A solution of 17 g. (0.106 mole) of IIIa was heated on the steam-bath in 40 ml. of acetic acid until a solid began to separate. This suspension was allowed to stand at room temperature overnight and the product was then collected on the filter and air-dried to give 16.5 g. (100%) of colorless crystals. The latter was recrystallized from two liters of ethanol to give 11 g. (67%) of a white solid, m.p. 231–233° dec. Comparison of the infrared and ultraviolet spectra showed this product to be identical with 2-amino-5-ethylidene-2-thiazoline-4-one (Va).

A solution of 4 g. of product Va was heated at reflux for 3 hours in 30 ml. of water containing 7 ml. of concentrated hydrochloric acid. The mixture was chilled and filtered to obtain 3.2 g. (80%) of white crystalline product of m.p. 115–117°. The mixed m.p. with an authentic sample of 5-ethylidenethiazolidin-2,4-dione (IVa) showed no depression.

Reaction between Ethyl 2,3-Epoxy-3-methylbutyrate (IIb), Thiourea and Sulfuric Acid.—Ethyl 2,3-epoxy-3-methylbutyrate (72 g., 0.5 mole) was treated with 40 g. (0.5 mole) of thiourea and 15 ml. (0.25 mole) of sulfuric acid following a procedure analogous to that used in the synthesis of Va. Neutralization gave a solid and an oil. The solid was collected on the filter, and recrystallized from water to give 2 g. (2%) of 2-amino-5-isopropylidene-2-thiazolin-4-one (Vb), m.p. 262–270° dec. Direct comparison of the infrared and ultraviolet spectra showed it was identical with the compound first prepared by Culvenor, *et al.*[5]

Anal. Calcd. for $C_6H_8N_2OS$: C, 46.13; H, 5.16; N, 17.79; S, 20.52. Found: C, 46.87; H, 5.23; N, 18.30; S, 20.24.

The oil from the above filtrate was extracted with methylene chloride, the solution dried over sodium sulfate and distilled twice to give ethyl 2,3-dihydroxy-3-methylbutyrate (VI), b.p. 70–71° (1.2 mm.), $n^{30}D$ 1.4391 (reported[6] b.p. 70–71° (2 mm.), $n^{20}D$ 1.4415).

Anal. Calcd. for $C_7H_{14}O_4$: C, 51.84; H, 8.70. Found: C, 52.04; H, 8.87.

The infrared spectrum supported the assigned structure. The yield of dihydroxy ester was 28 g. (35%).

A sample of Vb was refluxed in 10% hydrochloric acid solution to form 5-isopropylidenethiazolidin-2,4-dione (IVb).[5]

Ethyl 2,3-Epithio-3-methylvalerate (Ic).—A mixture of thiourea (23.4 g., 0.3 mole) and sulfuric acid (9 g., 0.3 equiv.) in 70 ml. of water was cooled at 0–10° and 47.4 g. (0.3 mole) of ethyl 2,3-epoxy-3-methylvalerate was added dropwise with stirring at such a rate that the temperature remained under 20° (about one hour). The mixture was stirred at room temperature for 20 hours and then treated with 0.3 mole of sodium carbonate in 70 ml. of water. The oil was separated and the aqueous layer extracted exhaustively with methylene chloride and ethyl ether. The organic layers were combined and fractionated to yield 6 g. (16%) of ethyl 3-methyl-2-pentenoate (VII), b.p. 51–71° (7 mm.), $n^{23}D$ 1.4323. Identification was made by both infrared and mass spectrometric studies; mol. wt. calcd., 142; found by mass spectrograph, 142. Nine grams (17%) of ethyl 2,3-epithio-3-methylvalerate (Ic), b.p. 77–79° (7 mm.), $n^{23}D$ 1.4683 was isolated. *Anal.* Calcd. for $C_8H_{14}O_2S$: C, 55.14; H, 8.10; S, 18.84; mol. wt., 174. Found: C, 55.18; H, 8.04; S, 17.59; mol. wt., by ebullioscopy (acetone), 169 ± 5%; by mass spectrograph 174. The infrared spectrum supported the assigned structure.

Acknowledgments.—The authors gratefully acknowledge the mass spectrometric analyses performed by Mr. H. R. Harless and the infrared and ultraviolet absorption studies conducted by Dr. H. F. White and Mr. C. M. Lovell. They also wish to thank Dr. Harry Wasserman of Yale University for his helpful advice in the preparation of this paper.

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[CONTRIBUTION FROM THE SECTION ON ENZYMES, LABORATORY OF CELLULAR PHYSIOLOGY, NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH]

Bacterial Degradation Products of Riboflavin. III. Isolation, Structure Determination and Biological Transformations of 1-Ribityl-2,3-diketo-1,2,3,4-tetrahydro-6,7-dimethylquinoxaline

BY H. TODD MILES, P. Z. SMYRNIOTIS AND E. R. STADTMAN

RECEIVED SEPTEMBER 25, 1958

A new compound, $C_{15}H_{20}N_2O_6$, has been isolated as a bacterial degradation product of riboflavin and shown to be 1-ribityl-2,3-diketo-1,2,3,4-tetrahydro-6,7-dimethylquinoxaline. This compound is in turn converted to the 3,4-dimethyl-6-carboxy-α-pyrone isolated previously.

