Table VI—Determination of 7-Hydroxy-3*H*-phenothiazine-3-one

Amount, μg	Recovery, %
16.4	89
18.2	93
18.4	94
19.6	96
24.6	93
27.3	95
27.8	94
29.4	99
Average ± *SD* = 94.1 ± 3.2%	

be carried out. This reactivity is also an explanation for the higher blank values obtained with the solvent acetone–6 *N* ammonia (100:2). One product formed, isopropylamine, also forms a complex with tropaeolin 00 that is extractable with chloroform. The use of freshly prepared solvent, careful cleaning of the glassware, and fast drying of the developed chromatograms were necessary to reduce the absorbance of the blank.

Although 7-hydroxy-3*H*-phenothiazine-3-one was the only compound formed during degradation of promethazine with an absorption maximum at 600 nm in an alkaline medium, the other products and promethazine itself interfered by reducing the compound to the colorless 3,7-dihydroxyphenothiazine. Therefore, the other compounds had to be removed by extraction with dichloromethane from the alkaline medium. The resulting water phase was diluted with 96% ethanol to get a clear solution. In the 5–40-μg range, Beer's law was obeyed. The molar absorptivity of the compound under this condition is 58,900. The results obtained with this method (Table VI) were quite satisfactory, provided that the ratio between water and ethanol in the solvent remained constant.

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Oxidative Degradation of Pharmaceutically Important Phenothiazines III: Kinetics and Mechanism of Promethazine Oxidation

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Abstract □ The kinetics of the thermal degradation of promethazine in an acidic medium under various conditions were investigated. The degradation of promethazine and the formation of some degradation products were studied under aerobic and anaerobic conditions. The influence of pH, metal ions such as copper(II) and iron(III), and antioxidants was investigated. In an oxygen-saturated medium, promethazine generally followed first-order kinetics. Increasing the pH increased the degradation rate to a limiting value at pH 5. Addition of copper(II) increased the degradation rate over the whole process, while iron(III) caused an increase for only a short time. Ascorbic acid sometimes increased the degradation rate, while higher concentrations of hydroquinone also accelerated the degradation. Pyrosulfite did not have any influence. Under anaerobic conditions, promethazine degraded only in the presence of copper(II) and iron(III) ions. As a result of the studies on the qualitative and quantitative aspects of the oxidation process, a mechanism for the oxidative degradation of promethazine is suggested. Promethazine 5-oxide and a number of degradation products without intact side chains are formed *via* a semiquinone free radical. The influence of several factors on the degradation process is discussed.

Keyphrases □ Promethazine—thermal degradation kinetics in acidic medium, effect of anaerobic and aerobic conditions, pH, metal ions, and antioxidants □ Degradation, thermal—promethazine, kinetics in acidic medium, effect of anaerobic and aerobic conditions, pH, metal ions, and antioxidants □ Oxidation—promethazine, thermal degradation kinetics in acidic medium, effect of anaerobic and aerobic conditions, pH, metal ions, and antioxidants □ Antiemetics—promethazine, thermal degradation kinetics in acidic medium, effect of anaerobic and aerobic conditions, pH, metal ions, and antioxidants □ Phenothiazines—promethazine, thermal degradation kinetics in acidic medium, effect of anaerobic and aerobic conditions, pH, metal ions, and antioxidants

Little information is available on the quantitative aspects of promethazine degradation. Chalabala *et al.* (1) mentioned the stability of a solution containing aminopyrine, trimecaine, and promethazine at pH 5.5–6.0 in the presence of sodium pyrosulfite, thiourea, or sodium formaldehyde sulfoxylate. One study (2) found that promethazine degradation followed first-order kinetics. The degradation rate decreased on changing the pH from 3.0 to 5.0. In another study, the degradation rate of the reaction was reported to be pH independent (3). Other studies (4, 5) also described first-order kinetics for the degradation reaction. However, at pH values over 5.5, degradation no longer followed first-order kinetics (5). Moreover, promethazine was most unstable at pH 4.3.

In this paper, the kinetics of promethazine degradation under varying conditions are described and the degradation mechanism is discussed.

EXPERIMENTAL

Materials—All materials used were described previously (6).

Methods—For the study of promethazine degradation under aerobic conditions, about 50 mg of promethazine hydrochloride, accurately weighed, was dissolved in a few milliliters of water. To the solution were added 12.5 ml of acetate buffer according to Walpole (7) to get the right pH (at 65°), any other necessary additive previously adjusted to the right pH, and water to a volume of 50.0 ml. The solution was saturated with oxygen, and 4 ml was transferred into 10-ml ampuls in an oxygen atmosphere according to a previous method (8). The ampuls were kept at 65° in the dark.

