TABLE III

Fraction	Rhizopterin, mg.	Fraction	Rhizopterin, mg.
1	48	6	73
2	240	7	30
3	310	8	18
4	210	9	9
5	140	10	5

Fractions 2, 3, 4 and 5 were combined and concentrated *in vacuo* to approximately 100 ml. The light yellow insoluble material which separated was collected by centrifugation, washed with 25 ml. of water followed by alcohol and acetone, and dried *in vacuo*. The precipitate weighed 1 g. and contained 830 mg. of rhizopterin by microbiological assay with *S. lactis R*.

Crystallization.—Crude rhizopterin (30 mg.) was dissolved in 1 ml. of 6 *N* ammonium hydroxide and freed of a small quantity of insoluble impurity by centrifugation. To the clear ammoniacal solution was added absolute ethanol (10 ml.). On standing, the ammonium salt of rhizopterin crystallized in small aggregates of fine yellow needles. Because the product lost ammonia on drying, the analyses were unsatisfactory.

The material was also crystallized as the free acid. Crude rhizopterin (200 mg.) was dissolved in approximately 125 ml. of dilute ammonium hydroxide. The solution was heated to boiling and acidified slowly to *p*H 6 with acetic acid. The hot solution was freed of an amorphous precipitate and treated with a small amount of norit, filtered, and further acidified to *p*H 4.5 with acetic acid. Rhizopterin crystallized from the cooled solution in the form of light yellow platelets. The compound did not melt below 300°.

Anal. Calcd. for $C_{15}H_{12}N_6O_4$: C, 52.92; H, 3.55; N, 24.68. Found: C, 53.14; H, 3.95.

Considerable difficulty was encountered in obtaining uniform nitrogen analyses. An average nitrogen value obtained from fourteen analyses on six separate samples was 24.74 with a standard error of ± 0.30.

Acknowledgment.—The authors wish to thank Dr. N. R. Trenner and associates for the ultraviolet absorption spectra and titration data, Mr. R. N. Boos and associates for the microanalyses, Dr. J. L. Stokes and associates for microbiological assays, and Drs. G. A. Emerson and W. H. Ott of the Merck Institute for Therapeutic Research for the animal experiments.

Summary

A procedure effecting a 200,000-fold purification has been described by which it was possible to isolate in crystalline form a new compound which promotes the growth of *S. lactis R* in a medium deficient in folic acid. The compound, which was previously referred to as the "S.L.R. factor," has been termed rhizopterin. Ultraviolet absorption spectra and potentiometric titration data indicate that rhizopterin is pterin-like in nature. The analytical data correspond closely to the formula $C_{15}H_{12}N_6O_4$.

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A Degradation Product of Rhizopterin

BY EDWARD L. RICKES, NELSON R. TRENNER, JOHN B. CONN AND JOHN C. KERESZTESY[1]

A study was made of the behavior of the microbiological activities of crude rhizopterin concentrates during treatment with acid and alkali. The *Streptococcus lactis R* activity was found to decrease upon treatment with these reagents, whereas, the *Lactobacillus casei* activity remained unchanged.

These preliminary observations indicated the possible formation of a substance less active for *S. lactis R* which was relatively stable to acid and alkali. When crystalline rhizopterin became available, it was subjected to similar alkali treatment. While under the influence of the alkali, the ultraviolet absorption spectrum was observed. Over a period of about twenty hours at room temperature, the normal ultraviolet absorption spectrum of rhizopterin in alkali was found to change in such a manner as to increase markedly in the region of 2800 Å. At the end of the twenty-hour period, the alkaline solution was acidified to a *p*H of approximately 1 and the ultraviolet absorption spectrum again observed (Fig. 1). Instead of the bands at 2525 and 3225 Å., characteristic of rhizopterin in acid solution, the new spectrum was characterized by bands at 2500 and 3025 Å.

On the basis of the foregoing observations, a larger quantity of crystalline rhizopterin was subjected to alkaline degradation and a product was isolated which appeared to have the empirical formula $C_{14}H_{10}N_6O_3Na_2$. A potentiometric titration of the free acid gave an equivalent weight of 150 and indicated it to be a weak dibasic acid.

The properties of this alkaline degradation product showed that it has a close relationship to rhizopterin and the pterins, and on this basis it was named aporhizopterin.

In Figs. 2 and 3 are presented the ultraviolet absorption spectra of aporhizopterin and vitamin B_c[2] (pteroylglutamic acid) at three *p*H values.[3] The ultraviolet absorption spectra of rhizopterin and xanthopterin have been presented.[4] The general similarity of the ultraviolet absorption spectra of these four compounds indicates a general class (pterin) relationship.

(1) Present address: National Institute of Health, Bethesda, Md.

(2) Bloom, Vandenbelt, Binkley, O'Dell and Pfiffner, *Science*, **100**, 295 (1944).

(3) The data in Fig. 3 were obtained with vitamin B_c isolated in this Laboratory.