Previous work in this study of the bacterial degradation of riboflavin showed that riboflavin is converted by anaerobic bacteria to 6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine and to free radical, quinhydrone-like complexes of the same compound.[1a,2]

During the oxidation of riboflavin to carbon dioxide and ammonia by an aerobic organism isolated from soil, a number of compounds accumulate in the culture medium as transitory degradation products. Absorption spectroscopy and paper chromatography of aliquots of the culture medium at various stages of growth reveal the appearance in early phases of growth of numerous substances which later disappear after the riboflavin has all been consumed. Among the substances that appear in the early phases are two strongly blue fluorescent compounds and a "quenching" compound that can be detected on paper chromatograms when viewed under ultraviolet light. With paper chromatography in a solvent system composed of butanol–acetic acid–water (160:40:75) the blue fluorescent compounds have R_f values of 0.58 (compound I) and 0.78 (compound II), and the "quenching" (compound III) has an R_f of 0.92. The isolation of III and its identification as 3,4-dimethyl-6-carboxy-α-pyrone was previously reported.[1b] Compounds I and II have been isolated as pure substances and are found to have identical absorption spectra[4]; the elemental composition of II ($C_{17}H_{20}N_3O_4$) was reported in a preliminary communi-

(1) (a) Preceding papers, H. T. Miles and E. R. Stadtman, THIS JOURNAL, **77**, 6747 (1955); (b) P. Z. Smyrniotis, H. T. Miles and E. R. Stadtman, *ibid.*, **80**, 2541 (1958).

(2) The authors are indebted to Dr. Edwin Becker for electron spin resonance measurements which confirm the free radical nature of the solid complexes isolated earlier.[1a]

cation.[3] This paper describes the isolation, structure determination and synthesis of I and presents evidence showing that it is an intermediate in the conversion of riboflavin to II and III by washed cell suspensions of the aerobic bacterium.

Cell-suspension Studies.—Since I accumulates only in trace amounts during the oxidation of riboflavin under growth conditions, studies were made with washed cell suspensions of the bacterium to determine if conditions could be found which would favor the accumulation of I. Of several metabolic inhibitors investigated, it was found that arsenite in concentrations of 5 to 10 $\times$ 10^{-3} M will selectively inhibit the decomposition of I without preventing its formation from riboflavin. Thus when riboflavin is incubated with washed cell suspensions in the absence of arsenite (Fig. 1), there is a gradual

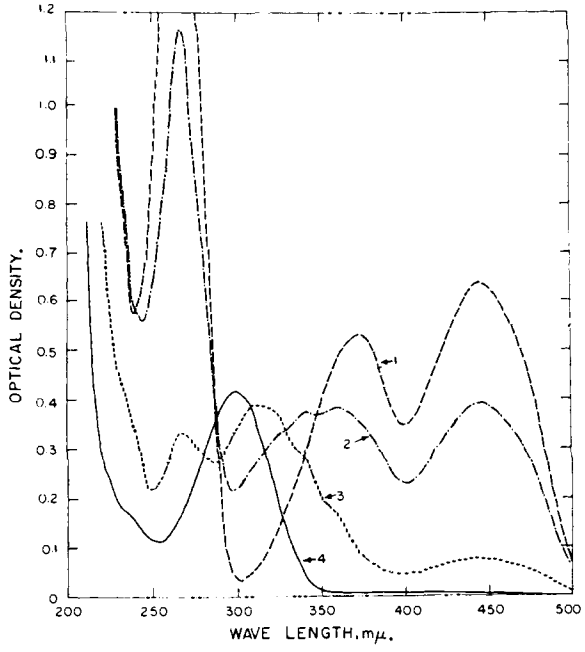


Fig. 1.—The bacterial conversion of riboflavin to 3,4-dimethyl-6-carboxy-α-pyrone (III). The reaction mixture (15 ml.) contained initially 1.5 ml. of bacterial cell suspension (in 0.05 M potassium phosphate buffer, pH 6.2) and 4.3 micromoles of riboflavin. The mixture was shaken in a 50-ml. flask in the dark at room temperature. After 0, 25, 55 and 160 minutes (curves 1, 2, 3 and 4, respectively), 0.5-ml. aliquots were centrifuged at 16,000 $\times$ g for 10 minutes (2°) to remove the bacteria, and 0.2 ml. aliquots of the supernatant solutions were diluted to 1.2 ml. and their spectra were determined in a Cary recording spectrophotometer. A control sample without riboflavin was used in the reference cuvette.

conversion of riboflavin to III as judged by the disappearance of the characteristic flavin spectrum and the appearance of a compound with an absorption maximum at 300 mμ. Paper chromatography of the incubation mixture at various periods of time revealed the presence of but small amounts of I and II at intermediate stages of incubation. On the other hand in the presence of 0.006 M arsenite, riboflavin is converted almost quantitatively to I and II as judged by the appearance of a characteristic absorption band with maximum at 323 mμ (*cf.* Fig. 2). From the molecular extinction coefficient of

