likely that this compound accounts for some of the biological activity in green plants. Our own limited experience with green plant sources indicates the presence therein of at least two microbiological growth factors both of which are active as antianemia agents in the chick.

Acknowledgment.—The authors wish to thank Mr. A. W. Spang for his microanalytical services and Mr. D. G. Calkins for assistance in much of the isolation work.

Summary

Methods are described for the isolation of crystalline vitamin Bc as the free acid and its dimethyl ester from hog liver.

The identical acid and ester were isolated from horse liver.

A method is described for the partial concentration of the chick antianemia activity in yeast extract. Following enzymatic digestion of such concentrates the identical acid and ester were isolated.

Some salts and derivatives of the vitamin are described.

Vitamin Bc was found to be identical with synthetic pteroylglutamic acid.

Evidence is presented for the occurrence in liver of a compound having biological properties similar to those of pteroylglutamic acid but differing from it chiefly in being very acid labile.

DETROIT, MICHIGAN RECEIVED FEBRUARY 17, 1947

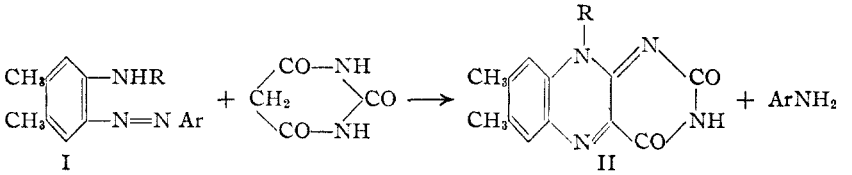
[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF MERCK AND CO., INC.]

The Reaction between o-Aminoazo Compounds and Barbituric Acid. A New Synthesis of Riboflavin

BY MAX TISHLER, KARL PFISTER, 3RD, R. D. BABSON, KURT LADENBURG[1] AND ANN J. FLEMING

Two methods have been reported previously for the syntheses of compounds having the isoalloxazine structure. These methods, designed chiefly for the synthesis of riboflavin, involve the reaction of *o*-phenylenediamines with alloxan[2] or with halogenated barbituric acids.[3]

A new synthesis of riboflavin[3a] is presented now consisting of the reaction between an appropriate *o*-aminoazo compound, I, and barbituric acid.[4]



(a) R = D-ribityl
(b) R = tetraacetyl-D-ribityl

This synthesis is more direct and simpler than the older methods particularly since barbituric acid is more readily accessible than alloxan or the halogenated barbituric acids. Moreover, the *o*-phenylenediamine required in the old procedures is difficult to prepare and is unstable in contrast to the corresponding *o*-aminoazo compounds.

The *o*-aminoazo compounds have been previously utilized for the preparation of phenazines[5] and benzimidazoles.[6] Crippa in a series of papers described the preparation of quinoxaline derivatives from *o*-aminoazo compounds and methyl ketones or ethyl acetoacetate.[7]

The reaction between barbituric acid and *o*-aminoazo compounds of the type I is catalyzed by weak organic acids, such as acetic acid. The optimum yields are obtained when a mixture of the reactants in glacial acetic acid or in acetic acid diluted with dioxane is refluxed until the azo compound is consumed. The presence of mineral acids not only inhibits the reaction but also rapidly destroys the azo compound. The *o*-aminoazo compound is also destroyed by refluxing a solution in acetic acid but the rate of decomposition is much less. If the azo compound in acetic acid is heated for several hours and then the barbituric acid is added, the yield of riboflavin is considerably decreased. The isolation of the by-product *p*-nitroaniline from the reaction of the *o*-aminoazo compound (Ib, Ar = p-$NO_2C_6H_4$) with barbituric acid suggests that riboflavin is formed in accordance with equation I → II.

With the intention of investigating the rela-

(1) Present address: Technical Enterprises Inc., 31 South Street, New York, New York.

(2) (a) Kuhn, Reinemund and Weygand, *Ber.*, **67**, 1460 (1934); (b) Kuhn and Weygand, *ibid.*, **67**, 1939 (1934); (c) Karrer, Solomon, Schöpp and Schlittler, *Helv. Chim. Acta*, **17**, 1165 (1934).

(3) Tishler, Wellman and Ladenburg, THIS JOURNAL, **67**, 2165 (1945).

(3a) *Cf.* Tishler and Carlson, U. S. Patent 2,350,376 (1944); *C. A.*, **38**, 4963 (1944).

