possessing a smaller total mass.[5] The mass difference, furthermore, is composed of essentially non-ultraviolet absorbing groupings. These conclusions have been substantiated by subsequent investigations.[6]

Experimental

Alkali Stability.—Rhizopterin (1.1 mg.) was dissolved in 0.1 *N* sodium hydroxide (2.5 ml.) and allowed to stand at room temperature nineteen to twenty hours. The ultraviolet absorption spectrum of this solution is shown in Fig. 1. The alkaline solution was then acidified to approximately *p*H 1 with hydrochloric acid and the ultraviolet absorption spectrum again noted (Fig. 1).

Alkaline Hydrolysis.—Rhizopterin (67.5 mg.) was dissolved in 1 *N* sodium hydroxide (1 ml.) and the solution allowed to stand at room temperature for forty-eight hours. The solution was then acidified with dilute hydrochloric acid and the precipitated crude aporhizopterin was recovered by centrifugation. The insoluble material (59.4 mg.) was dissolved in dilute sodium hydroxide and a mixture of alcohol and acetone was added until the solution was turbid. Small rosets of yellow needles crystallized from this solution on standing. This product was recrystallized by dissolving in sodium hydroxide and adding an alcohol–acetone mixture: yield, 52.8 mg.

Anal. Calcd. for $C_{14}H_{10}N_6O_3Na_2$: C, 47.21; H, 2.83; N, 23.58; Na, 12.91, equiv. wt., 156. Found: C, 46.68; H, 3.07; N, 23.8; Na, 13.37, equiv. wt., 150.

Acid Hydrolysis.—Rhizopterin (29.2 mg.) was suspended in 2.5 *N* hydrochloric acid (2 ml.) and heated on the steam-bath. The material dissolved and aporhizopterin crystallized from the solution in the form of yellow needles. This material was found to be one-tenth as active for *S. lactis R* as rhizopterin.

Acknowledgment.—The authors wish to thank Mr. R. Boos and his associates for the microanalyses.

Summary

Treatment of rhizopterin with alkali or acid gave a pterin-like degradation product, aporhizopterin, with the formula $C_{14}H_{12}N_6O_3$. A comparison of the physical properties of this compound with those of vitamin B_c indicated that these compounds appear to have identical chromophoric groups and differ only in that vitamin B_c contains additional non-ultraviolet absorbing mass.

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(5) The potentiometric titration of aporhizopterin gave a value of 150 for its equivalent weight and indicated it to be a dibasic acid. It was therefore assumed that its molecular weight is about 300. On this assumption then, it should be possible to evaluate the molecular weight of vitamin B_c from the above spectroscopic data. Taking the average value of the band ratios as 1.29 and the assumed molecular weight for aporhizopterin of 300, a value for E_0 of 390 is obtained. This is in fair agreement with the known value of 441 (see Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, SubbaRow, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith, *Science*, **103**, 667 (1946)).

(6) Wolf, Anderson, Kaczka, Harris, Arth, Southwick, Mozingo and Folkers, THIS JOURNAL, **69**, 2753 (1947).

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK AND CO., INC.

The Structure of Rhizopterin

BY DONALD E. WOLF, R. CHRISTIAN ANDERSON, EDWARD A. KACZKA, STANTON A. HARRIS, GLEN E. ARTH, PHILIP L. SOUTHWICK, RALPH MOZINGO AND KARL FOLKERS

The structure of rhizopterin, the "S.L.R. factor"[1] has been established by degradation and synthesis as *p*-[N-(2-amino-4-hydroxypyrimido[4,5-b]pyrazin-6-ylmethyl)-formamido]-benzoic acid (I).[2] Rhizopterin is active for the growth of *S. lactis R.* and inactive for the growth of *Lactobacillus casei.*[1]

Rhizopterin is a light yellow crystalline compound, insoluble in the common organic solvents and water, but soluble in mineral acids and alkali. Purification of rhizopterin was complicated by the fact that no suitable solvents were found for recrystallization. Impurities were difficult to remove.[3] At the suggestion of Dr. John B. Conn of this Laboratory, luteo ethylenediaminocobaltic chloride[4] was used as a reagent for purification. When the crude rhizopterin was dissolved in normal ammonium hydroxide and treated with luteo ethylenediaminocobaltic chloride, red crystals of the rhizopterin double salt separated. After recrystallization from hot water, the luteo ethylenediaminocobaltic salt of rhizopterin was converted into rhizopterin by treatment with dilute acetic acid.

The ultraviolet absorption spectrum of rhizopterin had indicated that this compound possessed a pteridin nucleus. The high melting point of rhizopterin, its insolubility in common organic solvents, and its solubility only in acid or alkaline solutions also were in agreement with the properties of known pterins.[3] The electrometric titration of rhizopterin indicated an equivalent weight of 167. Comparison of the titration curves of rhizopterin and xanthopterin showed definite similarities. When either compound was dissolved in alkali and titrated with acid, it was precipitated at about *p*H 7.