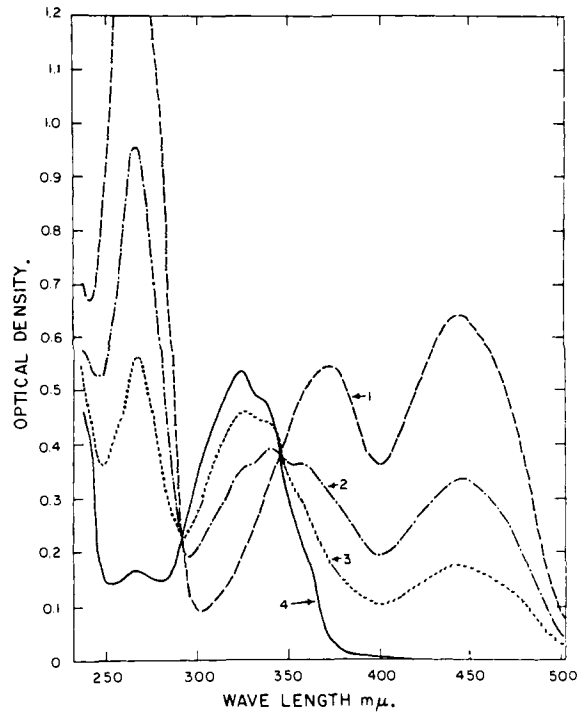


Fig. 2.—The bacterial conversion of riboflavin to compound I in the presence of arsenite. The experimental conditions were the same as for Fig. 1 except that potassium arsenite (0.0067 M) was added to the experimental and control samples. Aliquots were removed and the spectra measured after 0, 120, 220 and 325 minutes (curves 1, 2, 3 and 4, respectively).

riboflavin at 445 mμ (12,500) and of I at 325 mμ (10,700), it can be calculated that in this experiment the conversion of riboflavin to I is almost stoichiometric.[5] This is also indicated by the fact that the absorption spectra at various periods of time show isosbestic points at 292 and 346 mμ. Chromatography of the final reaction mixture showed it to contain mostly I and a small amount of II.

Conversion of I to II.—When I is incubated with washed cell suspensions in the presence of 0.006 M arsenite, paper chromatography reveals that it is converted to a second blue fluorescent compound with an R_f of 0.78 in the butanol–acetic acid–water solvent system. After elution with dilute alkali this second compound shows the same ultraviolet absorption spectrum as I, and acidification causes the changes in spectrum also observed in I. Evidence that this derivative of I is identical with II was obtained by showing that it was chromatographically indistinguishable in five solvent systems, from compound II, $C_{17}H_{20}N_3O_4$, reported previously.[3] Thus compounds I and II have identical absorption maxima but different R_f values and

(3) P. Z. Smyrniotis, H. T. Miles and E. R. Stadtman, *Bact. Proc.*, 120 (1958).

(4) Although the wave lengths of the absorption maxima are identical, the absorption intensity of II (ϵ_{325} = 17,200) on a molar basis is greater than that of I (ϵ_{325} = 10,700).

(5) There was some variation with different cell preparations, but the yield as determined by ultraviolet absorption usually was 70% or higher.

empirical formulas. Compound I appears to be a precursor of compound II (see Discussion).

Conversion of I to III.—The data of Fig. 3 show that when compound I is incubated with washed cell-suspensions in the absence of arsenite, it is converted to a substance with absorption maxima at 300 and 237 mμ and a minimum at 255 mμ, the same spectrum as that exhibited by the α-pyrone.[1b] From the molar extinction coefficients it can be calculated that the conversion is about 70%. Further evidence that the conversion product III is the α-pyrone was obtained by showing that the two materials had the same R_f values in several solvent systems (see Experimental) and that they were decomposed by alkali at similar rates to a substance with an absorption maximum at 235 mμ (*cf.* ref. 1b). It was also shown that the infrared spectra of 70 microgram samples in potassium bromide discs[6] of the conversion product and of the α-pyrone were the same after recovery by elution from paper chromatograms. Because of a fairly strong paper blank this identity does not by itself constitute conclusive evidence since differences in the spectra might be obscured in regions of strong absorption by impurities from the paper.

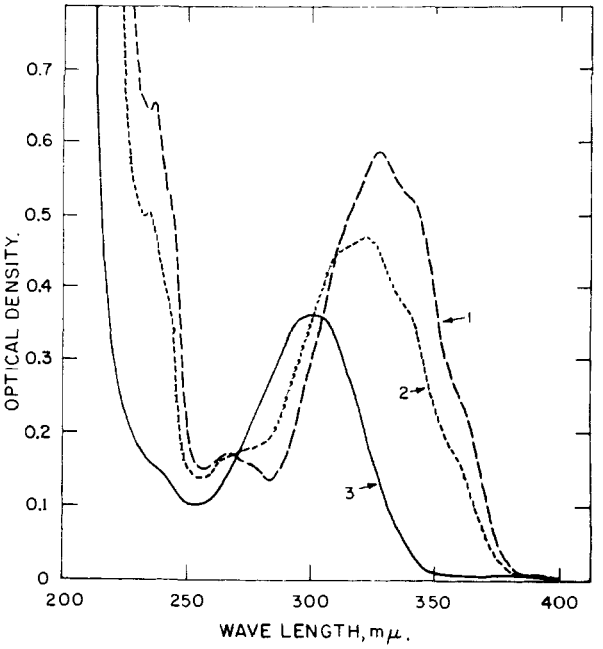


Fig. 3.—The conversion of compound I to 3,4-dimethyl-6-carboxy-α-pyrone (III). The reaction mixture (10 ml.) contained initially 1.0 ml. of bacterial suspension (in 0.05 *M* potassium phosphate buffer, *p*H 6.2) and 3.3 μmoles of compound I. A sample without compound I served as a control. After 0, 55 and 115 minutes, (curves 1, 2 and 3, respectively) aliquots were removed and the spectra were measured as described in Fig. 1.