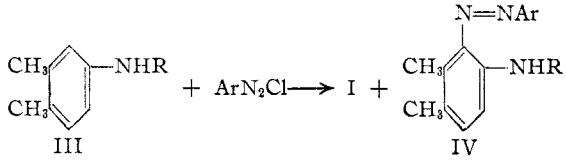
(4) Recently Bergel, Cohen and Haworth, British Patent 550,836 (1943); U. S. Patent 2,374,661 (1945), reported the preparation of riboflavin from 1-(D-ribitylamino)-2-phenylazo-4,5-dimethylbenzene (I, Ar = C_6H_5) and alloxantin in the presence of palladized charcoal. This reaction is an extension of the Kuhn and Karrer methods.[2] In the Bergel, *et al.*, procedure the azo group is undoubtedly reduced to the o-phenylenediamine by the alloxantin as the latter is dehydrogenated to alloxan.

(5) Witt, *Ber.*, **18**, 1119 (1885); **19**, 441 (1886); Krollpfeiffer, Müllhausen and Wolf, *Ann.*, **508**, 39 (1934).

(6) O. Fischer, *J. prakt. Chem.*, **107**, 16-49 (1924).

(7) G. B. Crippa, *Gazz. chim. ital.*, **59**, 330 (1929); **60**, 301 (1930); **62**, 394 (1932); **63**, 251 (1933); **66**, 649 (1936).

tionship of the nature of the azo grouping to reactivity, we synthesized a number of different *o*-aminoazo compounds and found, in contrast to Karrer and Meerwein's conclusion,[8] that the products were not pure 2-azo compounds, I, but contained significant amounts of the 6-azo compounds, IV. The amount of the 6-azo isomer formed varied with the nature of the arylazo radical; thus *p*-nitrobenzenediazonium chloride and benzenediazonium chloride formed the 6-azo isomers in 15 and 6% yields, respectively.

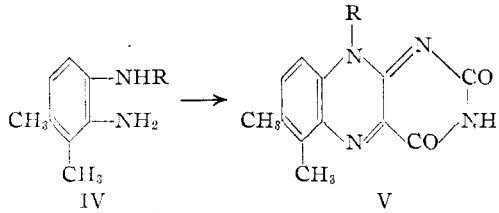


III IV

(a) R = D-ribityl
(b) R = tetraacetyl-D-ribityl

It is noteworthy that the 6-azo-1-ribitylamino compounds, IV, do not react with barbituric acid under the same conditions in which the isomeric azo compounds form riboflavin. In fact, the compounds IV are best isolated in pure form from the mixture obtained after the mixed azo compounds have reacted with barbituric acid and the riboflavin has been separated. The separation of the two isomeric *o*-aminoazo compounds by fractional crystallization is extremely difficult if the D-ribityl group is unacetylated. When the D-ribityl group is acetylated as in 1-(tetraacetyl-D-ribitylamino)-2-*p*-nitrophenylazo-4,5-dimethylbenzene, the purification is less difficult but still requires a number of recrystallizations.

The 6-azo-1-ribitylamino compounds, IV, have been converted into isoalloxazine derivatives by catalytic reduction of the azo grouping and condensation of the resultant *o*-phenylenediamine with alloxan[2] or with 5,5-dichlorobarbituric acid.[3]



IV V

(a) R = D-ribityl
(b) R = tetraacetyl-D-ribityl

The isoalloxazine Va was prepared from the *o*-aminoazo compound IVa (Ar = C_6H_5), whereas the corresponding tetraacetyl isoalloxazine Vb was obtained from IVb (Ar = *p*-$NO_2C_6H_4$). A comparison of the ultraviolet absorption spectra of riboflavin with that of the isomeric isoalloxazine is given (Fig. 1). Table I lists other properties of the two compounds.

The isoalloxazine, Va, 5,6-dimethyl-9-(D-1′-ribityl)-isoalloxaxine, previously named isoriboflavin, possesses the interesting biological property of suppressing growth in riboflavin deficient rats and in rats receiving suboptimal intake of the vitamin.[10] It has, however, less than 0.004 the activity of riboflavin when tested for promotion of growth of *L. casei*.[11]

TABLE I[9]

		Riboflavin	Isoriboflavin
1	$[\alpha]^{25}D$ in alkaline borate	+376 ± 1.5°	+237 ± 1.5°
	$[\alpha]^{25}D$ in acid solution	+53 ± 1°	+53 ± 1°
2	Fluorescence (*p*H 3.7)	Green	Brown (only about 25% as intense as riboflavin)
3	Polarograph $E^{1/2}$ (*vs.* S. C. E.)	−0.49	−0.47
	Polarograph I_d/c (μA. cc./mg.)	10.02 ± 0.05	9.71
4	Soly. in dil. NaOH or KOH	Soluble	Sl. soluble
5	Soly. in dil. LiOH	Soluble	Soluble