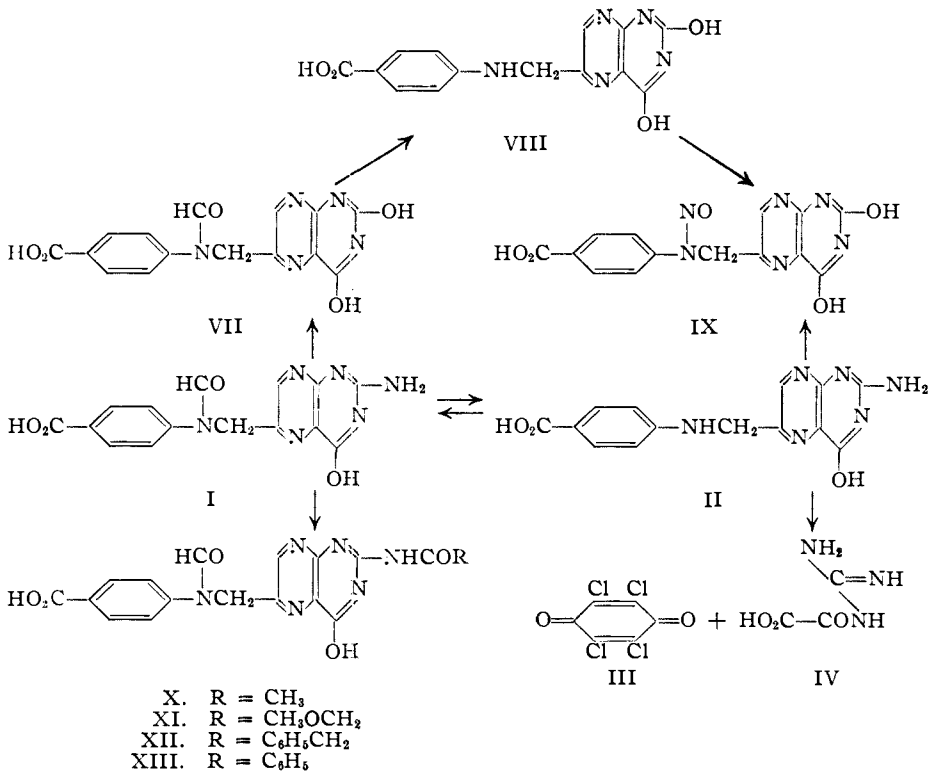
Determination of the molecular weight of rhizopterin by any of the usual methods was not feasible because of the extreme insolubility of the compound. In a search for more soluble derivatives it was found that rhizopterin could be acylated; several acyl derivatives were prepared for experiments in molecular weight determinations. The acetyl- (X), methoxyacetyl- (XI), phenylacetyl-

(1) Keresztesy, Rickes and Stokes, *Science*, **97**, 465 (1943).

(2) This systematic name is in accord with the Ring Index and has been recommended by Dr. E. J. Crane.

(3) Rickes, Chaiet and Keresztesy, THIS JOURNAL, **69**, 2749 (1947).

(4) Gmelin, "Handbuch der anorganischen Chemie," Vol. **58** [B], Verlag Chemie G. m. b. H., Berlin, 1930, p. 73.



(XII), and benzoyl- (XIII), derivatives of rhizopterin all were prepared. The molecular weight of acetylrhizopterin, determined ebullioscopically in glacial acetic acid, was 369 which, subtracting the weight of C_2H_2O for one acetyl group, corresponds to a molecular weight of 327 for rhizopterin. This latter value for rhizopterin is in good agreement with 334 obtained by doubling the titration equivalent weight of rhizopterin. These results with the analytical data indicated the molecular formula of $C_{15}H_{12}N_6O_4$, and a molecular weight of 340.

Potentiometric titration of acetylrhizopterin was more satisfactory than that of rhizopterin because the greater solubility of the derivative allowed a completion of the titration before precipitation occurred. The titration curve of acetylrhizopterin showed two spans, with midpoints at *p*H 7.46 and 3.86, indicating the presence of a carboxy group and a weakly acidic group in acetylrhizopterin.

Neither xanthopterin nor rhizopterin gave appreciable amounts of nitrogen by the usual van Slyke determination. However, when the sample of xanthopterin was dissolved in concentrated hydrochloric acid, rather than water or dilute acid, the determination gave nitrogen values which agreed well with the calculated value. When rhizopterin was treated similarly, the nitrogen value indicated the presence of one free amino group.

The conversion of rhizopterin to a hydrolysis product by treatment with either acid or alkali has been described.[5,6] To obtain the hydrolysis product (II), rhizopterin was treated with about 15% hydrochloric acid at 100°. The hydrolysis product (II) was obtained in nearly quantitative yield as a yellow microcrystalline compound. The hydrochloride and the free base gave analytical values in agreement with the formulas $C_{14}H_{13}N_6O_3Cl$ and $C_{14}H_{12}N_6O_3$, respectively; however, the results of nitrogen determination by the conventional methods were variable, a behavior previously reported for pterins.[7]

Acid hydrolysis of rhizopterin also yielded formic acid. When the filtrate after removal of hydrolysis product (II) was steam distilled, and the distillate was treated with *p*-bromophenacyl bromide, a crystalline *p*-bromophenacyl ester was obtained which melted at 137–139°. This ester and a sample of *p*-bromophenacyl formate were identical. Formic acid was also obtained when rhizopterin was hydrolyzed with alcoholic sodium hydroxide. The hydrolysis product (II) was treated with acetic anhydride to give a crystalline diacetyl derivative.

The hydrolysis product (II) was subjected to oxidation with potassium chlorate in the presence of excess hydrochloric acid in a manner similar to that used for the oxidation of xanthopterin.[8]

(5) Rickes, Trenner, Conn and Keresztesy, THIS JOURNAL, **69**, 2751 (1947).

(6) This compound was named aporhizopterin in these Laboratories. It is now known to be identical with pteroic acid.[11]

(7) Wieland and Purrmann, *Ann.*, **544**, 163 (1940).

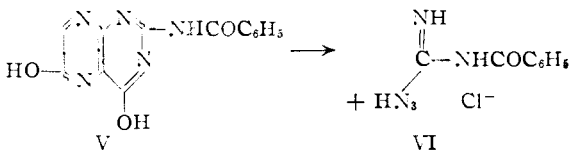
(8) Schöpf and Kottler, *ibid.*, **539**, 128 (1939).