The isolation and determination of promethazine, promethazine 5-oxide, 3*H*-phenothiazine-3-one, and 7-hydroxy-3*H*-phenothiazine-3-one were carried out as described previously (6, 9).

For the study of promethazine degradation under anaerobic conditions, the oxygen was removed by bubbling carbon dioxide through the solution for 5 hr. The degradation was carried out at 100°.

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Table I—Rate Constants k' of Promethazine Degradation at Various Temperatures at pH 4.6

Temperature, °K	k', sec^{-1}
318	3.3×10^{-7}
328	13.6×10^{-7}
338	54.4×10^{-7}
348	218.6×10^{-7}
363	1253.0×10^{-7}

RESULTS

Degradation of Promethazine under Aerobic Conditions—*Influence of Temperature*—At all temperatures, the degradation was first order with respect to promethazine. Table I gives the observed rate constants, k', at various temperatures at pH 4.6. The Arrhenius plot gives an E_a of 30.6 kcal/mole. This energy of activation includes a term for the variation of the oxygen solubility with temperature. The decreased solubility of oxygen with increasing temperature is partially compensated by an increase in solubility because of a higher oxygen pressure above the solution at higher temperatures.

Influence of pH—The pH of the solutions was varied from 1.5 to 6.3; in this region, the solubility of oxygen in water was independent of pH. The degradation followed first-order kinetics at all pH values. Figure 1 shows that the rate constant increased with increasing pH and remained constant above pH 5.

The formation of the degradation products that were quantitated was also pH dependent. Figure 2 shows that the formation of 3*H*-phenothiazine-3-one and promethazine 5-oxide showed maxima at pH 3.2 and 4.3, respectively; the formation of 7-hydroxy-3*H*-phenothiazine-3-one decreased with increasing pH. At pH values over 3, the formation of the latter compound was negligible. These phenomena were not primarily due to decomposition of these products; promethazine 5-oxide was stable at pH 6.3 (Table II), while 3*H*-phenothiazine-3-one only decomposed at low pH values. Evaluation of the intensity of the zone containing phenothiazine and 10-methylphenothiazine on chromatograms of degraded promethazine solutions led to the conclusion that the formation of these compounds increased with increasing pH.

Influence of Metal Ions—Of the metal ions investigated, only copper(II) and iron(III) ions influenced promethazine degradation. Figure 3 shows this influence, which was different for the two metals at pH 3.2. While the k' values increased up to a maximum with an increasing concentration of copper(II) (Fig. 4), only the initial degradation rate increased dramatically with an increasing iron(III) concentration, and after a short time the degradation curves had slopes equal to those obtained when no metal ions were added (Fig. 3). The decrease in the extrapolated intercepts at zero time of the latter part of these curves was proportional to the iron(III) concentration.

Figure 5 shows the influence on the formation of promethazine 5-oxide, and Figs. 6 and 7 show the influence on the 7-hydroxy-3*H*-phenothiazine-3-one and 3*H*-phenothiazine-3-one formation, respectively. From

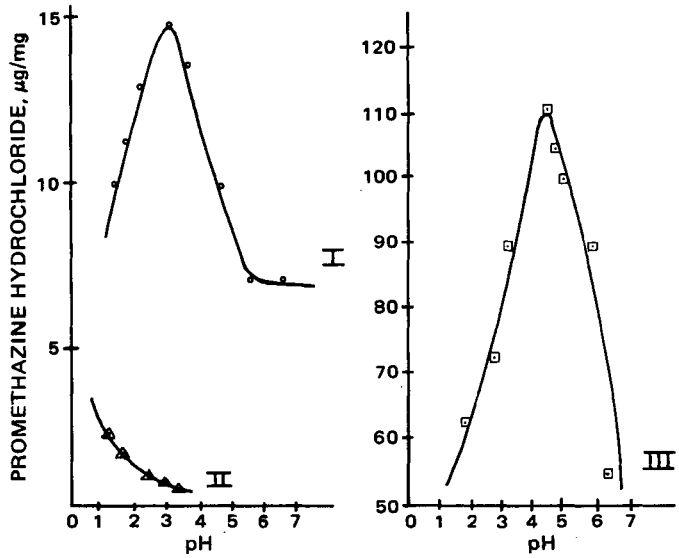


Figure 2—*Influence of pH on the formation of degradation products of promethazine after 25 hr at pH 3.2 under aerobic conditions at 65°. Key: curve I, 3H-phenothiazine-3-one; curve II, 7-hydroxy-3H-phenothiazine-3-one; and curve III, promethazine 5-oxide.*

Table II—Recoveries of 50 μg of Promethazine 5-Oxide after Storage during Specified Times at pH 6.3 and 65°

Time	Recovery, %
0	94
16 hr, 15 min	93
32 hr, 10 min	96
64 hr	98

Figs. 6 and 7, it can be seen that the formation of 3*H*-phenothiazine-3-one and 7-hydroxy-3*H*-phenothiazine-3-one increased with an increasing concentration of the metal ion. With iron(III), this increase only occurred in the initial phase of the process, but the influence of copper(II) was obvious during the whole process. In the presence of copper(II), the formation of promethazine 5-oxide increased (Fig. 5); in the presence of iron(III), the formation of promethazine 5-oxide decreased (Fig. 5).