Isolation and Characterization of Compound I.—With the discovery that washed cell suspensions catalyze the more or less quantitative conversion of riboflavin to I in the presence of arsenite, a large scale experiment was carried out to obtain sufficient material for isolation and identification purposes. The bacterial cells grown on riboflavin in the absence of arsenite were harvested and resuspended in riboflavin solution in the presence of arsenite (see Experimental). When the absorption spectrum of the incubation medium indicated the disappearance of riboflavin, the bacterial cells were centrifuged off, and I was isolated from the supernatant by column chromatography. The elementary analysis indicated the formula $C_{15}H_{20}N_2O_6$ after recrystallization from dimethylformamide. The absorption spectrum is given in Fig. 4.

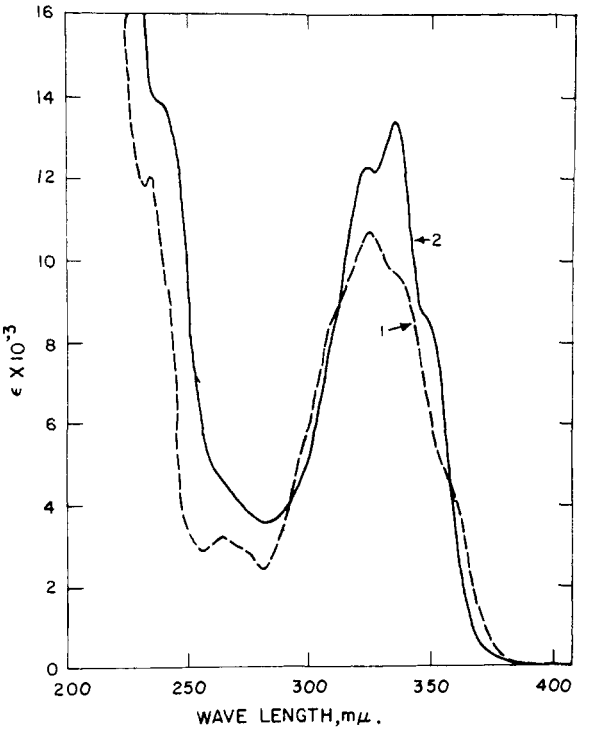


Fig. 4.—Influence of acid and alkali on the absorption spectrum of compound I. Curve 1, 0.004 *M* sodium hydroxide; curve 2, 0.004 *M* hydrochloric acid. The concentration of I is 0.001 *M*.

Determination of Structure.—The empirical formula shows that the conversion of riboflavin to I is associated with the loss of two carbons and two nitrogens; there is no change in hydrogen or oxygen.

It was found that one mole of I would reduce 3.2 moles of periodic acid, indicating that the ribityl side chain was still present at this stage of the bacterial degradation.

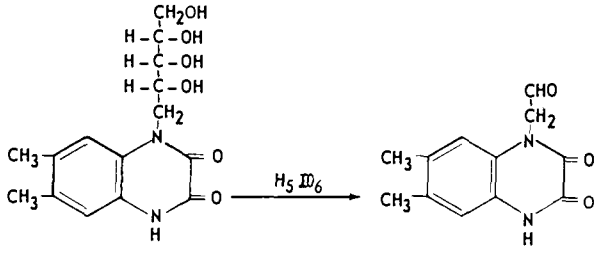
In the presence of arsenite, the degradation of 2-C^{14}-labeled riboflavin by washed cell suspensions leads to the accumulation of I containing no labeled carbon, together with a stoichiometric amount of C^{14}-labeled urea. No isotopically labeled carbon dioxide is formed. This experiment indicates that the nitrogen atoms and one carbon atom are lost as urea from the pyrimidine ring of riboflavin. Since the rapid consumption of 3 moles of periodate shows that no carbon has been lost from the side chain, the second carbon as well is probably lost from this ring.

The periodate oxidation was carried out on a large scale, and the crystalline oxidation product was isolated by extraction into ethyl acetate. This com-

(6) D. H. Anderson and N. B. Woodall, *Anal. Chem.*, **25**, 1906 (1953).

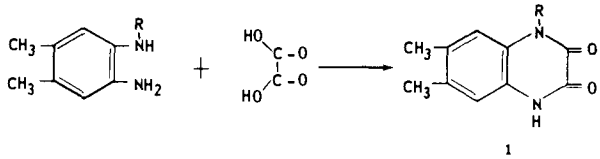
pound had the empirical formula $C_{12}H_{12}N_2O_3$ and ultraviolet spectrum identical with that of I. One of the oxygens of this compound must be the aldehyde group formed by the periodate oxidation. The most reasonable positions for the other two oxygens are the 2- and 3-carbons of a quinoxaline ring system.