Inasmuch as the coupling of 1-(D-ribitylamino)-3,4-dimethylbenzene, IIIa, or its tetraacetyl derivative, IIIb, with aromatic diazonium salts produces mixtures of the two *o*-aminoazo compounds, I and IV, separable with difficulty, caution is required in applying the previously described syntheses for riboflavin in which the *o*-aminoazo compounds are reduced to the *o*-phenylenediamines and condensed with alloxan or 5,5-dichlorobarbituric acid.[2,3] Although the requisite diamine was isolated in 50–55% yield and characterized by Karrer and Meerwein,[12] there is no indication in their publications that the pure diamine was employed in the synthesis of riboflavin. On the contrary, where the *o*-aminoazo compounds were used to prepare the required *o*-phenylenediamine, the latter was not isolated but was condensed directly with alloxan.[8] The use of intermediates free from isomers is particularly essential in the synthesis of riboflavin since the presence of even small quantities of the isomeric *o*-phenylenediamine would produce riboflavin contaminated by the antivitamin, isoriboflavin.

Riboflavin was synthesized by two procedures utilizing the barbituric acid reaction. 1-(D-Ribitylamino)-3,4-dimethylbenzene, IIIa, was coupled with an aromatic diazonium salt and the resulting *o*-aminoazo compound, Ia, was condensed with barbituric acid. Alternatively, 1-(tetraacetyl-D-ribitylamino)-3,4-dimethylbenzene, IIIb, was converted to the *o*-aminoazo compound, Ib, and after deacetylation to Ia was treated with barbituric acid. Tetraacetylriboflavin was prepared from the acetylated aminoazo compound, Ib, and barbituric acid.

Yields of riboflavin and tetraacetylriboflavin are listed in the Experimental Section, Tables II

(8) Karrer and Meerwein, *Helv. Chim. Acta*, **18**, 1130 (1935); **19**, 264 (1936). The melting points of the phenylazo- and *p*-nitrophenylazo-ribitylamines were not recorded in these papers.

(9) We are grateful to Dr. J. B. Conn, Mr. W. A. Bastedo, Jr., and Mrs. R. C. Anderson for carrying out the physical measurements.

(10) Emerson and Tishler, *Proc. Soc. Exptl. Biol. Med.*, **55**, 184 (1944).

(11) Determined by Dr. J. L. Stokes of this Laboratory.

(12) Karrer and Meerwein, *Helv. Chim. Acta*, **19**, 1190 (1936). One of the present authors (M. T.) has had occasion to prepare large amounts of the pure *o*-phenylenediamine from Ia, by the Karrer and Meerwein method in connection with a study of its reactivity toward halogenated barbituric acids.[3] The yield of sharply melting diamine from Ia never exceeded 70%.

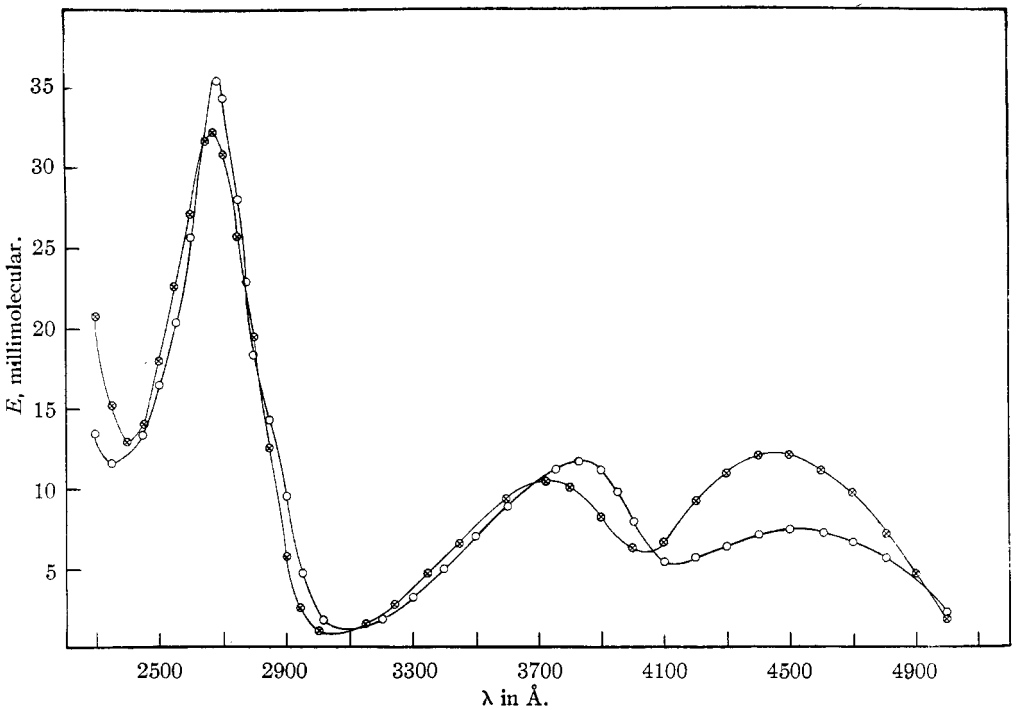


Fig. 1.—Ultraviolet absorption spectra of isoriboflavin, —○—, and riboflavin, —⊗—. Samples dissolved in 6 *N* hydrochloric acid, neutralized with sodium hydroxide and made up in *p*H 6 "Parstain" phosphate buffer.

