from the Union Carbide and Carbon Corp., and we wish to thank Dr. H. Mark for his encouragement of this work.

Summary

A kinetic study of acid- and alkaline-catalyzed hydrolysis of ethyl silicate reveals that the acid catalyzed hydrolysis is second order with respect to water and ethyl silicate, and that the rate is further directly proportional to the acid concentration used. The basic hydrolysis is first order with respect to ethyl silicate, proportional to the concentration of base and independent of the amount of water present. It is, moreover, complicated by silicate formation at higher concentrations.

The value of the activation energy of acid hydrolysis has been determined. The nature of the polymer formed and its stability in solution has been investigated. For the acid hydrolysis a mechanism intermediate between general acid and oxonium ion catalysis has to be postulated. A dimolecular as well as a termolecular reaction path appear possible. The base catalysis involves a nucleophilic displacement of the alkoxy group by the hydroxyl ion.

BROOKLYN, NEW YORK RECEIVED DECEMBER 21, 1949

[CONTRIBUTION FROM THE DIVISION OF PHARMACOLOGY AND TOXICOLOGY, UNIVERSITY OF ROCHESTER, SCHOOL OF MEDICINE AND DENTISTRY]

The Isolation and Characterization of Two Metabolic Products of Myanesin (3-(*o*-Toloxy)-1,2-propanediol)[1,2]

BY RICHARD F. RILEY[3]

Graves, Elliott and Bradley[4] have reported the isolation of β-(*o*-toloxy)-lactic acid, m. p. 146–147°, from the urine of man and rabbits which had received myanesin. Working independently, we isolated the same compound as well as a second metabolic acid, m. p. 168°, which is responsible for the positive Ehrlich diazo reaction[5] given by metabolic urines of man and experimental animals which have received the drug. The chromogenic substance, m. p. 168°, has now been identified as β-(2-methyl-4-hydroxyphenoxy)-lactic acid on the basis of its chemical and physical properties and by degradation to toluhydroquinone. A preliminary communication regarding this work has appeared.[6]

It is the purpose of the present paper to present (a) a method of isolation of these compounds, (b) pertinent data which have now led to a determination of the structure of the chromogen and (c) some information concerning the extent of metabolic conversion and excretion of the two substances.

Initially, we were successful in obtaining small quantities of the two compounds from human urine by a lengthy process involving adsorption on Darco G-60, elution with acetone and concentration of the carbon eluate followed by solvent and chromatographic fractionation. More recently, the counter-current distribution technique has been employed as a convenient means of isolation of both compounds, and has provided sufficient amounts of the chromogen to simplify degradation studies.

It was readily demonstrated, on the basis of carbon and hydrogen analyses, the neutral equivalent and ultraviolet absorption spectra, that the first metabolic product was β-(*o*-toloxy)-lactic acid.[6] That the chromogen was metabolically derived from β-(*o*-toloxy)-lactic acid was indicated since urine of rats which had received this compound showed the characteristic chromogenicity of urine of animals which had received myanesin. Combustion analyses on several different samples indicated that the empirical formula of the chromogen differed from that of the first metabolite by the addition of one oxygen atom. The second compound was, like the first, optically inactive. Electrometric titration showed the second compound to be a relatively strong acid, pK 3.5.

From these observations we concluded that the chromogen must be one of the four isomeric β-(hydroxy-2-methyl phenoxy)-lactic acids. Earlier observations that *o*-cresol, phenetol and diphenyl ether are metabolized to toluhydroquinone,[7] the glucuronide of *p*-ethoxyphenol[8] and *p*-hydroxydiphenyl ether,[9] respectively, led us to suspect that the chromogen was, in fact, β-(2-methyl-4-hydroxy phenoxy)-lactic acid, formed from β-(*o*-toloxy)-lactic acid by an analogous oxidation of the benzene ring para to the phenyl ether linkage. In further support

(1) Presented in part before the Division of Biological Chemistry of the American Chemical Society at the San Francisco, California, Meeting, 1949.

(2) Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

(3) University of California Atomic Energy Project, Post Office Box 31, Beverly Hills, California.

(4) Graves, Elliott and Bradley, *Nature*, **162**, 257 (1948).

(5) Hawk and Bergeim, "Practical Physiological Chemistry." 11th ed., P. Blakiston's Son and Co., Inc., Philadelphia, Pa., 1937, p. 669.

(6) Riley and Berger, *Arch. Biochem.*, **20**, 159 (1949).

(7) Preusse, *Z. physiol. Chem.*, **5**, 57 (1881).