From the foregoing experiments it is thus possible to deduce the following structures for I and its oxidation product



I

Synthesis of I.—Compound I was synthesized by fusing the hydrochloride of 2-(N-ribitylamino)-4,5-dimethylaniline with anhydrous oxalic acid. Two principal products were formed by the reaction, and these were separated by ion exchange chromatography.



I

R = Ribityl

The first blue fluorescent material eluted from the column was identical with compound I produced by bacterial action in respect to absorption spectrum (Fig. 4), R_f(0.58) and infrared spectrum. The mixed melting point was not depressed. The second blue fluorescent material from the column had the same absorption spectrum but a higher R_f (0.68). While this compound has not been investigated further as yet, it may be an oxalic ester of I.

Experimental[7]

Preparation of Bacterial Cell Suspensions.—Four 20-liter Pyrex bottles containing 15 l. of sterile riboflavin medium[8] were each inoculated with 400 ml. of a 52 hr. culture of a pure strain of the riboflavin decomposing aerobe (grown at 30° in medium of the same composition). A continuous vigorous stream of air was bubbled through the cultures to ensure aerobic conditions. After 45 hr. at 30° the bacteria were harvested in a Sharples centrifuge. The bacteria were washed by resuspending them in one liter of cold 0.05 *M* potassium phosphate buffer (*p*H 6.2). After centrifugation at 16,000 × *g* the washed bacteria were resuspended in one liter of fresh buffer solution and stored at 2–4°. They were used within 4 days of preparation.

Conversion of 2-C^{14} Riboflavin to Compound I and C^{14}-Labeled Urea.[9]—1.8 ml. of reaction mixture containing 3.16 μmoles of 2-C^{14}-labeled riboflavin (1400 counts per min. per μmole), 0.006 *M* arsenite and 0.5 ml. of bacterial cell suspension (*p*H 6.2) were shaken in air in a Warburg apparatus at 26°. The center well contained 0.2 ml. of 2 *N* sodium hydroxide to trap the evolved carbon dioxide. After 4.5 hr. the bacterial cells were removed by centrifugation at 16,000 × *g*. The supernatant solution was streaked on a sheet of filter paper and chromatographed in the butanol–acetic acid–water solvent system. 0.92 μmole of I (R_f 0.58) and 0.52 μmole of II (R_f 0.78), as determined by optical density measurements at 323 mμ, were eluted from the paper strips with 0.01 *N* sodium hydroxide.

Whereas no radioactive carbon was found in the eluate containing II, appreciable radioactivity was found in the compound I eluate. Since urea cannot be separated from I in the above chromatographic system, a further purification of I was achieved by chromatography on Dowex-1-acetate resin. To facilitate chromatography, 5.3 mg. of pure I was added to the eluate from the paper chromatogram (10 ml.), and the mixture was poured on a Dowex-acetate column (1.8 × 9.5 cm.). After washing the column with 160 ml. of water, I was eluted quantitatively from the resin with 100 ml. of 0.2 *N* acetic acid. The recovered I contained no radioactive carbon. All of the radioactivity was recovered in the effluent solution and in the water wash. The radioactive substance was identified as urea by showing that it gives the characteristic yellow color reaction with Ehrlich's reagent, that it is indistinguishable from urea by paper chromatography in the butanol–acetic acid–water solvent system (R_f 0.55), and that it is converted to $C^{14}O_2$ and two equivalents of ammonia when treated with the enzyme urease. Quantitative enzymatic analysis showed that 1.9 μmoles of urea was present in the reaction mixture after incubation. The specific radioactivity of the urea isolated was 1200 counts per min. per μmole, only slightly lower than that of the starting riboflavin (1400 counts per min. per μmole). Assuming that the difference in specific activities is due to the presence of endogenous urea in the bacterial suspension, it can be calculated that 1.6 μmoles of urea was formed from riboflavin. This corresponds well with 1.44 μmoles of compounds I + II that were formed. The present consideration of compounds I and II together is justified since they have qualitatively identical absorption spectra and therefore very probably contain the same diketoquinoxaline chromophore. Moreover, the evidence indicates that II is derived from I.

The carbon dioxide evolved during the incubation was devoid of radioactivity.

Conversion of I to II and to III.—To a solution of 25 mg. of I in 21 ml. of solution was added 18 ml. of bacterial cell suspension under the conditions indicated in Fig. 3. After 4.6 hr. the peak at 323 mμ had disappeared and a new one had formed at 303 mμ. The bacteria were removed by centrifugation, the supernatant solution was adjusted to *p*H 2, and the solution was extracted continuously with ether. Evaporation of the ether solution left a residue which was separated by trituration with ether into two fractions (A and B). Fraction A was less soluble in ether and had an R_f of 0.78 (blue fluorescence) in the butanol–acetic acid–water system. The absorption maximum in water was at 310 mμ, but on addition of alkali the spectrum changed to one characteristic of both I and II in alkali, and upon acidification the maximum reverted not to 310 mμ but to 323 mμ. It is possible that the initial 310 mμ maximum may be attributed to an enolic form of II.