Under these conditions the solid dissolved and yellow plate-like crystals appeared. This oxidation product was identified as chloranil (III) by analytical data, and by comparison of its characteristic sublimation behavior with that of a known sample. The formation of chloranil showed the presence of a substituted benzenoid nucleus in rhizopterin.

The aqueous oxidation mixture, which remained after the removal of the chloranil, was evaporated to dryness. Removal of the inorganic salts from the residue gave a white solid which did not melt below 275° and was identified as oxaloguanidine (IV). The oxaloguanidine was hydrolyzed to guanidine, which was identified as guanidine picrate.

When the hydrolysis product (II) was heated at 220–360° at very low pressure, *p*-aminobenzoic acid collected as a white sublimate. *p*-Acetamidobenzoic acid served as a characterization derivative. Similar thermal treatment of rhizopterin also gave *p*-aminobenzoic acid. Hydrolysis of rhizopterin in either hydrochloric acid or sodium hydroxide solution yielded *p*-aminobenzoic acid. It is evident that the chloranil was formed by oxidation of the *p*-aminobenzoic acid portion of rhizopterin. Chloranil is a known oxidation product of *p*-aminobenzoic acid.[9]

The isolation of guanidine from the oxidation of the hydrolysis product (II) provided chemical evidence for the presence of a 2-amino group on the pteridin nucleus. It was necessary to ascertain whether the formyl group in rhizopterin is attached to the 2-amino group of the pteridin nucleus or to the nitrogen atom of the *p*-aminobenzoic acid moiety. Benzoylxanthopterin (V) was prepared by allowing xanthopterin to react with a large excess of benzoic anhydride. Oxidation of benzoylxanthopterin according to the conditions used for the oxidation of the hydrolysis product (II) yielded benzoylguanidine hydrochloride (VI). The formation of benzoylguanidine and chloranil from benzoylrhizopterin indicated that the 2-amino group is the one benzoylated and, therefore, it is the unsubstituted amino group



V VI

in rhizopterin. This observation and the fact that rhizopterin contains one acylatable group while its hydrolysis product (II) contains two acylatable groups, shows that the formyl group in rhizopterin is attached to the nitrogen atom of the *p*-aminobenzoic acid moiety.

When rhizopterin was allowed to react with nitrous acid at room temperature, a colorless, water-soluble, crystalline compound (VII) was formed. This compound has the composition $C_{15}H_{11}N_5O_5$, and is the result of the replacement of an amino group by an hydroxyl group. It is designated desiminorhizopterin by analogy with desiminoleucopterin.[10]

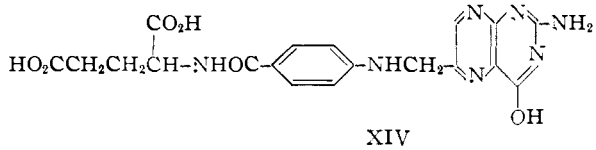
Acid hydrolysis of desiminorhizopterin gave a light yellow crystalline compound (VIII). This compound was the result of the hydrolytic removal of the formyl group from desiminorhizopterin (VII). It was also found that the hydrolysis product (II) as well as its desimino derivative (VIII), *p*-[(2,4-dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid, reacted with nitrous acid to give a stable N-nitroso derivative (IX), *p*-[N-(2,4-dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-nitrosamino]-benzoic acid. Thermal treatment of the N-nitroso derivative (IX) also yielded *p*-aminobenzoic acid as a pyrolysis product. The formation of an N-nitroso derivative of the desimino hydrolysis product (VIII) shows the secondary nature of the nitrogen atom of the *p*-aminobenzoic acid moiety.

The presence of a formyl group, a 2-amino-4-hydroxypteridin nucleus and a *p*-aminobenzoic acid moiety accounts for all of the elements in the molecular formula of rhizopterin except a methylene group. Since analyses for C-methyl, N-methyl and O-methyl had shown that none of these groups is present, the methylene group must be present as a bridge.

On the basis of the structural formula of rhizopterin (I) the degradation experiments are clearly interpretable. Substitution at the 6-position of the pteridin nucleus would appear to permit a quinoid-like form which might easily be hydrolyzed to give *p*-aminobenzoic acid.

Formylation of the hydrolysis product (II) allowed the resynthesis of rhizopterin. Formylrhizopterin, the postulated intermediate, apparently loses one formyl group readily in the dilute alkali employed in recrystallization.

The structure of the liver *L. casei* factor has been established as N-(*p*-[(2-amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoyl)-glutamic acid (XIV).[11] The name



XIV

pteroylglutamic acid has been used for this compound. The corresponding compound (II) without the glutamic acid group, *p*-[(2-amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid has also been synthesized and named pteroic acid.[11]

A sample of pteroic acid prepared as described,[11] from 2,4,5-triamino-6-hydroxypyrimi-

(9) Widnmann, *Ann.*, **193**, 202 (1878).

(10) Wieland, Metzger, Schöpf and Bülow, *ibid.*, **507**, 226 (1933).

(11) Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, Subba-Row, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith, *Science*, **103**, 667 (1946).

dine, dibromopropionaldehyde and *p*-aminobenzoic acid, was treated with formic acid. The product was recrystallized from dilute ammonium hydroxide by acidification with acetic acid. It was identical with rhizopterin, and thus completed the proof of the structure of rhizopterin.