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

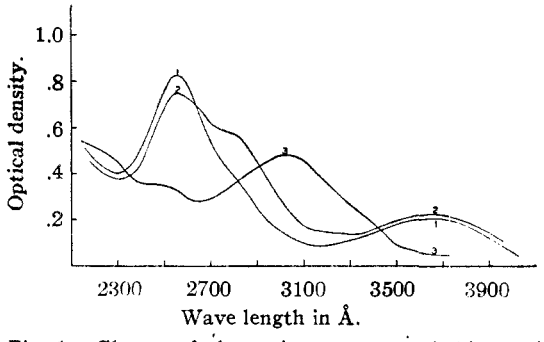

Fig. 1.—Change of absorption spectrum of rhizopterin on standing in 0.1 *N* sodium hydroxide: (1) 0 time, (2) after twenty hours, (3) after twenty hours (solution acidified).

The potentiometric titration of aporhizopterin proved to be somewhat difficult because of the low solubility of this substance in water. It was therefore necessary to carry out the titration by adding a known amount of alkali to the sample in order to obtain a solution of the compound in the form of its salt. The unbound (excess) alkali was then determined by back titration with hydrochloric acid. From these data, the equivalent weight could be evaluated. During the back titration, only a part of the titration curve of aporhizopterin could be obtained before precipitation of the compound occurred and therefore it was only possible to make an estimation of its acid strength. The general position of the mid-point of the binding span was found to be at a *p*H of about 7.5. The mid-point of the binding span for rhizopterin[4] was observed to have a *p*H value of 7.20.

In the case of vitamin B_c, it was possible to evaluate the first *pKa* value which was found to be 8.1. Again, the general similarity of the acid strengths of these compounds indicates a relationship.

The polarographic behavior of xanthopterin, rhizopterin, vitamin B_c and of aporhizopterin was also investigated. The data thus obtained are presented in Table I. The almost identical values of the half wave potentials for the four substances is striking, and further substantiates the common class relationship of these compounds.

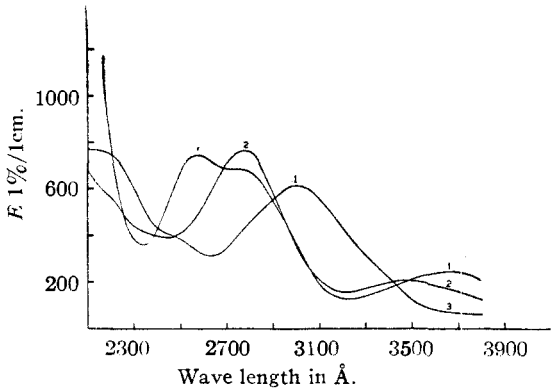

Fig. 2.—Absorption spectrum of aporhizopterin: (1) *p*H 12.6, (2) *p*H 7.0, (3) *p*H 1.3.

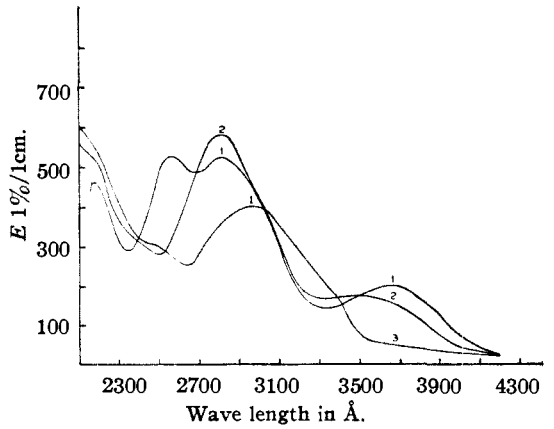

Fig. 3.—Absorption spectrum of vitamin Bc (pteroylglutamic acid): (1) 0.05 *N* sodium hydroxide, (2) *p*H 7.07, (3) 0.1 *N* hydrochloric acid.

TABLE I

The supporting electrolyte was 0.1 *M* $Li_2B_4O_7$ (*p*H 9.12); the concentrations of pteridins were such as to give approximately equal diffusion currents (*ca.* 0.0005 *M*); capillary constant, $m^{2/3}t^{1/6}$ = 1.65 mg.$^{2/3}$ sec.$^{-1/2}$.

	Xanthopterin	Rhizopterin	Aporhizopterin	Vitamin B_c
$(I_d/C)\mu$ A. ml./mg.	27.2	14.2	15.1	8.64
$E^1/_2$ *vs.* S C. E., v.	−0.80	−0.84	−0.81	−0.79

The correctness of the general conclusions arrived at above was again substantiated when it was observed that the ultraviolet absorption spectrum of aporhizopterin was quite similar to that of vitamin B_c at all *p*H values. The only difference between the two absorption spectra was in the intensity of the absorption bands. This relationship is illustrated by the data presented in Table II.

TABLE II

*p*H	Wave length (max.), Å.	B_c (peroylglutamic acid) $E^{1\%}_{1\,cm.}$	Aporhizopterin $E^{1\%}_{1\,cm.}$	Ratio
1	2975	404	600	1.49
7.07	3450	172	200	1.16
7.07	2825	581	750	1.30
12.5	3650	200	240	1.20
12.5	2850	519	625	1.20
12.5	2550	530	740	1.39

Thus it may be concluded that aporhizopterin possesses the same chromophoric grouping as is present in vitamin B_c and differs from it only in

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_6 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.