[CONTRIBUTION FROM THE STERLING-WINTHROP RESEARCH INSTITUTE]

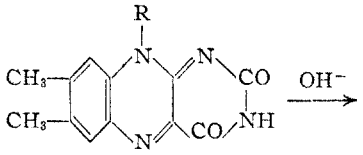
Alkaline Hydrolysis of Riboflavin

BY ALEXANDER R. SURREY AND FREDERICK C. NACHOD

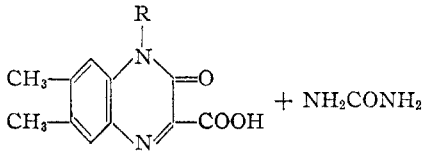
Riboflavin is hydrolyzed in alkaline solution to give urea and 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic acid. The latter has been shown to have a depressant action on cardiac and visceral muscles when injected intravenously in the dog. The kinetics of the alkaline degradation of riboflavin has been followed spectrophotometrically.

It has been found[1] that alkaline solutions of riboflavin, incubated at 60°, acquired the property of lowering the blood pressure of anesthetized dogs when administered intravenously. The present work was undertaken to isolate and identify the material responsible for this depressor action.

It is known that heating riboflavin in alkaline solution results in a decrease in its vitamin activity when tested in experimental animals.[2] Under similar conditions, the fluorescence associated with riboflavin gradually fades.[3] The hydrolysis of riboflavin was investigated by Kuhn and Wagner-Jauregg[4] who showed that urea was one of the products formed. Urea was also obtained[5] from the hydrolysis of lumiflavin (III) along with 1,2-dihydro - 2 - keto - 1,6,7 - trimethyl - 3 - quinoxalinecarboxylic acid (IV). The structure of the latter

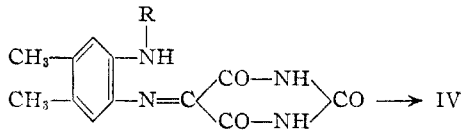


I, R is D-ribityl
III, R is CH_3



II, R is D-ribityl
IV, R is CH_3

was proven by synthesis[6] from the anil V, and later confirmed by Wellman and Tishler.[7] These



V, R is CH_3

authors prepared the quinoxaline compound, IV, by the condensation of 2-amino-4,5-dimethyl-1-methylaminobenzene and ethyl oxomalonate.

On the basis of similarity in structure of riboflavin (I) and lumiflavin (III) one would expect both compounds to be cleaved in the same manner by alkaline hydrolysis. This has now been found to be true. Riboflavin, on hydrolysis, yields urea and 1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic acid (II).

Attempts to isolate this degradation product (II) by extraction with an organic solvent according to the procedure employed by Kuhn and Rudy[5b] for IV were not successful. It was finally obtained by hydrolyzing the riboflavin in 1 *N* sodium hydroxide solution, neutralizing the resulting solution with acetic acid and allowing the mixture to stand at 0° for three days. The solid which separated from solution proved to be the sodium salt of 1,2-dihydro - 6,7 - dimethyl - 2 - keto - 1 - D - ribityl-3-quinoxalinecarboxylic acid monohydrate. Even at *p*H 5 the sodium salt could be isolated. Urea was isolated from the filtrate as dixanthylurea. The free acid II was obtained from an aqueous solution of the sodium salt by acidification with an excess of dilute sulfuric acid. After recrystallization from ethanol the product melted at 183–183.5° (cor.) with decomposition. The ultraviolet absorption spectra of the 1-D-ribitylquinoxaline (II) is almost identical (Fig. 1) with that reported for the 1-methylquinoxaline compound IV.

A search in the literature revealed that the riboflavin degradation product II had been synthesized by Tishler, Wellman and Ladenburg[8] during a study of methods of preparing isoalloxazines. These authors obtained II by hydrolysis of the anil (V, R is D-ribityl) prepared from 2-amino-4,5-dimethyl-1-D-ribitylaminobenzene and alloxan. The same product, m.p. 176°, was also prepared from the hydrolysis of the corresponding ester which was prepared from ribitylaminoxylidene and ethyl oxomalonate.