Experimental

Purification of Rhizopterin as the Luteo Ethylenediaminocobaltic Salt.—In a typical experiment, 441 mg. of crude rhizophterin was dissolved in normal aqueous ammonium hydroxide. To the alkaline solution was added 233 mg. of luteo ethylenediaminocobaltic chloride, $[Co\text{-}En_3]Cl_3 \cdot 3H_2O$. The solution was allowed to stand in the refrigerator overnight. The rhizopterin salt crystallized in red needle-like clusters. These were separated, washed with water and dried *in vacuo*. The first crop weighed 407 mg. A second crop was obtained by adding an excess of the luteo ethylenediaminocobaltic chloride and evaporating the solution to one-half volume *in vacuo*. The luteo ethylenediaminocobaltic salt of rhizopterin was purified by recrystallization from hot water. When pure, it melted at 247–250° (dec.).[12] A solution of 293 mg. of the luteo salt in hot water was acidified with acetic acid. Rhizopterin separated as light yellow leaf-like crystals. The product was collected in a centrifuge tube and washed with water. When dry, it weighed 226.5 mg.

To remove traces of ash, the rhizopterin was dissolved in *N* ammonium hydroxide. The solution was filtered, diluted to about ten volumes, warmed on a steam-bath, and acidified with acetic acid to *p*H 4 to 5. Light yellow crystals formed which were collected in a centrifuge tube and washed with water. Rhizopterin darkens at about 285°, but fails to melt below 300°.

The rhizopterin was dried in a weighing-pig at 140° *in vacuo* before the analyses.

Anal. Calcd. for $C_{15}H_{12}N_6O_4$: C, 52.94; H, 3.56; N, 24.70. Found: C, 52.86; H, 3.36; N, 24.90, C-methyl, N-methyl, and O-methyl, none.

At *p*H 11, rhizopterin showed *E* (1%, 1 cm.) maxima of 940 at 2550 Å. and 219 at 3650 Å.; at *p*H 7, *E* (1%, 1 cm.) maxima of about 550 at 2450 to 2750 Å. and 173 at 3500 Å.; at *p*H 3, *E* (1%, 1 cm.) maxima of 612 at 2700 Å. and 155 at 3450 Å.; at *p*H 1, *E* (1%, 1 cm.) maxima of 614 at 2525 Å., and 233 at 3250 Å.

Titration of rhizopterin was carried out by dissolving the sample in standard 0.1 *N* alkali and titrating with standard acid. Precipitation of rhizopterin occurred at about *p*H 7. The mid-point of the titration curve was approximately *p*H 7.2; equivalent weight, calcd., 170; found, 167.

Titration of xanthopterin was carried out similarly for comparison. Precipitation of xanthopterin occurred at about *p*H 7. The mid-point of the titration curve was approximately *p*H 9.0; equivalent weight, calcd., 89.5; found, 94.

Acetylrhizopterin.—Ninety-nine milligrams of rhizopterin was treated with 10 ml. of acetic anhydride. The mixture was refluxed until all the solid was in solution (about thirty minutes). The solution was cooled, filtered, and concentrated *in vacuo*. The residue of acetylrhizopterin was dissolved in 0.1 *N* ammonium hydroxide and the solution was filtered and diluted to ten volumes with water. The alkaline solution was warmed on a steam-bath and acidified with acetic acid. The acetylrhizopterin crystallized as nearly white filamentous crystals. After two recrystallizations, the pure product was collected and dried. It failed to melt below 300°. It weighed 92 mg. For analyses, samples were dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{17}H_{14}N_6O_5$: C, 53.40; H, 3.69; N, 21.98; mol. wt., 382. Found: C, 53.53; H, 3.83; N, 21.71; mol. wt. (ebullioscopic in glacial acetic acid), 369.

(12) All melting points have been determined using a micro-melting point block.

At *p*H 11, acetylrhizopterin showed *E* (1%, 1 cm.) maxima of 985 at 2575 Å. and 190 at 3530 Å.; at *p*H 7, *E* (1%, 1 cm.) maxima of 569 at 2575 Å. and 191 at 3400 Å.; at *p*H 1, *E* (1%, 1 cm.) maxima of 569 at 2725 Å. and 208 at 3300 Å.

Titration of acetylrhizopterin was carried out by dissolving the sample in 0.1 *N* alkali and titrating the solution with standard acid. The titration curve showed two spans with midpoints at *p*H 7.46 and 3.86; equivalent weight, calcd., 191; found, 175.

Methoxyacetylrhizopterin.—Nine milligrams of rhizopterin was treated with 1 ml. of methoxyacetic anhydride and heated on a steam-bath until all had dissolved (about two hours). The unchanged anhydride was decomposed with methanol and the solution was evaporated *in vacuo* to a heavy oil. When the oil was diluted with ether, an amorphous precipitate formed. The precipitate was collected in a centrifuge tube and washed with ether. The crude methoxyacetylrhizopterin was purified by dissolving it in dilute ammonium hydroxide and acidifying the solution with acetic acid. The product crystallized in nearly white micro-needles, m. p. 258–268° with effervescence. The yield was 7.1 mg. For analysis, the sample was dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{18}H_{16}N_6O_6$: methoxyl, 7.52. Found: methoxyl, 5.04, 5.12.

Phenylacetylrhizopterin.—Twenty and six-tenths milligrams of rhizopterin was treated with a large excess (about 0.5 g.) of phenylacetic anhydride and the mixture was heated on a steam-bath until the rhizopterin had dissolved in the melt. The unchanged anhydride was decomposed with methanol and the solution evaporated *in vacuo*. The heavy oil remaining was diluted with ether which caused the precipitation of the product as an amorphous solid. The crude phenylacetylrhizopterin was purified by dissolving it in dilute ammonium hydroxide, warming the solution on a steam-bath, and adding acetic acid to *p*H 2. From the acid solution, the phenylacetylrhizopterin crystallized in nearly white crystals. When dry the product weighed 23 mg. It melted at 276°.