and III. In the unacetylated series, the yields of riboflavin from the different pure *o*-aminoazo compounds are essentially the same. However, with the acetylated *o*-aminoazo compounds, the yields of riboflavin were influenced by the nature of the azo grouping. In this case it is of interest to note that the presence of the electronegative azo groupings is necessary for high yields.

The difference in reactivity of the 6- and 2-azo-amino compounds toward barbituric acid is noteworthy and merits further study. It would be interesting to know whether there is any connection between this observation and the behavior of the isomeric *o*-aminoazo compounds toward concentrated hydrochloric acid. The fact that the reactive 2-azo compounds, I, give a pronounced color change when treated with concentrated hydrochloric acid whereas the inactive 6-azo isomers form pale-colored solutions under the same conditions indicates that the former can rearrange into a stable resonating *o*-quinoidal system, whereas the latter compounds cannot. The difference in color behavior and in reactivity perhaps may be due to the same phenomenon, namely, steric inhibition of resonance.[13]

Experimental[14]

1-(D-Ribitylamino)-2-*p*-nitrophenylazo-4,5-dimethylbenzene (Ia, Ar = *p*-$NO_2C_6H_4$-).—The melting point of this compound was recorded previously[3] as 180–182°. Starting with 1-(tetraacetyl-D-ribitylamino)-2-*p*-nitrophenylazo-4,5-dimethylbenzene[15] recrystallized from acetic acid, methyl ethyl ketone and chloroform–methanol to a constant m. p. 170–171° (*cf.* reference 3) a purer product was obtained. Thus, 4 g. of the purified acetylated compound was added to a 20 cc. methanol solution of sodium methoxide equivalent to 0.95 g. of sodium and the mixture was boiled under reflux for thirty minutes. To the cooled solution was added 60 cc. of water and the precipitated solid was recrystallized from ethanol. The product was obtained as copper bronze platelets melting at 187–188°; wt. 2.6 g.; 92% yield.

Anal.[16] Calcd. for $C_{19}H_{24}O_6N_4$: C, 56.43; H, 5.98. Found: C, 56.37; H, 5.80.

The azo compound when dissolved in acetic acid produced a red purple solution which turned deep blue on the addition of concentrated hydrochloric acid.

A number of attempts to prepare the above *o*-aminoazo compound by the method of Karrer and Meerwein[8] gave only very impure and intractable products.

1-(Tetraacetyl-D-ribitylamino-2-*o*-nitrophenylazo-4,5-dimethylbenzene (Ib, Ar = *o*-$NO_2C_6H_4$-).—This compound was prepared from *o*-nitrobenzenediazonium chloride and 1-(tetraacetyl-D-ribitylamino)-3,4-dimethylbenzene by the same procedure used for the *p*-nitrophenylazo derivative.[3] The yield of crude azo compound melting at 122–125° was 65%. By two recrystallizations from 20 volumes of methanol the product was obtained as rods having a purple lustre, m. p. 131–132°. A solution in acetic acid turned deep purple on adding concentrated hydrochloric acid.

Anal. Calcd. for $C_{27}H_{32}O_{10}N_4$: C, 56.63; H, 5.63. Found: C, 56.85; H, 5.69.

(13) G. W. Wheland, "The Theory of Resonance and Its Application to Organic Chemistry," John Wiley and Sons, Inc., New York, N. Y., 1944, p. 185.

(14) All melting points were taken with a 360° rod-form thermometer and are uncorrected.

(15) The ribitylamine III used in coupling with *p*-nitrobenzene diazonium chloride was found by Dr. N. Trenner to be at least 99% pure by solubility analysis.

(16) We are indebted to Messrs. R. N. Boos, E. J. Thornton, L. Rosalsky, W. K. Humphrey and J. H. McGregor for all microanalytical data.

1-(D-Ribitylamino)-2-*o*-nitrophenylazo-4,5-dimethylbenzene (Ia, Ar = *o*-$NO_2C_6H_4$-) was prepared by deacetylating the above compound using sodium methoxide in methanol. It was obtained as purplish microscopic crystals melting at 152–153°.

Anal. Calcd. for $C_{19}H_{24}O_6N_4$: C, 56.43; H, 5.98. Found: C, 56.52; H, 5.92.