Fraction B (predominantly III) was more soluble in ether and had an R_f of 0.92 (quenching) and an absorption maximum at 300 mμ. This material was finally induced to crystallize but was contaminated by an oily material from which it could not be separated by extraction from different buffer solutions. When paper chromatograms were run in different solvents, the R_f values of the pure α-pyrone[1b] and fraction B (λ_{max} 300 mμ) were, respectively, as follows: ethanol–0.1 *M* sodium acetate buffer of *p*H 4.5 (1:1), 0.74 and 0.74; formic acid–*t*-amyl alcohol–water (35:65:5), 0.81 and 0.78; butanol–acetic acid–water (160:40:75), 0.92 and 0.90; *t*-butyl alcohol–triethylamine–water (100:20:30), 0.76 and 0.76; butanol saturated with water–concentrated ammonia (98:2), 0.23 and 0.23.

Parts of the two fractions were then purified by streaking out on washed sheets of filter paper. The butanol–acetic acid–water system was used for fraction A (less soluble in ether), and the butanol–ammonia system for the fraction B

(7) Microanalyses by W. Manser, Zurich, Switzerland.

(8) The medium contained per liter 1.0 g. of yeast extract, 1.0 g. of tryptone, 0.12 g. of riboflavin, 40 ml. of 1.0 *M* potassium phosphate buffer (*p*H 6.2), 200 mg. of $MgSO_4 \cdot 7H_2O$, 10 mg. of $CaCl_2 \cdot 2H_2O$, 10 mg. of $FeSO_4 \cdot 7H_2O$, 0.6 mg. of $MnSO_4 \cdot 4H_2O$, 0.6 mg. of $Na_2MoO_4 \cdot 2H_2O$. The medium was sterilized at 121° for 45 minutes.

(9) The authors are indebted to Dr. G. W. E. Plaut for a gift of riboflavin labeled in the 2-position. The compound was prepared by growing *Ashbya gossypii* in the presence of labeled formate (*J. Biol. Chem.*, **208**, 513 (1954)).

(more soluble in ether). From fraction A the compound with an R_f of 0.78 (max = 323 mμ) was eluted with 0.02 *N* potassium hydroxide. From fraction B the compound with an R_f of 0.92 (λ_{max} = 300 mμ) was eluted with water. Paper blanks were also eluted. Pure "323 compound"[3] (compound II, R_f 0.78) was also chromatographed in the first solvent and some of the pure α-pyrone[1b] was chromatographed in the second solvent in order to have reference compounds obtained from paper under identical conditions.

The ultraviolet spectra of the pyrone and the compound isolated from fraction B proved to be identical. Both were decomposed by dilute alkali[1b] at about the same rate, to a material absorbing at 235 mμ.

Aliquots of the eluates of the known and of the presumed α-pyrone containing 70 μg. of material (determined by optical density measurements) were lyophilized. The materials were then incorporated into potassium bromide discs[4] and the infrared spectra measured with a Beckman IR-7 spectrophotometer. The spectra so obtained were the same but had a number of bands (as indicated by separate measurement of a paper blank) which were probably caused by impurities eluted from the paper.

The "323 compound" (compound II; $C_{17}H_{20}N_3O_4$; R_f 0.78) and the compound from fraction A had the same ultraviolet spectra after elution from paper. The R_f values for the pure compound II and for the compound isolated from fraction A, respectively, are given in the solvent systems listed above in the same order: 0.58 and 0.58; 0.66 and 0.66; 0.78 and 0.78; 0.78 and 0.79; 0.55 and 0.54. Mixtures of the two materials traveled as a single spot in all solvents tested.

In the present case the paper blank was so large (perhaps as a result of elution with dilute alkali rather than with distilled water) that useful infrared spectra could not be obtained.

Isolation and Characterization of I.—Bacteria from 60 liters of growth medium were suspended in 15 liters of solution containing 5.0 grams of riboflavin, 0.006 *M* arsenite and 0.014 *M* potassium phosphate buffer (*p*H 6.2). The mixture was incubated at 30° in a 20-liter bottle. A vigorous stream of air was bubbled through the mixture to maintain aerobic conditions. When the riboflavin was nearly consumed (as determined by the disappearance of light absorption at 445 mμ), the incubation medium had a strong absorption band at 323 mμ, indicating an accumulation of I. The bacterial cells were then removed by centrifugation in a Sharples centrifuge, and the clear supernatant solution was passed through a Florisil column, 9 × 29 cm. After washing the column with 8 liters of water, I was eluted with 24 liters of 20% methanol in water and finally with 18 liters of 30% methanol. The methanolic eluates were passed over a Dowex-1-acetate column 9.5 cm. (diameter) × 4.0 cm. The resin was washed with 2.0 liters of water, and I and II were eluted with 500 ml. of 10 *N* acetic acid. Paper chromatography of the eluate in the butanol–acetic acid–water solvent system showed the presence of compounds I and II as well as a third substance. The latter was green fluorescent when viewed with ultraviolet light and had an R_f in the above solvent system of 0.71. The eluate was concentrated to dryness (room temperature) and the residue was dissolved in 150 ml. of 0.02 *N* sodium hydroxide and passed over a Dowex-1-acetate column 3 cm. (diameter) × 18 cm. The column was washed with 675 ml. of water with the elution of only trace amounts of I. I was eluted with 1.0 liter of 0.1 *N* acetic acid. This fraction contained neither II nor the green fluorescent compound with an R_f of 0.71. The 0.1 *N* acetic acid eluate containing I was evaporated to dryness. The solid residue (960 mg.) was dissolved in 60 ml. of hot glacial acetic acid and filtered. Upon cooling, 680 mg. of crystalline compound was obtained. After recrystallization from dimethylformamide, the compound melted at 257–260° (see discussion of m.p. in the section on synthesis of I).