Anal. Calcd. for $C_{23}H_{18}N_6O_5$: C, 60.26; H, 3.96; N, 18.34. Found: C, 60.51; H, 4.42; N, 18.55.

Benzoylrhizopterin.—Fifteen milligrams of rhizopterin was treated with 1 ml. of benzoic anhydride at 150° for one hour. The rhizopterin dissolved in the benzoic anhydride melt during the heating. The solution was cooled and diluted with ether to give an amorphous precipitate of benzoylrhizopterin. The precipitate was collected in a centrifuge tube and washed with ether. It was purified by dissolving it in *N* ammonium hydroxide, warming the solution, and acidifying with acetic acid. The benzoylrhizopterin was a nearly white crystalline product. It weighed 16 mg. For analyses, samples were dried in a weighing-pig at 140°. The compound melted at 250° (dec.).

Anal. Calcd. for $C_{22}H_{16}N_6O_5$: C, 59.46; H, 3.63; N, 18.91. Found: C, 59.70; H, 3.77; N, 19.07.

Determination of Free Amino Nitrogen in Rhizopterin.—Xanthopterin was used as a control in this determination. In a typical experiment, 12.3 mg. of xanthopterin was dissolved in 1 ml. of cold concentrated hydrochloric acid. This solution was added slowly to the nitrosating mixture in the conventional Van Slyke apparatus and the nitrogen collected by the usual procedure. A blank was run with 1 ml. of concentrated hydrochloric acid. Xanthopterin gave a value of 8.1% amino nitrogen (calcd. value 7.8%). Five to ten-milligram samples of rhizopterin were dissolved in 1 ml. of cold concentrated hydrochloric acid and subjected to analysis by the same procedure. Rhizopterin by this procedure gave values of 3.3 and 4.1% amino nitrogen (calcd. value 4.1).

Acid Hydrolysis of Rhizopterin (Formation of *p*-[(2-Amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic Acid (II)). Seventy-nine milligrams of rhizopterin was suspended in 5 ml. of water and heated on a steam-bath. The hot suspension was treated with 4 ml.

of concentrated hydrochloric acid which brought about complete solution. The hydrochloride of the hydrolysis product (II) separated as a yellow crystalline precipitate as the solution cooled. This was collected in a centrifuge tube, washed well with water, and dried over solid sodium hydroxide. The yield was 80.7 mg. The compound decomposed at high temperature. For analysis, the sample was dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{14}H_{13}N_6O_3Cl$: C, 48.21; H, 3.76. Found: C, 48.31; H, 3.82.

p-[(2-Amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid was obtained by dissolving the hydrochloride in concentrated ammonium hydroxide. The solution was diluted to ten volumes and filtered. The filtrate was heated on a steam-cone and acidified with acetic acid to *p*H 7. The yellow microcrystalline product was collected in a centrifuge tube, washed with water and dried. The compound decomposed at high temperature. For analyses, samples were dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{14}H_{12}N_6O_3$: C, 53.80; H, 3.87; N, 26.91. Found: C, 53.80; H, 3.80; N, 27.14.

Formic Acid from the Hydrolysis of Rhizopterin.—The filtrate from the acid hydrolysis of rhizopterin was steam-distilled to about one-third of the original volume. The distillate was made slightly alkaline and concentrated *in vacuo* to 1 ml. The *p*H was then adjusted to 6 with hydrochloric acid and the concentrate treated with a methanol solution containing 19 mg. of *p*-bromophenacyl bromide. The solution was allowed to reflux for one hour, then it was filtered and concentrated *in vacuo*. The residue was dissolved in methanol and allowed to crystallize in the refrigerator. Two types of crystals formed, needles and spherical clusters. The crystals were separated by the use of a spatula and each was resublimed under reduced pressure. The needles were unchanged *p*-bromophenacyl bromide; the spherical clusters resublimed to white needles melting at 137–139°. The melting point of a synthetic sample of *p*-bromophenacyl formate was 137–139°. A mixture of the two samples showed an unchanged melting point.

Confirmation of the formic acid as a hydrolysis product was obtained by a modified analysis for the formyl group. A sample of rhizopterin was hydrolyzed with alcoholic sodium hydroxide, the hydrolyzate acidified with sulfuric acid and steam distilled. The distillate was made alkaline with a known volume of standard alkali and concentrated to a suitable volume for titration. Potentiometric titration indicated the presence of formic acid. The mid-point of the titration was at *p*H 3.9 which is in good agreement with the value 3.67 found for a known sample for formic acid.

Alkaline Hydrolysis of Rhizopterin.—Eight milligrams of rhizopterin was hydrolyzed with 5 ml. of 2.5 *N* sodium hydroxide by refluxing for four hours. The hydrolyzate was neutralized with acetic acid; the yellow precipitate which formed was collected in a centrifuge tube and washed with water. The resulting crude hydrolysis product was extracted from silica with *N* ammonium hydroxide and the filtered solution acidified with acetic acid. The *p*-[(2-amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid was recrystallized twice, and a sample was dried in a weighing-pig at 140° for analysis.

Anal. Calcd. for $C_{14}H_{12}N_6O_3$: C, 53.80; H, 3.87. Found: C, 54.43; H, 3.96.

At *p*H 11, *p*-[(2-amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid showed *E* (1%, 1 cm.) maxima of 830 at 2560 Å., 770 at 2750 Å. and 260 at 3650 Å.; at *p*H 7, *E* (1%, 1 cm.) maxima of 890 at 2800 Å. and 230 at 3500 Å.