Evaluation of the zone containing phenothiazine and 10-methylphenothiazine led to the conclusion that the formation of these compounds increased with increasing concentration of copper(II) and iron(III) in the solutions.

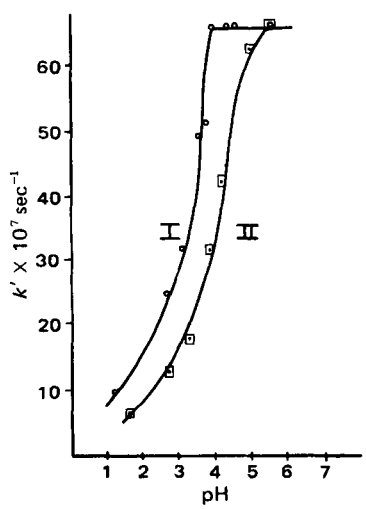


Figure 1—*Influence of pH on the degradation rate k' of promethazine under aerobic conditions at 65°. Key: curve I, no additive; and curve II, 2×10^{-3} M edetate disodium added.*

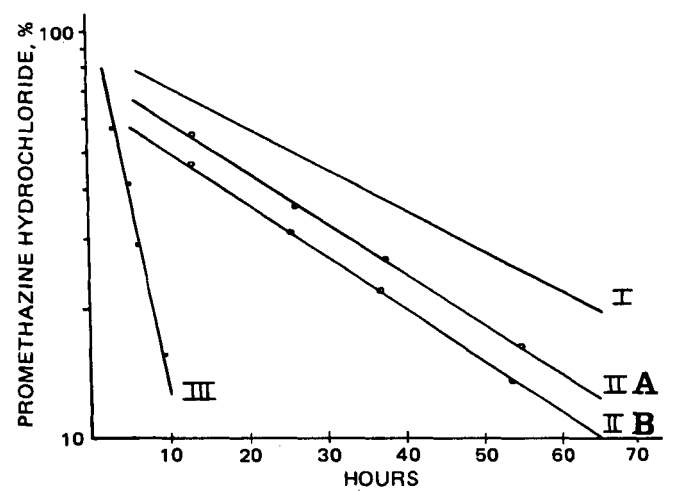


Figure 3—*Influence of copper(II) and iron(III) on the degradation of promethazine at pH 3.2 under aerobic conditions at 65°. Key: curve I, no additive; curve IIA, 10^{-3} M iron(III); curve IIB, 5×10^{-3} M iron(III); and curve III, 5×10^{-3} M copper(II).*

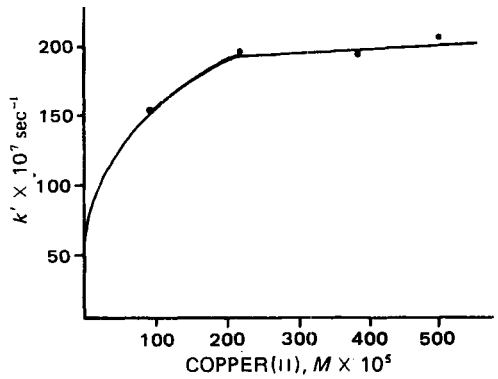


Figure 4—*Influence of copper(II) on the degradation rate* k′ *of promethazine at pH 3.2 under aerobic conditions at 65°.*

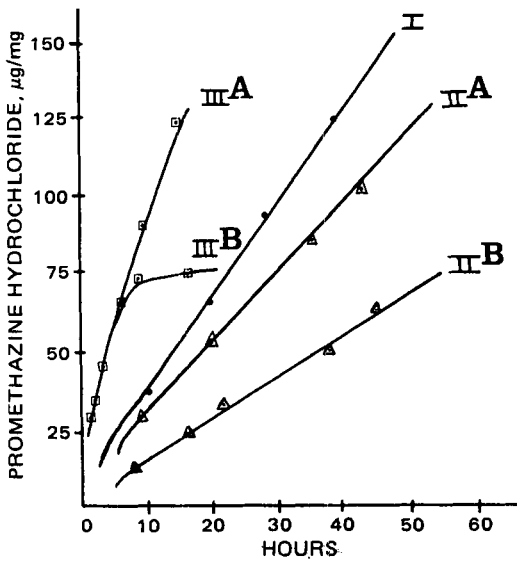


Figure 5—*Influence of copper(II) and iron(III) on the formation of promethazine 5-oxide at pH 3.2 under aerobic conditions at 65°. Key: curve I, no additive; curve IIA, 10^{-4} M iron(III); curve IIB, 10^{-3} M iron(III); curve IIIA, 10^{-4} M copper(II); and curve IIIB, 10^{-3} M copper(II).*