(8) Kossel, *ibid.*, **4**, 296 (1880); **7**, 292 (1883); Lehmann, *ibid.*, **13**, 181 (1889).

(9) Stroud, *J. Endocrinol.*, **2**, 55 (1940).

of this supposition was the observation that hydroquinone monomethyl ether gives the Ehrlich diazo reaction, although hydroquinone does not. No doubt hydroquinone reduces the benzene diazonium sulfonate to colorless benzene sulfonic acid while the monomethyl ether undergoes coupling to a red dye.[10]

Degradation of the chromogen with constant boiling hydrobromic acid gave a 53% yield of toluhydroquinone, identified by mixed melting point, ultraviolet absorption spectra, paper partition chromatography and conversion to toluquinone. These data assign the structure of the chromogen as β-(2-methyl-4-hydroxyphenoxy)-lactic acid.

Experimental[11]

1. Isolation of β-(*o*-Toloxy)-lactic Acid and β-(2-Methyl-4-hydroxyphenoxy)-lactic Acid from Metabolic Urine.—Seven and seven-tenths liters of urine were collected from a hospital patient who reportedly had received 19.0 g. of myanesin (1.5 g./day). The urine was saturated with sodium sulfate, the *p*H adjusted to 3.0 and extracted in a continuous extractor for 70 hours with 500 cc. of peroxide-free ether. The ether solution was extracted with an excess of 1 *M* sodium bicarbonate. To the bicarbonate solution of acids was added 1.8 mole of sodium dihydrogen phosphate, the whole diluted to 900 cc. and the *p*H adjusted to 4.5. This solution was re-extracted in a continuous extractor with peroxide-free isopropyl ether until a detectable amount of chromogen was present in the isopropyl ether (Fraction 1). The *p*H of the partially extracted aqueous concentrate was lowered to 3.0 by the addition of phosphoric acid and extraction continued with ethyl ether until no further chromogen was recovered (Fraction 2).

Fraction 1 was re-extracted three times with 20-cc. portions of 2 *M* potassium bicarbonate and carefully acidified to congo red with dilute phosphoric acid. The crystalline slurry of β-(*o*-toloxy)-lactic acid was heated to boiling with the addition of sufficient water to effect solution, decolorized with charcoal and filtered. Filtration and washing with cold water after 48 hours in the icebox gave 2.36 g. of faintly colored β-(*o*-toloxy)-lactic acid.

Fraction 2, volume about 200 cc., was permitted to stand in the icebox for two months during which time a considerable quantity of crystalline material separated. The supernatant ether was decanted and saved for fractionation by counter-current distribution. The crystals were dissolved in a minimum of boiling water, decolorized with Norite, to yield on cooling 1.13 g. of a white crystalline compound, m. p. 186°, which was identified as hippuric acid.

Anal. Calcd. for $C_9H_9O_3N$; C, 60.33; H, 5.06; neut. eq., 179.2. Found: C, 60.16; H, 4.90; neut. eq., 181.1.

The supernatant ether, containing 56 milliequivalents of acid, was concentrated to 50 cc. and submitted to a 21-tube separation against 50 cc. of saturated phosphate buffer, *p*H 3.5, employing the separatory funnel method of Craig and co-workers.[12] By evaporating the stationary ether phase of funnels 0 to 3, inclusive, and recrystallizing the residue from water, there was obtained 0.71 g. more of pure white β-(*o*-toloxy)-lactic acid, m. p. 144°. The melting point was raised by further recrystallization to 146° and showed no depression on admixture with the synthetic compound.

Anal. Calcd. for $C_{10}H_{12}O_4$: C, 61.20; H, 6.16. Found: C, 60.92; H, 6.32; another sample gave: C, 61.05; H, 5.82.

The total recovery of β-(*o*-toloxy)-lactic acid was 3.07 g. which is equivalent to 15% of the ingested drug.

The contents of funnels 4, 5 and 6 were discarded. Both phases of funnels 7 to 12, inclusive, which by qualitative colorimetric analysis contained most of the chromogen, were pooled, the buffer phase adjusted to *p*H 5.5, and the chromogen passed to the buffer by shaking out. The buffer was acidified to *p*H 2.8 with phosphoric acid and the chromogen extracted into 50 cc. of ethyl ether in a small continuous liquid-liquid extractor. Thirty cc. of benzene was added to the ether solution of chromogen and the solvents removed by fractional distillation until about 15 cc. of a benzene solution remained. The volume of benzene was adjusted to 16.3 cc., 8.7 cc. of isobutyl alcohol was added and the chromogen again distributed, this time against 25 cc. of water in a 21-funnel system, employing isobutyl alcohol–benzene, 35:65, as the stationary phase.