Acetylation of the Hydrolysis Product (II) (Formation of *p*-[N-(2-Acetamido-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-acetamido]-benzoic Acid).—Ten milligrams of the hydrolysis product (II) was refluxed with a large excess (about 2 ml.) of acetic anhydride until solution was complete. The solution was concentrated to dryness *in vacuo*. The residue of the acetylated product was dissolved in *N* ammonium hydroxide. The solution was filtered and acidified with acetic acid which caused precipitation of *p*-[N-(2-acetamido-4-hydroxypyrimido[4,5-*b*]-pyrazin-6-ylmethyl)-acetamido]-benzoic acid as a nearly white granular precipitate. Recrystallization, with careful warming of the alkaline solution and slow addition of the acetic acid, produced a micro-crystalline product. The compound decomposed at elevated temperature. For analysis, the sample was dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{18}H_{16}N_6O_5$: C, 54.54; H, 4.07. Found: C, 54.44; H, 3.98.

At *p*H 11, the acetylation product showed *E* (1%, 1 cm.) maxima of 1000 at 2550 Å. and 210 at 3500 Å.; at *p*H 7, *E* (1%, 1 cm.) maxima of 560 at 2550 Å., a shoulder of about 420 at 2750 Å. and 209 at 3400 Å.; at *p*H 1, *E* (1%, 1 cm.) maxima of 600 at 2350 Å., 465 at 2800 Å. and 219 at 3300 Å.

Oxidation of *p*-[(2-Amino-4-hydroxypyrimido[4,5-*b*]-pyrazin-6-ylmethyl)-amino]-benzoic Acid.—One hundred two milligrams of the hydrolysis product (II) as the hydrochloride was divided into four approximately equal parts. To each was added 1.6 ml. of water and 0.6 ml. of concentrated hydrochloric acid. The suspension was heated on a steam-bath and 0.5 *M* potassium chlorate solution added in 0.1-ml. portions. The material dissolved after addition of approximately 0.3 ml. of the oxidizing agent and chloranil was formed as a light yellow flaky precipitate. The oxidation mixtures were combined and the precipitate collected on a filter. The chloranil weighed 29.4 mg. It was purified by recrystallization from methanol in yellow plates which sublimed without melting.

Anal. Calcd. for $C_6O_2Cl_4$: C, 29.31; H, 0.00; Cl, 57.68. Found: C, 29.05; H, 0.78; Cl, 60.98.

The aqueous oxidation mixture after removal of chloranil was concentrated to dryness *in vacuo* leaving a white solid residue. The inorganic salts were extracted with cold water leaving oxaloguanidine as a white solid. This was collected in a centrifuge tube, washed with water, and dried; weight, 18 mg. It was purified by recrystallization from 0.2 *N* hydrochloric acid. The recrystallized oxaloguanidine failed to melt up to 275°.

Anal. Calcd. for $C_3H_5N_3O_3$: N, 32.06. Found: N, 31.72.

The recrystallization of oxaloguanidine from dilute acid resulted in some hydrolysis of this compound. The mother liquor from the recrystallization was treated with alcoholic picric acid. The solution was concentrated to dryness leaving guanidine picrate as a yellow residue. It was recrystallized from hot water. It failed to melt below 300° but decomposed at about that temperature.

Anal. Calcd. for $C_7H_8N_6O_7$: N, 29.17. Found: N, 28.38.

***p*-Aminobenzoic Acid from the Pyrolysis of Rhizopterin and of *p*-[(2-Amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic Acid.**—Both rhizopterin and its hydrolysis product (II) gave *p*-aminobenzoic acid on pyrolysis *in vacuo*. A tube containing 10 mg. of the hydrolysis product (II) was evacuated by a mercury vapor pump at about 0.01 mm. As the sample was heated above 220°, decomposition occurred and a white sublimate appeared. Pyrolysis was discontinued at 360° and the melting point of the sublimate determined. Its characteristic behavior on a hot stage of resubliming at 140° and melting at 182–187° suggested that it might be *p*-aminobenzoic acid. A test for this substance, made according to the Bratton and Marshall[13] procedure, was positive for *p*-aminobenzoic acid. Resublimation of the crude *p*-aminobenzoic acid gave pure material which on a hot stage resublimed at about 140° and melted at 187–188°. The melting behavior of a mixture of this material and an authentic sample of *p*-aminobenzoic acid was unchanged.

For further identification, the *p*-aminobenzoic acid was converted to its acetyl derivative by dissolving it in acetic anhydride. The unused acetic anhydride was evaporated

(13) Bratton and Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

under reduced pressure. The acetyl derivative melted at 256–258°. A mixture of the compound with an authentic sample of *p*-acetamidobenzoic acid melted without depression.

Isolation of *p*-Aminobenzoic Acid from Rhizopterin by Hydrolysis.—Two and six-tenths milligrams of rhizopterin was heated in 2 ml. of 1% hydrochloric acid at 200° for three hours. The solution was allowed to cool and an aliquot taken for the determination of *p*-aminobenzoic acid by the method of Bratton and Marshall.[13] By this determination, 0.3520 mg. of *p*-aminobenzoic acid was present. This corresponds to about 34% of the theoretical value.

In another experiment, 8 mg. of rhizopterin was heated with 5 ml. of 2.5 *N* sodium hydroxide at reflux temperature for four hours. The solution was acidified with acetic acid which caused precipitation of a yellow product. The filtrate from this precipitate gave a positive test for *p*-aminobenzoic acid by the method of Bratton and Marshall.[13]

Benzoylxanthopterin.—Five hundred milligrams of xanthopterin was mixed with about 4 g. of benzoic anhydride in a tube protected from moisture and heated at 200° for one and one-half hours. The xanthopterin dissolved in the melt. The mixture was allowed to cool and the benzoic acid and unused benzoic anhydride were extracted with ether. The residue of benzoylxanthopterin was dissolved in dilute ammonium hydroxide, the solution was treated with a little decolorizing carbon and filtered. The alkaline solution was heated on a steam-bath and acidified with acetic acid. The benzoylxanthopterin precipitated in microcrystals. It was collected and washed with water. The recrystallization was repeated twice and the benzoylxanthopterin dried in a weighing-pig at 140° for analysis. The compound melted at 270–272° (dec.).

