alkylated amino nitro compounds decrease markedly with increasing size of the alkyl groups. The melting points of the trihalogeno derivatives of aniline, monomethylaniline and dimethylaniline show the same decrease; thus, the melting points for the trichloro derivatives are 78.5, 32 and < 25°; for the tribromo, 122, 39 and < 25°, respectively; and for the triiodoaniline and dimethylaniline, 185 and 69–70°, respectively. The authors believe that these decreases are another example of the same phenomenon recorded by Arnold, Pierce and Barnes.


## Summary

1. 2,4,6-Triiododimethylaniline has been synthesized.

2. *p*-Iodoaniline, *p*-iododimethylaniline, 2,4,6-triiodoaniline and 2,4,6-triiododimethylaniline have been examined from the standpoint of halogen reactivity, in reactions with acid stannous chloride, bromine and nitrous acid.

3. As predicted from the theory of damped resonance, the halogen atoms in 2,4,6-triiododimethylaniline show a relatively low order of reactivity.


EDMONTON, ALBERTA, CANADA RECEIVED APRIL 28, 1947

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

# Isolation of Rhizopterin, A New Growth Factor for *Streptococcus Lactis R*

BY EDWARD L. RICKES, LOUIS CHAIET AND JOHN C. KERESZTESY[1]

Experiments performed in this Laboratory very early demonstrated the presence, in some natural materials, of more than one factor capable of supporting the growth of *Streptococcus lactis R*[2] on a "folic acid" deficient medium.[3] Differential assays of concentrates from several natural sources indicated that some possessed a much higher activity for *S. lactis R* than for *Lactobacillus casei*.[4] This property was shown to a striking degree by a charcoal adsorbate[5] derived from the purification of *Rhizopus nigricans* fumaric acid fermentation liquors. The fractionation of an eluate of this adsorbate by means of chromatographic adsorption procedures led to the isolation, in crystalline form, of a compound highly active for *S. lactis R* and substantially inactive for *L. casei*.[6]

Subsequent investigations have shown that this new growth factor is a pterin.[7,8] Because of the nature and source of the factor, the name rhizopterin has been adopted for this compound, formerly referred to as the "S.L.R. factor."[9]

It was found that rhizopterin was relatively weakly adsorbed on alumina and appeared in the initial eluates. It could be distinguished from succeeding more strongly adsorbed fractions, which were active for both organisms. The more strongly adsorbed material which appeared in the later eluate fractions corresponded closely in chromatographic adsorption behavior and biological activity to other concentrates of vitamin $B_c$ (pteroylglutamic acid).

To obtain rhizopterin in crystalline form, it was necessary to effect a 200,000-fold purification from the initial charcoal eluate. This was accomplished through the following sequence of steps: elution of the charcoal adsorbate, readsorption by norit A and elution, adsorption by fuller's earth and elution, precipitation at *p*H 7, chromatographic adsorption on alumina, and crystallization as the free acid or ammonium salt.

The microanalytical data for rhizopterin are in best agreement with the formula $C_{15}H_{12}N_6O_4$. The pterin-like nature of the compound is indicated by a comparison of the absorption spectra of rhizopterin and xanthopterin (Figs. 1 and 2). The pterin nature is further demonstrated by the potentiometric titration data for these two compounds (Table I). An equivalent weight of 167.5

TABLE I

| | Rhizopterin | Xanthopterin |
|---|---|---|
| Initial *p*H | 4.3 | 5.0 |
| Midpoint | 7.2 | 9.0 |
| Neutralization point | 10.3 | 10.8 |

was calculated for rhizopterin. Since the potentiometric titration indicated that it is a dibasic acid, one may assume a molecular weight of 335, which is in agreement with the value of 340 calculated for $C_{15}H_{12}N_6O_4$. Other physical properties of rhizopterin, such as its insolubility in water and common organic solvents, its solubility in acid and alkali, and the failure of the compound to melt up to 300° further indicate the correlation of rhizopterin with the pterins. Later chemical and physico-chemical studies confirmed the pterin-like nature of the compound.[7,8]

It was found that 0.000034 $\gamma$ of rhizopterin per ml. of culture medium is necessary to produce half maximum growth of *S. lactis R*. It is essentially inactive for *L. casei*.