1-(Tetraacetyl-D-ribitylamino)-2-*p*-tolylazo-4,5-dimethylbenzene (Ib, Ar = *p*-$CH_3C_6H_4$-).—To a mixture of 59.0 g. of *p*-toluidine, 1000 cc. of acetic acid, 125 cc. of water and 112 cc. of concentrated hydrochloric acid was added 35 g. of sodium nitrite maintaining the temperature at 3–5°. After the nitrite was consumed, 106 g. of 1-(tetraacetyl-D-ribitylamino)-3,4-dimethylbenzene was added followed by the addition of a solution of 36 g. of sodium hydroxide in 150 cc. of water. The latter was added in a half-hour period at 0–5°. The mixture was aged at 8–10° for about two hours and then diluted with 1 liter of ether. The mixture was shaken with water and the ether layer was washed with water and with an aqueous solution of sodium bicarbonate. After drying with anhydrous sodium sulfate, it was concentrated to dryness and the orange-red residue was dissolved in 400 cc. of hot methanol, filtered and refrigerated. The product separated as a bright orange needle melting at 86–89°. After two recrystallizations from methanol the product melted at 90–92° and weighed 78.6 g. The addition of concentrated hydrochloric acid to an acetic acid solution of the azo compound produced a deep blue-green color.

Anal. Calcd. for $C_{28}H_{35}O_8N_3$: C, 62.09; H, 6.51. Found: C, 61.91; H, 6.37.

1-(D-Ribitylamino)-2-*p*-tolylazo-4,5-dimethylbenzene (Ia, Ar = *p*-$CH_3C_6H_4$-) was prepared by deacetylating the compound described above. It gave the same color reaction with concentrated hydrochloric acid and melted at 171–172°.

Anal. Calcd. for $C_{20}H_{27}O_4N_3$: C, 64.32; H, 7.29. Found: C, 64.44; H, 7.39.

Azo compounds from four other diazonium compounds were prepared by the above general method.

1-(Tetraacetyl-D-ribitylamino)-2-phenylazo-4,5-dimethylbenzene (Ib, Ar = C_6H_5-).—The deep red oil obtained (92% yield) resisted crystallization and was used without purification.

Anal. Calcd. for $C_{27}H_{33}O_8N_3$: C, 61.48; H, 6.26. Found: C, 61.20; H, 6.39.

1-(D-Ribitylamino)-2-phenylazo-4,5-dimethylbenzene (Ia, Ar = C_6H_5-) was prepared by deacetylating the above oil (95% yield, m. p. 168–170°) and from the unacetylated ribitylamine IIIa according to the procedure of Karrer and Meerwein[8] (89% yield, m. p. 167–170°). After several recrystallizations from 1-butanol the azo compound was obtained as brick-red needles of m. p. 174–175°.

Anal. Calcd. for $C_{19}H_{25}O_4N_3$: C, 63.51; H, 6.96; N, 11.70. Found: C, 63.39; H, 7.15; N, 11.74.

1-(Tetraacetyl-D-ribitylamino)-2-*p*-chlorophenylazo-4,5-dimethylbenzene (Ib, Ar = *p*-ClC_6H_4-).—The product, which separated on dilution of the reaction mixture with an equal volume of water, melted at 94–97° (97% yield). After several recrystallizations from methanol the m. p. was 101–103°.

Anal. Calcd. for $C_{27}H_{32}O_8N_3Cl$: C, 57.70; H, 5.74. Found: C, 57.71; H, 5.57.

Catalytic deacetylation of the crude azo compound gave the unacetylated azo compound, m. p. 154–157°.

1-(Tetraacetyl-D-ribitylamino)-2-(2′,4′-dimethylphenylazo)-4,5-dimethylbenzene (Ib, Ar = 2,4-$(CH_3)_2C_6H_3$-) was obtained crude (m. p. 76–82°) in 90% yield. Recrystallization from methanol gave a product of m. p. 83–85°.

Anal. Calcd. for $C_{29}H_{37}O_8N_3$: C, 62.09; H, 6.71. Found: C, 62.35; H, 6.41.

The deacetylated compound of m. p. 147–149° was obtained from the crude tetraacetyl compound.

1-(Tetraacetyl-D-ribitylamino)-2-*p*-methoxyphenylazo-4,5-dimethylbenzene (Ib, Ar = *p*-$CH_3OC_6H_4$-) was obtained crude (m. p. 106–107°) in 92% yield. After recrystallization from methanol the product melted at 111–112°.

Anal. Calcd. for $C_{28}H_{35}O_9N_3$: C, 60.31; H, 6.33. Found: C, 60.42; H, 6.14.

The unacetylated compound derived from the above crude melted at 158–160°.