The preliminary pharmacological investigation of the quinoxaline compound II has shown the substance to have a depressant action on cardiac and visceral muscles. Intravenous injection in the dog is most often characterized by a marked fall in blood pressure, brachycardia and relaxation of the intestine.

Having found that the keto acid II was the depressant agent formed from riboflavin, it seemed desirable to investigate the conditions for its formation and also to determine whether any other similar compounds showed this activity. In this connection, solutions of D- and L-arabinoflavin in 1 *N* sodium hydroxide were warmed on a steam-bath for a few minutes, neutralized with acetic acid and submitted for testing. The hypotensive effect was similar to that caused by the riboflavin degradation product II. Apparently hydrolysis of these isoalloxazines occurs very rapidly. The

(1) Personal communication, Miss Estelle Ananenko of the Pharmacology Department of this Institute.

(2) H. Chick and M. H. Roscoe, *Biochem. J.*, **24**, 105 (1930).

(3) R. Kuhn and G. Moruzzi, *Ber.*, **67**, 888 (1934).

(4) R. Kuhn and T. Wagner-Jauregg, *ibid.*, **66**, 1577 (1933).

(5) (a) R. Kuhn, H. Rudy and T. Wagner-Jauregg, *ibid.*, **66**, 1950 (1933); (b) R. Kuhn and H. Rudy, *ibid.*, **67**, 892 (1934); (c) R. Kuhn, K. Reinemund and F. Weygand, *ibid.*, 1460 (1934).

(6) R. Kuhn and K. Reinemund, *ibid.*, **67**, 1932 (1934).

(7) J. W. Wellman and M. Tishler, THIS JOURNAL, **69**, 714 (1947).

(8) M. Tishler, J. W. Wellman and K. Ladenburg, *ibid.*, **67**, 2165 (1945).

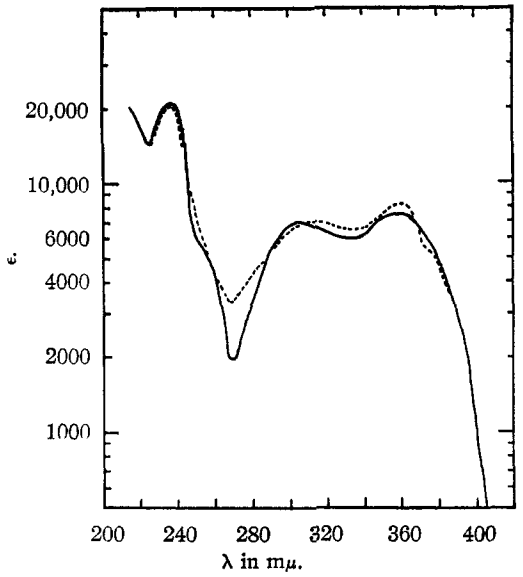


Fig. 1.——, 3,4-Dihydro-6,7-dimethyl-3-keto-4-D-ribityl-2-quinoxalinecarboxylic acid in 0.01 *N* NaOH; - - -, 3,4-dihydro - 6,7 - dimethyl - 3 - keto - 4 - methyl - 2 - quinoxalinecarboxylic acid in 0.1 *N* NaOH; redrawn (calcd. on decadic log extinction coefficients) from Kuhn and Rudy, *Ber.*, 67, 894 (1934).

same is true for riboflavin. A sample of this vitamin was dissolved in alkali at 20° and the solution was tested immediately. No hypotensive effect was observed. However, after standing at 25° for one hour the solution was retested. A sharp drop in blood pressure in the dog was observed, indicating that hydrolysis occurs even at room temperatures. If the riboflavin was dissolved in alkali and immediately buffered with boric acid, the resulting solution showed no hypotensive activity even after prolonged standing.