Analysis, Fig. 1, showed that funnels 4 to 7 contained mainly residual hippuric acid mixed with chromogen, funnels 9 to 11 contained fairly pure chromogen and funnels 13 to 15 contained a third, at present unidentified, acid contaminated with chromogen.

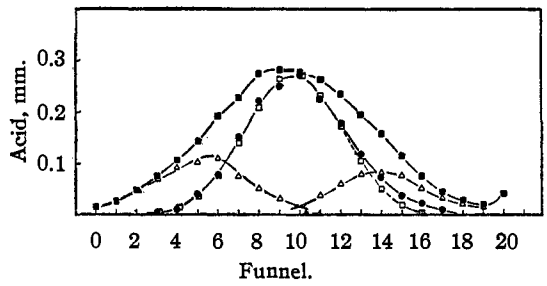

Fig. 1.—Counter-current distribution of a concentrate of chromogen: ■—■, total acid by titration; ●—●, chromogen determined colorimetrically; □—□, theoretical distribution curve, $K = 0.86$ for chromogen; Δ—Δ, difference between total acid by titration and chromogen by colorimetry; for system isobutyl alcohol–benzene (35:65)–water.

By colorimetric analysis (see below) the system contained 3.45 millimoles of chromogen which represents 3.3% of the ingested myanesin.

The contents of funnels 9 to 11 were evaporated to a small volume, charcoaled and the filtrate evaporated to dryness. Recrystallization, effected from 15 cc. of boiling benzene containing just sufficient butyl alcohol to dissolve the solid, gave 41.7 mg. of pure chromogen. Another 68.5 mg. of slightly yellow chromogen was obtained by evaporating the filtrate and recrystallizing the residue from hot 2-nitropropane. Some chromogen was also obtained by similar crystallizations of material contained in funnels 11–13, to give a total recovery of 150 mg. of chromogen, m. p. 165–166°, insoluble in benzene and petroleum ether, very soluble in water, alcohol, acetone and ether.

Anal. Calcd. for $C_{10}H_{12}O_4$: C, 56.60; H, 5.70. Found: C, 56.62; H, 5.75. The first sample prepared by adsorption on carbon and chromatographic fractionation of the eluates gave: C, 57.00; H, 5.60.

Electrometric titration of 24.05 mg. of chromogen with 0.01075 *N* sodium hydroxide showed a single inflection. The neutral equivalent, calculated for $C_9H_{11}O_3COOH$: 212.1; found, 208. The *p*H at half neutralization was 3.5.

The ultraviolet absorption spectrum was determined on a Beckman Model DU spectrophotometer. The spectrum of the chromogen, dissolved in 95 per cent. ethanol, showed a maximum at 290 mμ and a minimum at 251 mμ. $E^1_{1\,cm}$ 2955 at 290 mμ. The rotation in water was

(10) Oreton and Everatt, *J. Chem. Soc.*, **93**, 1010 (1908).

(11) All melting points were determined on a micro melting point stage and are uncorrected. Microanalyses by C. W. Beazley, Skokie, Illinois.

(12) Craig, Golumbic, Mighton and Titus, *J. Biol. Chem.*, **161**, 321 (1945).

$[\alpha]^{28}D$ + 3.13° (c = 1.63) (0.163 g. in 10 cc. of water gave α + 0.051, P.E. = 0.041, in a 1-dm. tube).

2. **Degradation of the Chromogen.**—In model degradation experiments, carried out on β-(*o*-toloxy)-lactic acid, it was found that fusion with anhydrous pyridine hydrochloride[13] gave 40 to 50% yields of *o*-cresol. Degradation with constant boiling hydrobromic acid gave *o*-cresol in yields of 25 to 30%. Degradation of β-(*o*-toloxy)-lactic acid with concentrated hydrochloric acid or 48% hydriodic acid at elevated temperatures in sealed tubes gave poor yields of *o*-cresol.

When the pyridine hydrochloride procedure was applied to chromogen, considerable unidentified non-phenolic material was obtained together with a small amount of toluhydroquinone, provisionally identified by paper chromatography.[14] Degradation of the chromogen with anhydrous aluminum bromide in benzene[15] was unsuccessful. Since degradation with constant boiling hydrobromic acid appeared to be the next method of choice, it was applied to 134 mg. of analytically pure chromogen.