Anal. Calcd. for $C_{15}H_{20}N_2O_8$: C, 55.55; H, 6.22; N, 8.64; O, 29.60. Found: C, 55.46; H, 6.25; N, 8.64; O, 29.56.

The compound is strongly fluorescent when viewed under ultraviolet light and has an R_f of 0.58 when chromatographed on paper in the butanol–acetic acid–water solvent system. The ultraviolet absorption (Fig. 4) shows maxima at 213 mμ (ε 46,000), 234 mμ (ε 12,100), 263 mμ (ε 3,190) and 323 mμ (ε 10,700) in 0.004 *N* hydrochloric acid. A shift in spectrum is observed in alkali (0.004 *N* sodium hydroxide) with maxima at 222 mμ (ε 39,000), 239 mμ (ε 14,000) 322 mμ (ε 12,200), 334 mμ (ε 13,300), 348 mμ (shoulder, ε 8,550). The compound showed no change upon treatment with 1.0 *N* hydrochloric acid or with 1.0 *N* potassium hydroxide for 8 hr. at 100°, and it is stable to ultraviolet and visible light.

Analytical Periodate Oxidation.—A spectrophotometric procedure[10] was used, the reaction being followed by decrease in absorption at 265 mμ as the periodic acid was consumed (even though the absorption of periodate is much less at this wave length than at its maximum at 222 mμ, it is still high enough at the concentration used to give satisfactory analyses. This wave length was chosen to avoid the intense peaks of I). The reaction was carried out with 0.001 *M* periodic acid in phosphate buffer at *p*H 4.5 and was complete in three or four minutes. Glycerol was used as a standard. The average of three determinations was 3.2 μmoles of periodate consumed per μmole of I.

The excess periodate was decomposed with potassium arsenite, and the solution was allowed to react with an acid solution of 2,4-dinitrophenylhydrazine.[11] After standing 5 min. the solution was made basic. A strong carbonyl test was observed with a Klett colorimeter using a no. 54 filter.[11] Extraction of the basic solution with ethyl acetate removed all color showing that no aldehydo or keto acids were formed.

Preparative Periodate Oxidation of I.—To a solution of 200 mg. of I in 10 ml. of 0.1 *N* sodium hydroxide was added slowly 21 ml. of 0.1 *N* periodic acid, the *p*H being kept at or above 5 by simultaneous addition of 0.1 *N* sodium hydroxide. The solution was allowed to stand 15 min. The precipitate (which formed from the supersaturated solution upon stirring) was dissolved by addition of 1 *N* NaOH. The solution was adjusted to *p*H 8 by addition of sulfuric acid and extracted ten times with ethyl acetate, the extraction being followed by ultraviolet measurements (in ethyl acetate λ_{max} 319 mμ).

The ethyl acetate solution was dried with anhydrous magnesium sulfate and evaporated to a volume of 180 ml.; when cooled it deposited 83 mg. of crystals. The material was recrystallized three times from glacial acetic acid and dried 20 hr. at 60° and 0.1 mm. The compound had the same ultraviolet spectrum as I and an R_f of 0.76 in the butanol–acetic acid–water solvent system (although the R_f is the same as that of compound II, the elementary composition shows that they are not the same).

Anal. Calcd. for $C_{12}H_{12}N_2O_3$: C, 62.96; H, 5.21; N, 12.06; O, 20.67. Found: C, 61.90; H, 5.26; N, 11.95; O, 20.92.

Synthesis of Compound I.—A dry mixture of 50 mg. of 2-(N-ribitylamino)-4,5-dimethylaniline hydrochloride[12] and 26 mg. of anhydrous oxalic acid was heated at 160° in an oil-bath. There was a vigorous evolution of gas from the melt, and the reaction was complete in 10 or 15 min. On trituration with ether in methanol the green glass crystallized. The ultraviolet spectrum of the crystals in both acid and alkali was that of the bacterial product, I, but paper chromatograms showed two fluorescent spots, one at R_f 0.58 (that of the natural material) and one at 0.68.

