 11.1 m*M* dextrose), oxygenated with 95% O_2–5% CO_2, was pumped over the preparation at a rate of 3–4 ml/min with a peristaltic pump[4]. The superfusion fluid was maintained at room temperature since the ileum displayed less spontaneous activity at 25 than at 37°.

Drugs to be tested were dissolved in saline (0.9% NaCl) and injected directly into the stream of superfusion fluid. Doses were increased in a logarithmic fashion while drug concentrations were adjusted to keep the volume of each dose less than 0.1 ml. The tension developed by the muscle during contraction was recorded on a polygraph[5]. The maximum response of each muscle was taken as 100%, and all smaller responses were ex-

[1] Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] City Chemical Corp., New York, N.Y.
[3] Myograph B, Narco Bio-Systems, Houston, TX 77017.
[4] Polystaltic pump, Buchler Instruments Co., Fort Lee, NJ 07024.
[5] Physiograph model Four-A, Narco Bio-Systems, Houston, TX 77017.