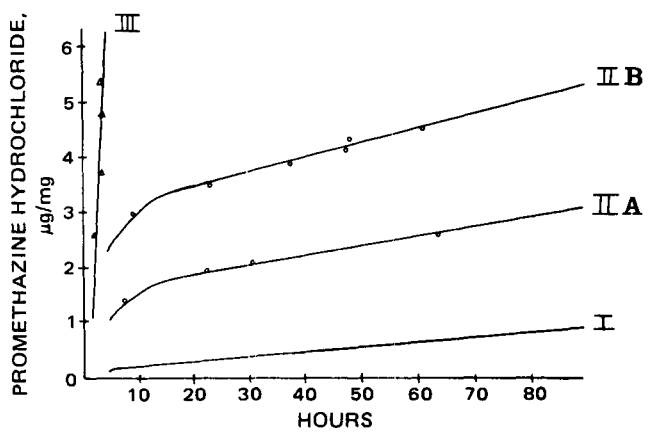


Figure 6—*Influence of copper(II) and iron(III) on the formation of 7-hydroxy-3H-phenothiazine-3-one at pH 3.2 under aerobic conditions at 65°. Key: curve I, no additive; curve IIA, 10^{-3} M iron(III); curve IIB, 5×10^{-3} M iron(III); and curve III, 5×10^{-3} M copper(II).*

Figure 3 shows that the extrapolated intercepts at zero time of the second part of the curves decreased with an increasing iron(III) concentration. For solutions without added iron(III), the intercepts were also below 100%, probably because of contamination of the reagents with very

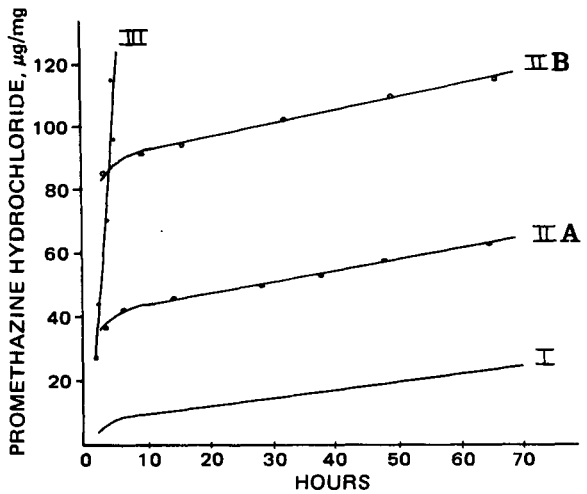


Figure 7—*Influence of copper(II) and iron(III) on the formation of 3H-phenothiazine-3-one at pH 3.2 under aerobic conditions at 65°. Key: curve I, no additive; curve IIA, 10^{-3} M iron(III); curve IIB, 5×10^{-3} M iron(III); and curve III, 5×10^{-3} M copper(II).*

Table III—Influence of Edetate Disodium on the Rate Constant *k′* of Promethazine Degradation at pH 3.2 and 65°

Edetate Disodium, *M*	k', sec^{-1}
0	28.9×10^{-7}
2×10^{-3}	13.9×10^{-7}
10^{-2}	12.8×10^{-7}

low concentrations of iron(III). Promethazine degradation in the presence of edetate disodium was compared with the degradation without this additive. Table III shows that the rate constant decreased on addition of edetate disodium but that the concentration of the additive had no further influence on it. The "zero-time" value of the curves in the presence of edetate disodium was 100%. Moreover, the formation of 3*H*-phenothiazine-3-one was greatly reduced while the amount of 7-hydroxy-3*H*-phenothiazine-3-one was negligible. The formation of promethazine 5-oxide was hardly influenced. The rate constant of promethazine degradation was reduced by edetate disodium at low pH values; at pH 6.3, *k′* had the same value as the standard (Fig. 1). The data available led to the conclusion that the stabilizing effect of edetate disodium on promethazine is due to removal of metal ions, primarily iron(III), from the solutions.

Influence of Antioxidants—Of the antioxidants investigated at pH 3.2, only hydroquinone and ascorbic acid interfered in the oxidation process. Sodium pyrosulfite had no effect under these circumstances.