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

(2) This organism is also known as *Streptococcus faecalis R*.

(3) Assay method of Mitchell, Snell and Williams, THIS JOURNAL, **63**, 2284 (1941).

(4) Norit eluate factor assays using *L. casei*: Hutchings, Bohonos and Peterson, *J. Biol. Chem.*, **141**, 521 (1941).

(5) This adsorbate was obtained from Chas. Pfizer and Co. *Cf.* J. H. Kane, A. Finlay, P. F. Amann, U. S. Patent 2,327,191 (1943).

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

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

(8) Rickes, Trenner, Conn and Keresztesy, *ibid.*, 2751 (1947).

(9) Stokes, Keresztesy and Foster, *Science*, **100**, 522 (1944).

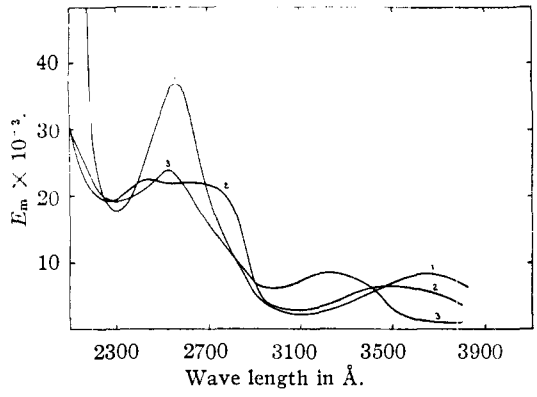


Fig. 1.—Absorption spectrum of rhizopterin: (1) *p*H 12.6, (2) *p*H 7.0 and (3) *p*H 1.3.

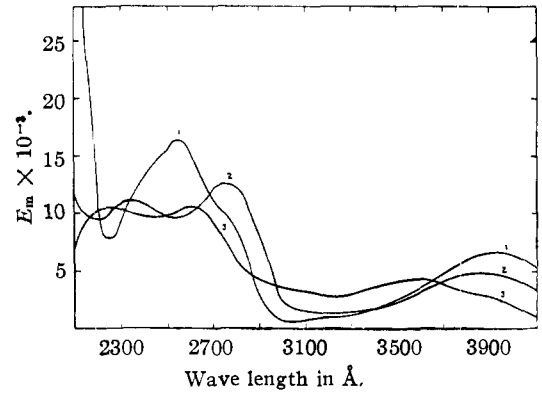


Fig. 2.—Absorption spectrum of xanthopterin: (1) *p*H 12.6, (2) *p*H 7.0 and (3) *p*H 1.3.

Rhizopterin was found to be inactive for hemoglobin formation and growth promotion in chicks on a diet containing all known nutrients except members of the folic acid group. When the compound was fed at the rate of 1 mg. per rat per day, the granulocytopenia which was induced in rats on a folic acid deficient diet containing succinylsulfathiazole was not cured. Vitamin $B_c$ (pteroylglutamic acid) was effective at 20 γ per day.

## Experimental

**Elution of Adsorbate.**—Moist charcoal adsorbate[5] (180 lb.) was stirred for one hour with 150 gal. of 40% ethanol containing 0.25% sodium hydroxide. The eluate showed an activity for *S. lactis R* corresponding to 0.004 to 0.007 γ of rhizopterin per mg. of solids, or a total of 140 to 200 mg. of rhizopterin, and an activity for *L. casei* corresponding to a total of 20 mg. of pteroylglutamic acid.

**Readsorption on Norit.**—The eluate was acidified with dilute sulfuric acid to *p*H 2 to 3. Norit A (5 lb.) was added and the mixture stirred for one hour and then filtered.

**Elution of Readsorbate.**—The combined readsorbates from five accumulated batches were suspended in 150 gal. of 30% ethanol containing 0.5% sodium hydroxide. The suspension was stirred for one hour and filtered. This eluate contained the equivalent of approximately 1.0 g. of rhizopterin and 100 mg. of pteroylglutamic acid.