Anal. Calcd. for $C_{13}H_9N_5O_3$: C, 55.12; H, 3.20. Found: C, 54.89; H, 4.19.

Oxidation of Benzoylxanthopterin.—Twenty-three milligrams of benzoylxanthopterin was suspended in 2 ml. of 4.5 *N* hydrochloric acid. This suspension was heated at 60–70° and 0.75 ml. of 0.2 *M* potassium chlorate solution was added dropwise. After heating for thirty minutes, the mixture was clear and colorless. The solution was then cooled and saturated with ether. The aqueous solution on standing gave long needle-like crystals which were separated by centrifugation, washed with water and then ether. These crystals of benzoylguanidine hydrochloride melted at 200–205° (dec.).

Anal. Calcd. for $C_8H_{10}N_3OCl$: C, 48.13; H, 5.05. Found: C, 48.70; H, 5.07.

Oxidation of Benzoylrhizopterin.—Six and seven-tenths milligrams of benzoylrhizopterin was suspended in 1 ml. of 15% hydrochloric acid and the mixture heated on a steam cone. Aqueous 0.5 *M* potassium chlorate solution was added dropwise to the hot solution until the benzoylrhizopterin had dissolved. Chloranil crystallized from the solution immediately. The solution was cooled and the chloranil collected on a filter. The filtrate was concentrated to about 0.5 ml. and saturated with ether. This caused crystallization of the benzoylguanidine hydrochloride. The melting point was 200–207°. A mixture of this product with a known sample of benzoylguanidine hydrochloride melted without depression.

Desiminorhizopterin.—Five milligrams of rhizopterin was dissolved in 1 ml. of concentrated hydrochloric acid and the solution added immediately to a mixture of 5 ml. of 25% sodium nitrite solution and 5 ml. of glacial acetic acid. The two solutions were mixed thoroughly and allowed to stand at room temperature for one hour. The reaction mixture was then concentrated to dryness *in vacuo*. The residue was washed with cold water to remove inorganic salts. A light yellow precipitate of crude desiminorhizopterin remained. Purification was accomplished by recrystallization from hot water. A small amount of yellow *p*-[(2,4-dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid was formed but was separated by its insolubility in hot water. The desiminorhizopterin seems to be dimorphous; slow cooling of its aqueous solution produced small square plate-like crystals. Rapid cooling gave white needle-like crystals which melted at 321–323° (dec.). For analyses, samples were dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{15}H_{11}N_5O_5$: C, 52.79; H, 3.25; N, 20.52. Found: C, 52.78; H, 3.36; N, 21.44.

At *p*H 11 desiminorhizopterin showed *E* (1%, 1 cm.) maxima of 720 at 2425 Å., 620 at 2700 Å. and 175 at 3550 Å.

***p*-[(2,4-Dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic Acid (VIII).**—Eleven milligrams of desiminorhizopterin was suspended in 10 ml. of 15% hydrochloric acid and warmed on a steam-bath for fifteen minutes. The solid dissolved in the hot acid. As the solution cooled, a canary yellow crystalline precipitate formed. It was collected in a centrifuge tube and washed with water. The product was recrystallized by dissolving it in 1 *N* ammonium hydroxide, diluting with three volumes of water and acidifying the solution with acetic acid at about 90°. The yellow crystalline product failed to melt below 300°. For analysis the sample was dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{14}H_{11}N_5O_4$: C, 53.67; H, 3.54. Found: C, 53.83; H, 3.52.

At *p*H 11 *p*-[(2,4-dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid showed *E* (1%, 1 cm.) maxima of 492 at 2385 Å., 920 at 2780 Å. and 220 at 3550 Å.; at *p*H 7, *E* (1%, 1 cm.) maxima of 600 at 2780 Å. and 262 at 3300 Å.

***p*-[N-(2,4-Dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-nitrosamino]-benzoic Acid (IX).**—Five milligrams of the desiminorhizopterin hydrolysis product (VIII) was dissolved in 2 ml. of concentrated hydrochloric acid. The solution was cooled in ice and 3 ml. of cold 25% aqueous sodium nitrite solution added slowly. The reaction mixture was allowed to stand at room temperature overnight. A nearly white precipitate of the nitroso derivative formed on standing. The mixture was diluted with three volumes of water and the precipitate collected in a centrifuge tube, washed with water and dried at room temperature over phosphoric anhydride for analysis. The compound gave a strong Liebermann nitroso color test with phenol and sulfuric acid.

Anal. Calcd. for $C_{14}H_{10}N_6O_6$: C, 49.12; H, 2.95. Found: C, 48.91; H, 3.27.

In another experiment, 5 mg. of the hydrolysis product (II) was dissolved in about 2 ml. of concentrated hydrochloric acid by gentle heating. The solution was cooled and added to a cold mixture of 2 ml. each of glacial acetic acid and 25% sodium nitrite solution. The mixture was stirred well and allowed to react for one hour. It was concentrated to dryness under reduced pressure. The residue was extracted with water to remove inorganic salts. The insoluble portion was light yellow; it was separated and washed again with water. The product was dissolved in very dilute (about 0.01 *N*) ammonium hydroxide. When this was acidified with acetic acid the nitroso derivative (IX) crystallized from solution. It was collected in a centrifuge tube, washed with water and dried. The compound gave a strong Liebermann test for the nitroso group. Its ultraviolet absorption spectrum was identical with that of the same compound above.