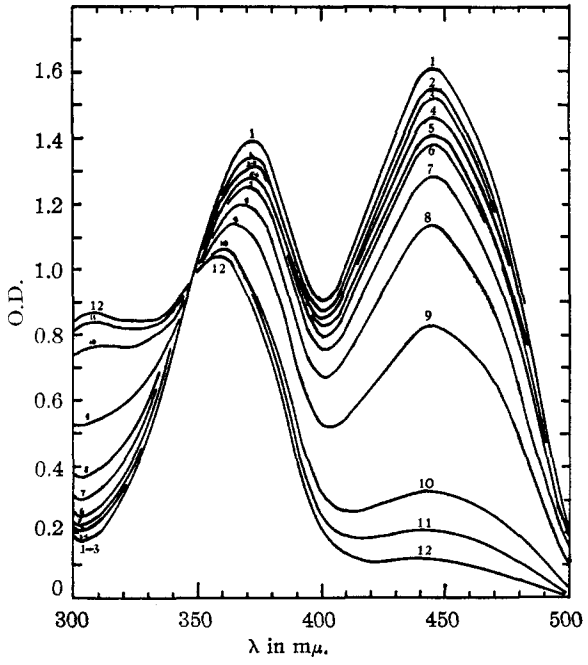


Fig. 2.—Kinetics of the alkaline degradation of riboflavin in 0.25 *N* NaOH at 25°: curves scanned at: 1 min. (1), 16 min. (2), 1 hr. (3), 3 hr. (4), 5 hr. (5), 8 hr. (6), 14.5 hr. (7), 24 hr. (8), 48 hr. (9), 120 hr. (10), 168 hr. (11), 482 hr. = t_8 (12).

The kinetics of the alkaline degradation were followed spectrophotometrically. A 5% solution of riboflavin in 0.25 *N* NaOH was prepared and kept in a thermostat in a dark red bottle at 25.0 ± 0.05°. Samples of 1 ml. were withdrawn at specified intervals and diluted in a boric acid–Na borate buffer *p*H 7.95 The spectrophotometric data are shown in Fig. 2.[9] They were obtained by means of a Cary recording spectrophotometer (Model 11, Serial No. 37) using a slit schedule of 50. It can be seen readily that the reactions under the prevailing conditions are quite rapid, and that as much as 4% change occurs in the first 16 minutes.

By replotting the optical density figures at λ = 445 mμ, reduced for O.D.$_\infty$, it can be shown graphically (Fig. 3) that we are dealing with a first order reaction. The velocity constant is calculated from the change of the absorbancy at λ_{445} as k_{25} = 1.62×10^{-2} hr.$^{-1}$.

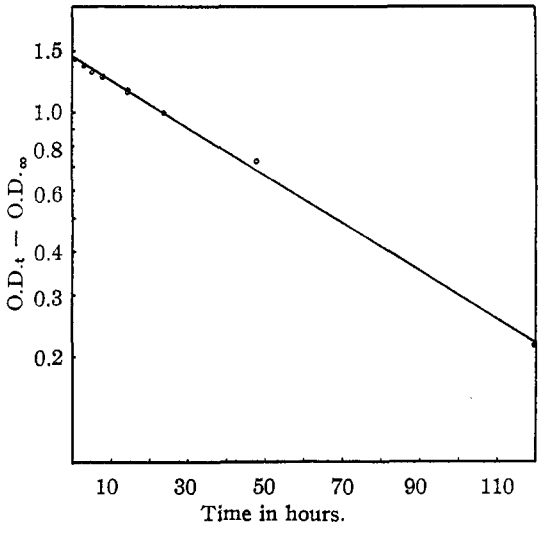


Fig. 3.—Plot of O.D.$_t$ − O.D.$_\infty$ *versus* time to demonstrate first order reaction of the alkaline degradation of riboflavin.