Condensation of *o*-Aminoazo Compounds, Ia, with Barbituric Acid; Riboflavin.—A mixture of 27 g. of 1-(D-ribitylamino)-2-*p*-nitrophenylazo-4,5-dimethylbenzene (m. p. 187–188°), 14.3 g. of barbituric acid, 180 cc. of dioxane and 34 cc. of acetic acid was boiled under reflux for five hours. At the end of the heating period, a sample gave a very pale green color with concd. hydrochloric acid.[17] The mixture was cooled and then filtered. The solid product was washed well with hot water and dried, wt. 21.0 g. The crude riboflavin was recrystallized from boiling water or was purified by the elegant procedure developed by Pasternack and Brown,[18] consisting of dissolving the crude in 4 volumes of 18% hydrochloric acid, adding a few drops of superoxol, to bleach impurities, filtering and diluting with water. The yield of pure riboflavin from the above quantities of reagents was 16.5 g. (65.8% yield).

Starting with a less pure *p*-nitrophenylazo compound, m. p. 167–170°, the yield of pure riboflavin was 46.9%.

The condensation of other *o*-aminoazo compounds, Ia, with barbituric acid was carried out and worked up in the manner described above. A summary of the yields obtained with these azo compounds is recorded in Table II.

TABLE II

RIBOFLAVIN FROM BARBITURIC ACID AND *o*-AMINOAZO COMPOUNDS, Ia.

Azo grouping	M. p., °C., of azo cpd.	Quality of azo cpd.	Riboflavin yield, %
$C_6H_5N_2$—	174–175	pure	71
$C_6H_5N_2$—	168–170	[19]	62[20]
p-$CH_3C_6H_4N_2$—	171–172	pure	69
p-$CH_3C_6H_4N_2$—	166–167	[19]	67
o-$NO_2C_6H_4N_2$—	152–153	pure	70
p-$NO_2C_6H_4N_2$—	187–188	pure	66
p-$NO_2C_6H_4N_2$—	171–174	[19]	47
o-$ClC_6H_4N_2$—	153–158[21]	poor	43
p-$ClC_6H_4N_2$—	154–157	fair	44
p-$CH_3OC_6H_4N_2$—	158–160	fair	53
2,4-$(CH_3)_2C_6H_3N_2$—	147–149	fair	59
p,p′-$N_2C_6H_4C_6H_4N_2$—	120–125[21]	poor	14

Condensation of *o*-Aminoazo Compounds, Ib, with Barbituric Acid; Tetraacetylriboflavin.—A mixture of 50 g. of 1-(tetraacetyl-D-ribitylamino)-2-*p*-nitrophenylazo-4,5-dimethylbenzene, m. p. 170–171°, 14.6 g. of barbituric acid and 500 cc. of acetic acid was boiled under reflux for two hours. The mixture was concentrated nearly to dryness under reduced pressure. The residue was extracted with hot ether until the ether extracts were almost colorless. The combined ether extracts were saved for

(17) In all condensations of *o*-aminoazo compounds with barbituric acid, heating was continued until a test sample treated with concd. hydrochloric acid no longer produced a deep color.

(18) Pasternack and Brown, U. S. Patent 2,324,800; *C. A.*, **38**, 221 (1944).

(19) These aminoazo compounds analyze well but contain significant quantities of the 6-azo isomer.

(20) The same yield was obtained with this quality azo whether prepared from the acetylated azo compound or by Karrer and Meerwein's procedure.[8]

(21) These aminoazo compounds were prepared in the usual manner. Although the quality of each is poor, they are recorded for practical interest.

isolation of the 6-azo isomer, IVb. The residue was extracted with chloroform and the extracts were concentrated to dryness. The solid, crude tetraacetylriboflavin was dissolved in 850 cc. of hot methanol and the solution was concentrated to 450 cc. After a few hours refrigeration, the pure tetraacetylriboflavin was separated and dried, wt. 34.6 g. (73% yield); m. p. 236–238°.

Anal. Calcd. for $C_{25}H_{28}O_{10}N_4$: C, 55.14; H, 5.14. Found: C, 55.06; H, 5.41.

Starting with an unrecrystallized *p*-nitrophenylazo, m. p. 161–162°, the yield of pure tetraacetylriboflavin was 56.3%.

Riboflavin was readily prepared from the tetraacetyl derivative by catalytic deacetylation in methanol. Thus, to a solution of 5 g. of tetraacetylriboflavin in 100 cc. of methanol was added 10 cc. of methanol in which 0.1 g. of sodium was dissolved. The mixture was heated at 50° for one hour during which time pure riboflavin separated. The yield was over 95%.

The other acetylated *o*-aminoazo compounds, Ib, were condensed with barbituric acid and worked up in the same manner. A summary of the yields of tetraacetylriboflavin obtained with the acetylated azo compounds is found in Table III.