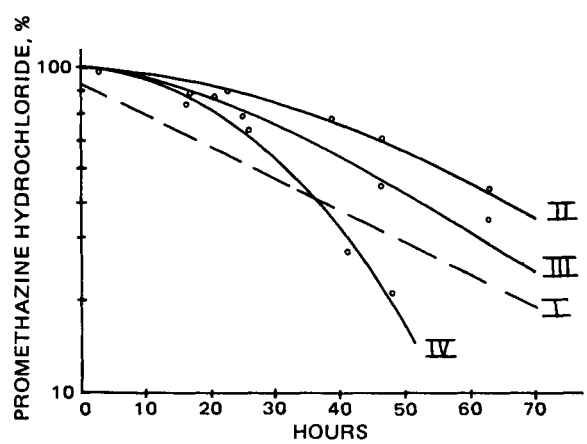


Figure 8—*Influence of hydroquinone on the degradation of promethazine at pH 3.2 under aerobic conditions at 65°. Key: curve I, no additive; curve II, 0.1% hydroquinone; curve III, 0.5% hydroquinone; and curve IV, 1.0% hydroquinone.*

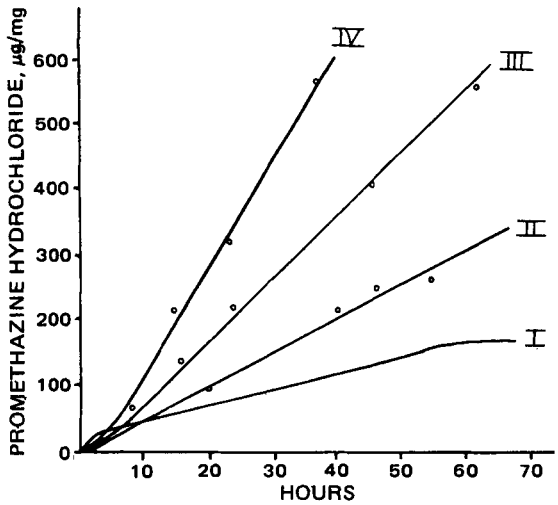


Figure 9—*Influence of hydroquinone on the formation of promethazine 5-oxide at pH 3.2 under aerobic conditions at 65°. Key: curve I, no additive; curve II, 0.1% hydroquinone; curve III, 0.5% hydroquinone; and curve IV, 1.0% hydroquinone.*

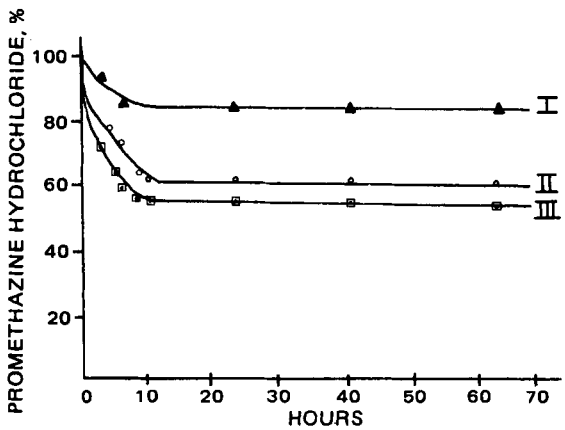


Figure 10—*Influence of copper(II) and iron(III) on the degradation of promethazine at pH 3.2 under anaerobic conditions at 100°. Key: curve I, no additive; curve II, 10^{-3} M iron(III); and curve III, 10^{-3} M copper(II).*

Ascorbic acid had an unpredictable effect on promethazine stability. Sometimes stabilization occurred, but the degradation increased in other cases. This phenomenon is possibly due to the instability of the antioxidant itself at this pH (10).

Figure 8 shows that low concentrations of hydroquinone protected promethazine from degradation. On increasing the concentration, the degradation accelerated and the reaction was not first order.

Figure 9 shows that the formation of promethazine 5-oxide increased dramatically in the presence of hydroquinone while other degradation products hardly were formed.

Degradation of Promethazine under Anaerobic Conditions—Only some initial degradation occurred, probably due to traces of oxygen left in the solution. When this oxygen was consumed, no further degradation took place. This degradation could be suppressed by adding antioxidants such as sodium pyrosulfite, hydroquinone, and ascorbic acid.

Degradation of promethazine under anaerobic conditions occurred in the presence of copper(II) and iron(III) only when these metals were present in the highest oxidation state (Fig. 10). The degradation products formed under these conditions were 10-methylphenothiazine, phenothiazine, and 3*H*-phenothiazine-3-one.

DISCUSSION

Scheme I represents the most probable mechanism for the oxidative degradation of promethazine (I). The first step is the loss of an electron from I to give a semiquinone free radical (II) (11–17). This reaction is reversible. Compound II is only stable under certain conditions. It can lose another electron to give the phenazothionium ion (III), and it can disproportionate to give I and III. Compound III hydrolyzes to promethazine 5-oxide (IV).