Experimental

Sodium Salt of 1,2-Dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic Acid Monohydrate.—A solution of 50 g. of riboflavin in 1.2 l. of 1 *N* sodium hydroxide was heated at 80° for one hour. After cooling, the solution was neutralized with acetic acid and allowed to stand three days at 0°. The solid which separated was filtered off, washed with alcohol and dried at 90°; yield 40.5 g., m.p. 228–231° (dec.). The product was recrystallized by dissolving in hot water, filtering with charcoal and adding an equal volume of alcohol to the filtrate. After drying for two hours at 95°, the material melted at 242.0–243.0° dec. (cor.).

Anal. Calcd. for $C_{16}H_{19}N_2O_7Na \cdot H_2O$: C, 48.98; H, 4.89; N, 7.08; Na, 5.75; H_2O, 4.61. Found: C, 48.98; H, 5.40; N, 7.14; Na, 5.86; H_2O, 4.59.

The original filtrate was distilled *in vacuo* until 300 cc. remained. A 5-cc. sample of this solution was diluted with

(9) A somewhat similar series of curves was reported by C. Daglish, N. Baxter and F. Wokes, *Quart. J. Pharm.*, **21**, 344 (1948), to show the effect of irradiation on absorption curve of riboflavin in alkaline solution. Inasmuch as our work was carried out in a flask protected from all incident irradiation, one must conclude that the changes shown are due to hydrolysis rather than irradiation.

35 cc. of 50% acetic acid, and 0.4 g. of xanthydrol in 8 cc. of methanol was added. After standing overnight, the dixanthylurea was filtered off, washed with 50% acetic acid and dried; 0.25 g., m.p. 270–274°. A mixed melting point determination with an authentic sample was not depressed.

1,2-Dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxalinecarboxylic Acid (II).—The sodium salt was dissolved in about ten volumes of hot water and an excess of dilute sulfuric acid was added. After cooling in ice, the yellow colored solid was filtered off and then recrystallized from 95% alcohol. After drying *in vacuo* at 60° and then at 100° to constant weight, the product melted at 183–183.5° dec. (cor.).

Anal. Calcd. for $C_{16}H_{20}N_2O_7$: C, 54.54; H, 5.72; N, 7.95. Found: C, 54.68; H, 5.64; N, 7.90.

Acknowledgment.—The authors are indebted to Dr. F. P. Luduena and Miss Estelle Ananenko of the Pharmacology Department of this Institute for the pharmacological data, and to Mr. M. E. Auerbach and Mr. K. D. Fleischer for the analyses reported, and to Miss Catherine M. Martini for the spectrophotometric determinations.

RENSSELAER, N. Y. RECEIVED NOVEMBER 7, 1950

[CONTRIBUTION FROM THE GEORGETOWN UNIVERSITY MEDICAL CENTER]

Bromination of Allopregnane-3,20-dione[1,2]

BY MARTIN RUBIN, HENRY WISHINSKY[1] AND FRANCA BOMPARD

The stepwise bromination of allopregnane-3,20-dione has been shown to proceed on carbons 2, 17 and finally 4. The direct tribromination yielded the 2,4,17-tribromide which on dehydrobromination gave the corresponding triene, a suitable intermediate for selective Ring A thermal aromatization to aromatic analogs of progestational and cortical steroids.