**Fuller's Earth Adsorption and Elution.**—The eluate (150 gal.) from the norit readsorption was diluted to 300 gal. with water and promptly adjusted to *p*H 2.5 with sulfuric acid. Fuller's earth (6.5 kg.) was added slowly and the *p*H maintained at 2.5 by the addition of 50% sulfuric acid. The mixture was stirred for one hour and filtered. The filtrate was treated with a second equal amount of fuller's earth and then discarded.

The combined fuller's earth adsorbates were suspended in a mixture of 15 gal. of ethanol (95%), 5 gal. of concentrated (28%) ammonium hydroxide and 30 gal. of water and stirred for one hour. The suspension was filtered and the filtrate concentrated *in vacuo* to 3 to 5 liters. On standing in the cold this solution, the *p*H of which was approximately 7, deposited a precipitate containing the active material. Additional active material was recovered from the filtrate in the form of a precipitate which separated on acidification to *p*H 2.5.

***p*H 7-Precipitate.**—The precipitate was separated from the liquor by centrifugation, washed with 6% acetic acid followed by alcohol and acetone, and dried *in vacuo*. This precipitate (90 to 150 g.) contained 600 mg. of rhizopterin of approximately 0.5% purity. Six batches of this material totaling 885 g. were combined for further purification.

**Chromatographic Adsorption.**—A portion of the combined *p*H 7-insoluble fractions (126 g.) was extracted three times by stirring for one-half hour with 1 liter of 0.5 *N* hydrochloric acid. The insoluble residue was removed by centrifugation and discarded. The extract was stirred with 5 volumes of 95% ethanol, the *p*H was adjusted to 4.5 with 30% sodium hydroxide and the precipitate which formed was removed by filtration and discarded. The clear filtrate (18 liters) was stirred with aluminum oxide[10] (100 to 150 g.) for one hour. The alumina was removed and the filtrate chromatographed under pressure on a column 72 mm. in diameter containing 200 g. of alumina. The column was washed with 2 liters of 95% ethanol and the filtrate discarded. Elution was then carried out with a mixture containing 20 volumes of methanol, 10 volumes of concentrated (28%) ammonium hydroxide and 70 volumes of water. The *p*H of the effluent was carefully followed after the addition of the ammoniacal eluant. Immediately following a rise in the *p*H of the eluate, fractions were taken as indicated in Table II.

TABLE II

| Fraction | Volume, ml. | Rhizopterin, mg. | Solids, mg. | Purity, % |
|---|---|---|---|---|
| 1 | 100 | 2.2 | 70 | 3.0 |
| 2 | 100 | 100.0 | 840 | 11.9 |
| 3 | 100 | 50.0 | 710 | 7.0 |
| 4 | 100 | 13.6 | 470 | 2.9 |
| 5 | 250 | 7.3 | 320 | 2.3 |
| 6 | 250 | 4.2 | 250 | 0.68 |

A total of 7 columns similar to that described above was required for processing the combined batch of *p*H 7-insoluble material. The first four or five fractions from each of the seven chromatograms were combined. The resulting solution contained approximately 60 γ of rhizopterin per mg. of solids and represented a total of 1.2 g. of rhizopterin. This solution also showed an activity for *L. casei* corresponding to approximately 20 mg. of pteroylglutamic acid. The combined fractions were concentrated *in vacuo* to 2 liters. The solution was made alkaline (*p*H 12) with ammonium hydroxide and treated with five volumes of 95% ethanol. The precipitate which formed was removed by filtration and discarded, and after removal of excess ammonia by distillation *in vacuo*, the filtrate was acidified to *p*H 4.5 with glacial acetic acid. This solution was then chromatographed on a column 72 mm. in diameter which contained 400 g. of alumina. The column was washed thoroughly with 80% ethanol and eluted with 50% methanol containing 3% concentrated ammonium hydroxide. Fractions (Table III) of 100 ml. each were taken in the manner described for the first chromatogram. The material showing *L. casei* activity was eluted from the column in later fractions not illustrated in the table.

(10) Merck–Brockman alumina was used throughout this work.

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 $C_{15}H_{12}N_6O_4$.

RAHWAY, NEW JERSEY RECEIVED MAY 13, 1947

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

# A Degradation Product of Rhizopterin

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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).