Radical II is subjected to another reaction, as shown by Waaler (18). Cleavage of the side chain passes *via* II since IV is the only product with an intact side chain. Waaler (18) found that the products of this reaction are 10-methylphenothiazine, acetaldehyde, and dimethylamine. Previously (6), it was shown that phenothiazine and formaldehyde also are formed. Roseboom and Perrin (19) showed that degradation of 10-methylphenothiazine does not lead to the formation of these products, so it is postulated that cleavage of the side chain of II results in the formation of the intermediate V*a*. This compound can be reduced to give 10-methylphenothiazine (VI); from the rest of the side chain, acetaldehyde (XVI) and dimethylamine (XVII) are formed *via* the carbonium ion XV.

Radical V*a* can undergo mesomerization to give V*b*. This intermediate is subjected to oxidation to give the carbonium ion VII, which can be hydrolyzed to phenothiazine VIII. The rest of the side chain is split off during this process, and formaldehyde (XVIII) is formed. Carbonium ion VII also can be oxidized to give the intermediate IX, which can be hydrolyzed to give the semiquinone radical X and XVIII. Compound X disproportionates to give VIII and phenazothionium ion XI*a*, as shown by Roseboom and Perrin (19). From XI*a*, 3*H*-phenothiazine-3-one (XIII) and 7-hydroxy-3*H*-phenothiazine-3-one (XIV) are formed *via* mesomer XI*b*. Compound XI*a* also undergoes hydrolysis to give phenothiazine 5-oxide (XII).

The influence of such factors as pH, metal ions, and antioxidants on the degradation process will be different for the two parallel reactions of radical II. An increase in pH causes an increase in the value of k'. At lower pH values, the formation of IV also increases. At pH 4.3, a maximum occurs; a further increase in pH greatly reduces the formation of IV. This phenomenon is not due to decomposition of IV, since IV is stable. Moreover, the formation of VI and VIII seems to increase with increasing pH. The explanation is that the cleavage of the side chain, *i.e.*, the formation of V*a* from II, is catalyzed by hydroxide ions in agreement with the findings of Waaler (18). An increase in pH decreases the formation of XIII and XIV, indicating that these compounds are formed under the influence of protons, as also shown by Roseboom and Perrin (19).

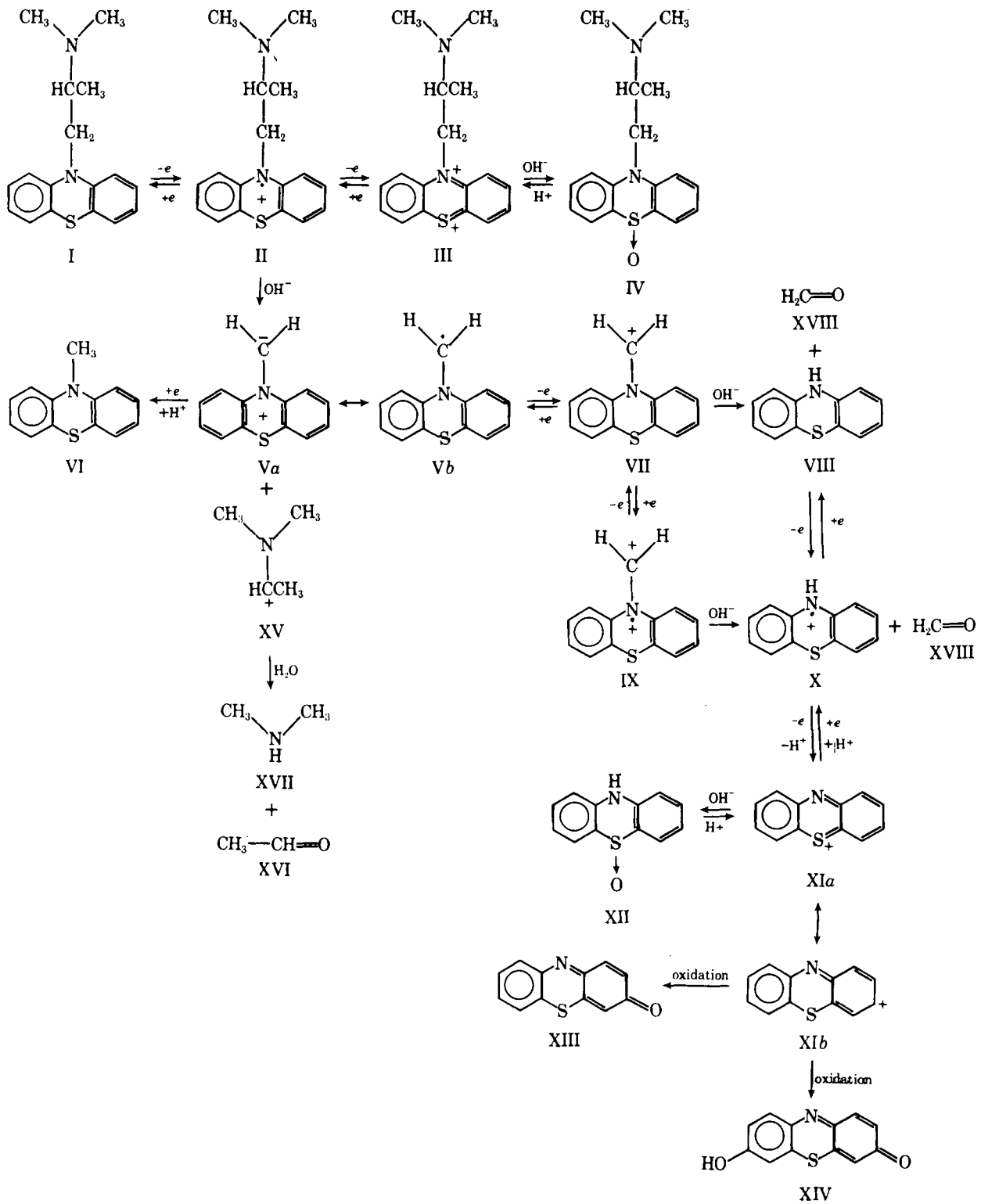
The degradation process is highly influenced by copper(II) and iron(III), but other metals do not interfere. Copper(II) catalyzes the formation of radical II from I, shown by the increase of k' and the increase in the formation of IV. Copper(II) ions also influence the formation of radical V*a* from II, shown by the increase of XIII and XIV. Since addition of copper(II) to a solution of I causes the formation of VIII and XIII under anaerobic conditions, copper(II) also promotes the formation of VII and the oxidation of this compound.