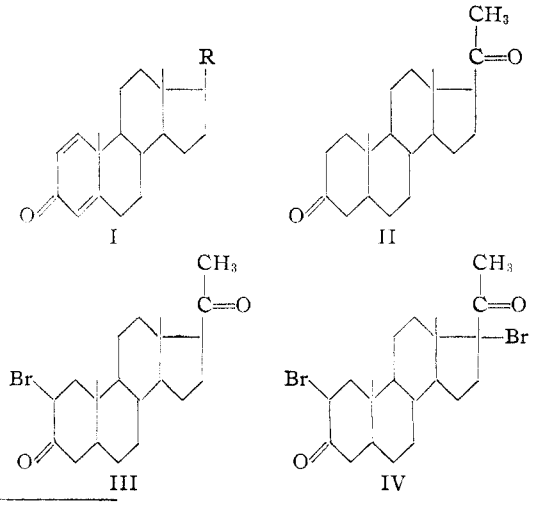
The conversion of steroidal sapogenins to estrogenic hormones and phenolic analogs of the progestational and cortical hormones[3] by the selective thermal aromatization procedure developed by Inhoffen, *et al.*,[4] and recently improved by us[5] required the preparation of Ring A dienones, I. Structures of this type have been formed by the 2,-4-dibromination and dehydrobromination of 3-ketoallosteroids.[6] The usual course of the bromination of allopregnane-3,20-dione (II), the readily available 3-ketoallosteroid derived from the sapogenins, may be expected to be complicated by the presence in the molecule of a second active center at the C-20 carbonyl group. Marker, *et al.*, have shown[7] that the hydrogen at C-17 in pregnane-3β-ol-20-one and allopregnane-3β-ol-20-one is replaceable by bromine in acetic acid at room temperature, and that the C-21 hydrogen can be replaced by bromine at 40°. It is of interest, therefore, that Butenandt and Mamoli have reported[8] that the monobromination of (II) resulted in the formation of the 2-bromo derivative (III). This compound was characterized by its dehydrobromination to Δ^1-pregnane-3,20-dione, ultraviolet absorption maximum at 230 mμ (ethanol).

We have extended the study of the monobromination of (II) to include the dehydrobromination of the products remaining in the mother liquors after removal of the crystalline 2-bromo fraction. By comparison of the yields of the Δ^1-fraction and the Δ^4-fraction formed in the course of the collidine dehydrobromination from pure 2-bromoallopregnane-3,20-dione it has been possible to estimate that at least 90% of the monobromination occurs at the 2-position. This calculation is based on the reasonable assumption that the only precursor of the Δ^1-allopregnane-3,20-dione is the 2-bromo derivative in the mother liquor material.

The introduction of a second bromine atom in (III) takes place at C-17 primarily with the formation of (IV). Proof of this fact has been obtained by preparation of the identical compound by further bromination of 17-bromoallopregnane-3,20-dione (V).[7]

The direct dibromination of (II) under the present conditions has been found to be a complicated reaction. Depending on the molar relation of bromine and (II), on the concentration of hydrogen bromide in the reaction mixture, and on other unknown factors it has been possible to isolate two dibromides, (IV) and its 2,4-isomer (VI), as well as two tribromides, the 2,4,17-compound, (VII) and a third tribromide of unknown structure.

Proof of the structure of the 2,4-dibromo compound (V) has been obtained by its independent synthesis from allopregnane-3-one-20β-ol (VIII), by dibromination in the 2,4-positions and subsequent oxidation to the dione (VI). The preparation of (VIII)[9] has been improved by utilization



I II III IV

(1) From the Ph.D. Thesis of Henry Wishinsky to the Graduate School of Georgetown University.

(2) Supported by grants-in-aid from Chemical Specialties Co., Inc., and the Geschickter Fund for Medical Research, Inc.

(3) C. Djerassi, G. Rosenkranz, *et al.*, THIS JOURNAL, **73**, 1523 (1951).

(4) H. Inhoffen, *Angew. Chem.*, **59**, 207 (1947).

(5) E. Hershberg, M. Rubin and E. Schwenk, *J. Org. Chem.*, **15**, 292 (1950).

(6) H. Inhoffen, *Naturwissenschaften*, **26**, 756 (1938).

(7) R. Marker, *et al.*, THIS JOURNAL, **64**, 210, 2089 (1942).

(8) A. Butenandt and L. Mamoli, *Ber.*, **68**, 1850 (1935).

(9) R. Marker, *et al.*, THIS JOURNAL, **59**, 164 (1937).