At *p*H 11 *p*-[*N*-(2,4-dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-nitrosamino]-benzoic acid (IX) showed *E* (1%, 1 cm.) maxima of 635 at 2385 Å., 710 at 2775 Å. and 190 at 3550 Å.; at *p*H 7, *E* (1%, 1 cm.) maxima of 615 at 2325 Å., 395 at 2775 Å. and 260 at 3300 Å.

Pyrolysis of *p*-[N-(2,4-Dihydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-nitrosamino]-benzoic Acid (IX).—Three milligrams of the nitroso compound (IX) was evacuated to 0.001 mm. and heated to 200°. A white sublimate was formed which gave a positive color test for *p*-aminobenzoic acid by the method of Bratton and Marshall.[13] A portion of the sublimate was resublimed onto a slide for a micro-melting point determination. On the microblock the compound resublimed to needles at 150° and melted at 181–

184°. A mixture of this sample with *p*-aminobenzoic acid resublimed to needles at 150° and melted at 187–188°.

Formylation of the Hydrolysis Product (II) (Resynthesis of Rhizopterin (I)).—Twenty-five milligrams of the hydrolysis product (II) was treated with a mixture of 2 ml. of formic acid (88%) and 0.7 ml. of acetic anhydride. The mixture was heated at reflux temperature until all dissolved (about twenty minutes). The solution was filtered and concentrated to dryness *in vacuo*. The solid residue was dissolved in 1 *N* ammonium hydroxide; the solution was filtered and diluted to about ten volumes with water. It was then warmed on a steam-bath and acidified slowly with acetic acid to about *p*H 4. The rhizopterin crystallized in yellow platelets. It was collected in a centrifuge tube, washed with water, and dried. Microbiological assays of the product, which was resynthesized in this manner, using *S. lactis* R. showed it to have the biological activity of rhizopterin. For analyses, samples were dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{15}H_{12}N_6O_4$: C, 52.94; H, 3.56; N, 24.70. Found: C, 52.57; H, 3.24; N, 24.70.

Formylation of Pteroic Acid (II) (Synthesis of Rhizopterin (I)).—One hundred fifty milligrams of pteroic acid (II), prepared as described,[11] was treated with 10 ml. of formic acid (98%) and heated on a steam-bath. All solid dissolved after five minutes, but heating was continued for one hour. The solution was concentrated to dryness under reduced pressure. The residue was dissolved in 10 ml. of 1 *N* ammonium hydroxide. The solution was diluted to about 10 volumes, warmed to 70° and acidified with acetic acid to *p*H 4. Rhizopterin (I) crystallized from the solution in light yellow leaves. For analyses, samples were dried in a weighing-pig at 140°.

Anal. Calcd. for $C_{15}H_{12}N_6O_4$: C, 52.94; H, 3.56; N, 24.70. Found: C, 52.76; H, 3.85; N, 24.45.

The biological activity was determined using *S. lactis* R. and found to be the same as that of rhizopterin isolated from natural sources.

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Summary

The structure of rhizopterin, the S.I.R. factor, is *p*-[N-(2-amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-formamido]-benzoic acid (I). Rhizopterin has the characteristic ultraviolet absorption spectrum and solubility properties of pterins. It is biologically active for *S. lactis* R. but inactive for *Lactobacillus casei*.

Rhizopterin has been hydrolyzed under mild conditions to give *p*-[(2-amino-4-hydroxypyrimido[4,5-*b*]pyrazin-6-ylmethyl)-amino]-benzoic acid (II) and formic acid. Oxidation of this hydrolysis product (II) with a mixture of hydrochloric acid and potassium chlorate produced oxaloguanidine and chloranil. More drastic hydrolysis or pyrolysis of rhizopterin or its hydrolysis product (II) produced *p*-aminobenzoic acid. Oxidation of benzoylrhizopterin gave benzoylguanidine hydrochloride and chloranil. Desiminorhizopterin was obtained by the action of nitrous acid on rhizopterin.

Rhizopterin was obtained by formylation of the hydrolysis product (II) and also by formylation of the same compound prepared by synthesis.

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Calcium Ion Activities in Supersaturated Solutions Stabilized by Sodium Metaphosphate as Determined by Clay Membrane Electrodes

BY R. F. REITEMEIER[1] AND A. D. AYERS[2]

Introduction

During the past decade, the retardation and prevention of precipitation from solutions presumably supersaturated with calcium carbonate has been accomplished by the addition of about 2 p. p. m. of glassy sodium hexametaphosphate. Systems thus stabilized include irrigation waters containing free ammonia,[3] alkaline soil solutions,[4] industrial and municipal water supplies,[5] and oil-field brines.[6] The efficacy of this minute concentration of hexametaphosphate in preventing the normal deposition of the excess calcium carbonate from irrigation waters was first demonstrated by Ayers in 1935 (which discovery was the basis for the first patent in this field[3]) and was an outgrowth of its use in stoichiometric proportions to soften hard water by formation of a soluble complex anion containing calcium.[7] Although the mechanism of the process has been investigated by various workers,[8,9,10] no completely satisfactory explanation has yet been proposed. A possible mechanism, which has not been directly investigated previously, is that the hexametaphosphate somehow reduces the activity of the calcium ions so that they cannot condense with carbonate

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(3) Rosenstein, U. S. Patent 2,038,316 (1936); reissues 20,360 (1937) and 20,754 (1938).

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