The influence of iron(III) on the degradation process is apparently different. There is a dramatic increase in the initial degradation rate on adding iron(III) ions to a solution of I, but the degradation rate after a short time decreases and a first-order reaction occurs with a rate constant equal to k' of the degradation without the addition of iron(III). The initial concentration of iron(III) causes a high oxidation potential and, thus, a high reaction rate. On reduction of iron(III) to iron(II), the oxidation potential decreases, causing a decrease in the degradation rate to the level of the degradation without added iron(III).

This phenomenon cannot be explained by assuming that hydrolysis of iron(III) at pH 3.2 causes precipitation of this ion since solutions of iron(III) with this pH remain clear even after storage. The difference between iron(III) and copper(II) is probably due to the much higher reaction rate of the oxidation of copper(I) to copper(II) under aerobic conditions in comparison to the oxidation of iron(II) to iron(III), which causes a higher oxidation potential in the copper-containing solutions during the whole degradation process.

Iron(III) promotes the cleavage of the side chain and the formation of radical V*a*, as shown by the decrease in the formation of IV, the increase in the formation of products without a side chain, and the occurrence of the latter products under anaerobic conditions. Both the formation of radical V*a* and carbonium ion VII are promoted.

Addition of edetate disodium decreases the rate constant k' of the degradation reaction, especially at lower pH values, while the speed of the formation of IV is almost the same and hardly any XIII and XIV are formed. The explanation of this phenomenon is that low concentrations of iron(III) in the medium catalyze the formation of radical II and the split off of the side chain and, thus, the formation of radical V*a* and other intermediates. The removal of iron(III) from the solution by addition of edetate disodium decreases the degradation and suppresses the cleavage



Scheme I

of the side chain so that IV is the main degradation product under these conditions.

Under the influence of hydroquinone, promethazine degradation initially decreases; but when the concentration of the antioxidant is increased, the degradation rate increases. Apparently, the oxidation products of hydroquinone, also being radicals (20), interfere in the redox process by promoting the formation of II and III. Since the formation of radical V*a* is by hydrolysis, the influence of hydroquinone on this reaction is negligible and IV is the main degradation product.

The influence of ascorbic acid on promethazine degradation probably can be explained in the same manner. Kassem *et al.* (10) found that ascorbic acid rapidly decomposes at lower pH values. During this process, radicals are formed that might interfere in the degradation of promethazine.

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Determination of Ethinyl Estradiol in Human Urine by Radiochemical GLC

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Received June 6, 1977, from the *Department of Drug Metabolism, Warner-Lambert Research Institute, Morris Plains, NJ 07950.* Accepted for publication December 1, 1977. *Present address: ICI Americas, Wilmington, DE 19897. ‡Present address: Hoffmann-La Roche Inc., Nutley, NJ 07111. §Present address: Office of Toxic Substances, U.S. Environmental Protection Agency, Washington, DC 20460.