TABLE III

TETRAACETYLRIBOFLAVIN FROM BARBITURIC ACID AND *o*-AMINOAZO COMPOUNDS, Ib

Azo grouping	M. p., °C. of azo cpd.	Quality of azo cpd.	Tetra-acetyl-riboflavin yield, %
$C_6H_5N_2$—	oil	[19]	50
p-$CH_3C_6H_4N_2$—	90–92	pure	49
p-$CH_3C_6H_4N_2$—	oil	[19]	36
o-$NO_2C_6H_4N_2$—	131–132	pure	69
p-$NO_2C_6H_4N_2$—	170–171	pure	73
p-$NO_2C_6H_4N_2$—	160–163	[19]	56
o-$ClC_6H_4N_2$—	oil[21]	poor	45
p-$ClC_6H_4N_2$—	94–97	fair	47
p-$CH_3OC_6H_4N_2$—	106–107	fair	32
2,4-$(CH_3)_2C_6H_3N_2$—	76–82	fair	45
p,p'-$N_2C_6H_4C_6H_4N_2$—	77–100[21]	poor	37

Isomeric Aminoazo Compounds, IVb and a.—The acetylated 6-azo compounds were isolated from the ether extracts mentioned above. In general, the ether extracts were concentrated to dryness and the residues were dissolved in methanol and allowed to crystallize. None of these *o*-aminoazo compounds reacted with barbituric acid using longer reaction times than were required for their isomers.

1-(Tetraacetyl-D-ribitylamino)-6-*p*-nitrophenylazo-4,5-dimethylbenzene (IVb, Ar = *p*-$NO_2C_6H_4$-) was isolated in 10% yield from the reaction mixture starting with the *p*-nitrophenylazo compound melting at 161–162°. The purer 2-*p*-nitrophenylazo compound, m. p. 170–171°, contained 1% of the 6-*p*-nitrophenylazo isomer. The latter was obtained as reddish-purple needles melting at 137–138°.

Anal. Calcd. for $C_{27}H_{32}N_4O_{10}$: C, 56.64; H, 5.63; N, 9.79. Found: C, 56.71; H, 5.80; N, 9.97.

The addition of concentrated hydrochloric acid to an acetic acid solution of the above azo compound produced a pale-orange color. On treatment with ice-cold red fuming nitric acid, and pouring the mixture onto ice a flocculent yellow precipitate was formed which would not crystallize. The filtrate on treatment with an alkaline solution of β-naphthol formed α-(*p*-nitrophenylazo)-β-naphthol identified by mixed m. p. determination.

1-(D-Ribitylamino)-6-*p*-nitrophenylazo-4,5-dimethylbenzene (IVa, Ar = *p*-$NO_2C_6H_4$-) was prepared from the above tetraacetyl compound by deacetylation in methanol by sodium methoxide. The product separated as bronze colored needles melting at 207–208°.

Anal. Calcd. for $C_{19}H_{24}O_6N_4$: C, 56.43; H, 5.98; N, 13.86. Found: C, 56.54; H, 6.38; N, 13.82.

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1-(D-Ribitylamino)-6-phenylazo-4,5-dimethylbenzene (IVa, Ar = C_6H_5-) was obtained in about 4% yield from the dioxane–acetic acid filtrates from the reaction between the phenylazo compound, m. p. 168–170°, and barbituric acid. The product was obtained as orange needles melting at 196–198°. The same product was obtained in comparable yields using the azo compound (m. p. 167–170°) prepared by Karrer and Meerwein's[8] procedure.

Anal. Calcd. for $C_{19}H_{25}O_4N_3$: C, 63.49; H, 7.01; N, 11.69. Found: C, 63.83; H, 7.20; N, 11.95.

1-(D-Ribitylamino)-6-*p*-tolylazo-4,5-dimethylbenzene (IVa, Ar = *p*-$CH_3C_6H_4$-) was obtained from methanol as orange rosets melting at 202–203°.

Anal. Calcd. for $C_{20}H_{27}O_4N_3$: C, 64.32; H, 7.29. Found: C, 64.09; H, 7.04.