The above preparation was repeated on a large scale (1.5 g. of diamine hydrochloride and 0.47 g. of oxalic acid), and a solution of the reaction products (on the basis of 323 mμ absorption the solution contained about 500 mg. of I or its derivatives) was poured on a column of water-washed Dowex 1 (acetate form), 6.7 cm. in diameter and 17 cm. in height. The early fractions contained the 323 mμ absorbing material contaminated with a substance absorbing at 276 and 285 mμ. The fractions were also followed by paper chromatography since the third component of the mixture (R_f 0.68) had the same ultraviolet spectrum as the second. The pooling of the fractions was done to achieve purity at the expense of yield. The eluting solvents were added as follows: water, 2300 ml.; 0.01 *N* acetic acid, 1100 ml.; 0.03 *N* acetic acid, 5200 ml.; 0.05 *N* acetic acid, 100 ml.; 0.08 *N* acetic acid, 100 ml.; 0.20 *N* acetic acid, 1700 ml.

(10) C. E. Crouthamel, H. V. Meck, D. S. Martin and C. V. Bank, THIS JOURNAL, 91, 3031 (1949).

(11) T. E. Friedeman and G. E. Haugen, *J. Biol. Chem.*, 147, 415 (1943). Because of a fading observed with aldehydes under the usual conditions of the Friedeman and Haugen keto acid analysis, the value observed had no quantitative significance.

(12) The authors are indebted to Dr. J. A. Aeschlimann of Hoffman-La Roche for the generous gift of this intermediate.

The fractions from 4100 to 5000 ml. effluent contained only the 323 mμ absorbing material of R_f 0.58. These were pooled, evaporated under vacuum to 20 ml. and allowed to stand overnight. The crystals which separated weighed 100 mg. and melted at 255–262° after being washed and dried. When the cover slips were pressed together on the Kofler hot stage, new crystals formed which melted at about 285–295°. The ultraviolet spectrum was the same as that of I. The 100 mg. of material of m.p. 255–262° was recrystallized from dimethylformamide and dried. This material melted at 262–266° after sintering at 259°. As before, new crystals could be obtained from the melt, and these melted at 292–300°.[13] There was some variation in the melting ranges of these crystals depending on the rate of heating.

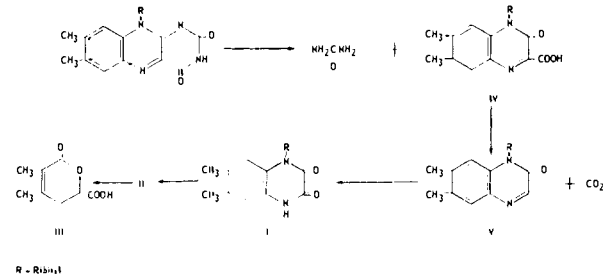
A sample of the natural material, compound I, melted at 257–260° and at 289–292°. A mixture of the two melted at 261–264° and again at 278–292°.

The infrared spectra of Nujol mulls of these samples measured with a Perkin–Elmer model 21 Spectrophotometer were identical. The higher melting crystals from both natural and synthetic samples were removed from the hot stage below their m.p., and micro potassium bromide discs were prepared using about 60 μg. of sample. The infrared spectra of these higher melting forms were measured with a Beckman IR-7 spectrophotometer and found to be identical, though different from the lower melting form.

Discussion

It has been shown above that riboflavin is converted by an aerobic strain of bacteria to I and urea. It is obvious that a one carbon fragment must also be produced at some point in the transformation and that this might be carbon dioxide, though there is no evidence on the form in which this carbon is detached from the ring.

A plausible course for this reaction is the production from riboflavin of urea and the quinoxalone carboxylic acid, IV. This in turn might be converted to I, either directly or from the lactam V, as indicated.



It was found, in experiments to be reported later, that the quinoxalone carboxylic acid can be converted chemically to I by treatment with hydrogen peroxide in acetic acid. Preliminary experiments, however, have failed to demonstrate an analogous biological transformation. Neither the acid, IV, nor the lactam V, (obtained from the acid by decarboxylation and sublimation) was converted to I when incubated with washed cells in the presence of arsenite. The lactam did undergo transformation to a substance different from either I or II, but the acid did not appear to be metabolized. This evidence is not conclusive since it is possible that an actual metabolite may be unchanged because of its inability to pass through an intact cell wall, but in view of the ready biochemical transformations of similar compounds by the cells, this explanation does not appear probable in this instance. It is possible that a ureido derivative of the acid IV (or other derivative) may be formed initially from riboflavin and that for reasons of enzyme specificity only this derivative could be further converted to I.

It has been found that when I is added to a suspension of cells in the presence of arsenite compound II is formed but that no I is formed when II is added. The sequence of formation of these intermediates is thus in the biosynthetic direction of I → II rather than the reverse, as one might suppose merely from an inspection of the empirical formulas.

Although very little work has been done on the structure of II ($C_{17}H_{20}N_3O_4$),[3] the fact that this compound has the same absorption maxima as I in both acid and base indicates that the diketoquinoxaline chromophore is also present in II and hence suggests that the addition of one carbon and one nitrogen and the loss of two oxygens have occurred in the ribityl side chain.

A possible mode of formation of the α-pyrone, III, from earlier intermediates has been proposed in the second paper of this series.[1b]

BETHESDA, MARYLAND

(13) The ultraviolet spectrum of the higher melting crystals was identical with that of the lower melting form (Fig. 4), but the R_f values were different in four of the five solvent systems cited previously. These observations constitute presumptive evidence that the high-melting material was a reaction product rather than a polymorph but that the reaction had not affected the diketoquinoxaline chromophore.