Abstract □ A radiochemical GLC analysis was developed for ^{3}H-labeled ethinyl estradiol in human urine. The technique was applied to the unconjugated and aglycone fractions of urine collected from women who were dosed orally with: (*a*) single capsules containing 2.0 mg of ^{3}H-quinestrol (900 μCi) and 2.5 mg of unlabeled quingestanol acetate dissolved in sesame oil and (*b*) single tablets containing 100 μg of ^{3}H-quinestrol (86 μCi). Unconjugated ethinyl estradiol in Day 1 urine collections represented means of 0.02% of the high quinestrol dose and 0.12% of the low dose. Ethinyl estradiol glucuronide in the same collections represented means of 0.55% of the high drug dose and 1.35% of the low dose. The method could detect 1-ng quantities of ^{3}H-ethinyl estradiol and ^{3}H-quinestrol.

Keyphrases □ Ethinyl estradiol—radiochemical GLC analysis in human urine □ Radiochemical GLC—analysis, ethinyl estradiol in human urine □ GLC, radiochemical—analysis, ethinyl estradiol in human urine □ Estrogens—ethinyl estradiol, radiochemical GLC analysis in human urine

Radiochemical GLC (1–14), a system of monitoring the eluates from GLC columns for radioactive compounds, has the capability of detecting only the labeled compounds in complex mixtures of predominantly unlabeled components. Moreover, this system offers flexibility, convenience, specificity, and high sensitivity.

This paper reports a drug metabolism study using radiochemical GLC. Single low doses of ^{3}H-quinestrol (17α-ethinyl estradiol 3-cyclopentyl ether) were administered to women, and their urine was assayed for quinestrol and its metabolite ethinyl estradiol. This biotransformation was described previously (15, 16), but information on the extent of the conversion is lacking.

EXPERIMENTAL

Radioactive Ethinyl Estradiol—Merrill and Vernice (17) described the synthesis of 6,7-^{3}H-ethinyl estradiol with a specific activity of 275 mCi/mmole. Its chemical purity and radiochemical purity were approximately 99%.

Radioactive Quinestrol—The synthesis of this compound (mol. wt. 364.5) from ^{3}H-ethinyl estradiol also was reported previously (17). The oral contraceptive dose consisted of a capsule containing 2.0 mg of ^{3}H-quinestrol (900 μCi) and 2.5 mg of unlabeled quingestanol acetate in 0.2 ml of sesame oil stabilized with 0.05% piperidine. The estrogen replacement dose was a tablet containing 100 μg of ^{3}H-quinestrol (86 μCi).

Radioactivity Measurements—The scintillation counting solution was prepared by dissolving 6 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene in 1 liter of ethanol–toluene (17:100). By using internal standardization, 200-μl samples of urine were counted in 20 ml of this solution with a liquid scintillation spectrometer[1] at 4°.

Radiochemical GLC—An oven and proportioning temperature controller[2] were connected to a gas proportional counter[3] by a heated glass-lined metal tube. The operating conditions for the gas chromatograph were: column, 1.8 m coiled glass, 2 mm i.d., 20% SE-30 on 80–100-mesh Gas Chrom Q; carrier gas (helium) flow rate, 50 ml/min; oven temperature, 215°; and injector temperature, 245°. The operating conditions for the gas proportional counter were: quench gas (propane) flow rate, 2.5 ml/min; hydrogen flow rate, 10 ml/min; transfer line temperature, 237°; and oxidizer and reduction furnace temperature, 750°.

Preparation of Standard Curves—Quantitation of ^{3}H-ethinyl estradiol peaks was accomplished by the following absolute calibration method. A stock solution of ^{3}H-ethinyl estradiol in methanol was prepared; its concentration was 140,380 dpm/4.0 μl. From this stock solution, serial twofold dilutions were made to give a set of standards corresponding to 70,190, 35,100, 17,550, and 8775 dpm/4.0 μl. Aliquots (4.0 μl) of the standards were injected into the gas chromatograph so that a standard curve could be constructed by integration (planimetry) of the resultant peaks. Data plotted in this manner yielded acceptable straight lines. However, it was necessary to generate a new curve each day because the slope varied from day to day.

The standard curves from one column are shown in Fig. 1; curve A was obtained on Day 1, curve B on Day 2, curve C on Day 3, and curve D on

[1] Tri-Carb model 3320, Packard Instrument Co., Downers Grove, Ill.
[2] Warner-Chilcott, Morris Plains, N.J.
[3] Model 894, Packard Instrument Co., Downers Grove, Ill.

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