The chromogen was weighed into a small heavy-walled tube sealed at one end, 0.5 cc. of constant boiling hydrobromic acid was added, the tube was flushed with nitrogen and sealed off and heated 3 hours at 130°. The reactants were transferred to a separatory funnel with 10 cc. of water and the dark brown solution, containing only traces of chromogen extracted five times with 5- to 10-cc. portions of peroxide-free ether. The pooled ether extracts were washed once with 3 cc. of saturated potassium bicarbonate, and the light orange ether solution dried with anhydrous sodium sulfate. One-tenth of this solution was retained for examination by partition chromatography on paper.[14] The remainder was evaporated to a dark semi-crystalline gum in a small sublimation unit. This residue was sublimed at 20 mm. at 125–150°, to give 36.7 mg. of nearly white sublimate or 53%, calculated as toluhydroquinone, melting sharply at 126.5° and showing no depression on admixture with authentic toluhydroquinone. A few mg. of less pure toluhydroquinone melting at 124-125° was also recovered.

Examination of the crude degradation product by paper partition chromatography showed only the presence of toluhydroquinone and traces of unhydrolyzed chromogen. No other phenols were indicated.

Ten mg. of the degradation product was oxidized with chromic acid to yield 4.6 mg. of toluhydroquinone, lemon yellow needles, m. p. 65.5° after resublimation. The mixed m. p. with authentic toluquinone showed no depression.

3. **Colorimetric Determination of Chromogen.**—Several attempts were made to develop a quantitative colorimetric method of determination of chromogen in metabolic urine, employing either Ehrlich diazo reagent or the modification described by Hunter.[16] These attempts were unsuccessful. Solutions of nearly pure chromogen were found, however, to react in predictable fashion with Ehrlich reagent when treated by the following procedure.

To 25 to 125 μg. of chromogen contained in 5 to 15 cc. of water are added 2.0 cc. of fresh Ehrlich reagent and 2.0 cc. of concentrated ammonium hydroxide and the whole diluted to 25 cc. The pink color which develops is read after one hour in a Klett photoelectric colorimeter, employing a Farrand filter with maximum transmittancy at 525 mμ. Color intensity was found to be proportional to concentration over the range indicated.

Normal urine apparently contains something, perhaps catechol and hydroquinones, which partially inhibit color development and lead to erratic results when the method is applied to metabolic urines. The procedure given above served to follow the chromogen in counter-current distributions of reasonably pure material.

4. **Synthesis of β-(*o*-Toloxy)-lactic Acid.**—The preparation of this compound has been reported but without details.[4,6] The method of synthesis was similar to that employed by Koelsch[17] for the preparation of β-phenoxylactic acid. To 2.16 g of freshly distilled *o*-cresol dissolved in 10 cc. of 12% sodium hydroxide was added 1.25 g. β-chlorolactic acid and the mixture refluxed two hours. The reactants were adjusted to *p*H 6.5 to 7.0 with dilute hydrochloric acid and steam distilled. The crude acid remaining behind was precipitated from the cooled solution by acidification with hydrochloric acid. The filtered product was recrystallized with charcoaling from hot water to give 1.04 g., 53%, of pure β-(*o*-toloxy)-lactic acid, m. p. 146–146.5°.

Anal. Calcd. for $C_{10}H_{12}O_4$: C, 61.20; H, 6.16. Found: C, 61.37; H, 6.22.

Acknowledgment.—The author wishes to acknowledge the coöperation of Dr. Elmer Stotz in providing us with several facilities of the Department of Biochemistry.

Summary

A method of isolation of two acidic metabolic products of myanesin (3-(*o*-toloxy)-1,2-propanediol) from human urine is described. The first metabolic product, β-(*o*-toloxy)-lactic acid, was recovered in a yield equivalent to 15% of the ingested drug. The second metabolic compound, which gives the Ehrlich diazo reaction, was characterized by degradation to toluhydroquinone, as β-(2-methyl-4-hydroxyphenoxy)-lactic acid. The latter compound, recovered in a yield equivalent to 3% of the ingested drug, is undoubtedly derived from the first and provides another example of a biochemical oxidation of the benzene ring para to a phenyl oxygen linkage.

ROCHESTER 20, NEW YORK RECEIVED MARCH 13, 1950

(13) Prey, *Ber.*, **75B**, 350 (1942).

(14) Riley, THIS JOURNAL, **72**, 5782 (1950).

(15) Pfeiffer and Loewe, *J. prakt. Chem.*, **147**, 293 (1936).

(16) Hunter, *Biochem. J.*, **19**, 25 (1925).

(17) Koelsch, THIS JOURNAL, **52**, 2430 (1930).