9-(Tetraacetyl-D-1'-ribityl)-5,6-dimethyl-isoalloxazine; Tetraacetylisoriboflavin (Vb).—A mixture of 50 g. of 1-(tetraacetyl-D-ribitylamino)-6-*p*-nitrophenylazo-4,5-dimethylbenzene, 250 cc. of pure dioxane and 10 g. of Raney nickel was shaken with hydrogen at 20 lb. pressure. The hydrogenation was complete in three hours, 5 moles of hydrogen per mole of azo compound being adsorbed. The filtered mixture was concentrated to dryness under reduced pressure in a nitrogen atmosphere. A slurry of 41.5 g. of alloxan monohydrate and 41.5 g. of boric acid in 1000 cc. acetic acid was added to the residue and the mixture was stirred at 50° for two hours. The mixture was filtered from the dianil of alloxan and *p*-phenylenediamine and the filtrate was concentrated to 150 cc. volume, diluted with ten volumes of water and extracted with chloroform. The chloroform extract was washed well with water, dried over anhydrous sodium sulfate, and concentrated to dryness. The residue was dissolved in methanol, treated with Norit, and the filtrate was refrigerated, wt., 24.6 g. (51.5%); m. p. 223–225°. The product was recrystallized from methanol from which it separated as small rosets of needles melting at 228–230°. A sample mixed with tetraacetylriboflavin (m. p. 234–236°) melted at 206–215°.

Anal. Calcd. for $C_{25}H_{28}O_{10}N_4$: C, 55.15; H, 5.11; N, 10.29. Found: C, 55.22; H, 5.14; N, 10.01.

The isomers are indistinguishable from each other in color and general appearance. In concentrated sulfuric acid tetraacetylriboflavin produces an orange red color whereas the isomer forms a brownish yellow color.

Tetraacetylisoriboflavin was also prepared by condensing the residue from the catalytic hydrogenation of the 6-*p*-nitrophenylazo compound with 5,5-dichlorobarbituric acid in pyridine in the manner developed by us previously.[3] The formation of *p*-phenylenediamine in the reaction rendered the isolation of the product very difficult. The yield was poor (13%).

9-(D-1'-Ribityl)-5,6-dimethyl-isoalloxazine; Isoriboflavin (Va).—To a solution of 15 g. of the tetraacetylisoriboflavin (m. p. 226–228°) in 1,250 cc. of methanol at reflux temperature was added 2.5 cc. of methanol solution of sodium methoxide (prepared from 0.07 g. of sodium). Isoriboflavin started to precipitate after ten minutes and boiling under reflux was continued for two hours. The mixture was made slightly acidic by addition of acetic acid, cooled and filtered. The dried product, m. p. 261–262° dec., weighed 9.95 g. (95.7% yield). The product was further purified by dissolving in 18% hydrochloric acid, and adding superoxol in the manner described for riboflavin[18]; recovery yield 70%; m. p. 265–266° dec. sintering at 260°. A sample mixed with riboflavin (m. p. 268–270° dec.) was completely decomposed at 255–258°.

Anal. Calcd. for $C_{17}H_{20}O_6N_4$: C, 54.25; H, 5.32; N, 14.89. Found: C, 54.00; H, 5.36; N, 14.92.

Although the sodium and potassium salts of isoriboflavin are sparingly soluble in water, the lithium salt is freely soluble.

The optical rotation of a 0.50% solution in *N*/20 potassium hydroxide half saturated with borax gave $[\alpha]^{25}D$ +237 ± 1.5° whereas riboflavin under the same conditions showed $[\alpha]^{25}D$ +376 ± 1.5°. In 0.5% solution in *N*/2 lithium hydroxide half saturated with borax: isoriboflavin, $[\alpha]^{25}D$ +220 ± 2°; riboflavin, $[\alpha]^{25}D$ +386° ± 2°.

The polarograms were made in 0.1 *M* lithium tetraborate at concentration equal to 0.130–0.190 mg./cc. Capillary constant $m^{2/3}t^{1/6}$ = 2.10 mg.$^{2/3}$ sec.$^{-1/2}$. As indicated in Table I, polarographic constants of isoriboflavin were nearly identical with those of riboflavin.

Isoriboflavin was also prepared from 1-(D-ribitylamino)-6-phenylazo-4,5-dimethylbenzene by reducing the azo compound to the corresponding diamine, and condensing the latter with alloxan in acetic acid containing boric acid. The product was isolated in 67% yield and found identical with the isoriboflavin described above.

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Summary

1. A new synthesis of riboflavin has been described consisting of the reaction between 1-(D-ribitylamino)-2-arylazo-4,5-dimethylbenzene and barbituric acid.

2. The coupling of 1-(D-ribitylamino)-3,4-dimethylbenzene with a number of different aryl diazonium salts produced predominantly the 2-arylazo compounds contaminated with significant quantities of the isomeric 6-arylazo derivative. The latter did not react with barbituric acid to form an isoalloxazine.

3. The isomeric 6-arylazo derivative was catalytically reduced to an *o*-phenylenediamine derivative which was condensed with alloxan to isoriboflavin.

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[CONTRIBUTION FROM THE DIVISION OF PLANT BIOLOGY OF THE CARNEGIE INSTITUTION OF WASHINGTON]

Organic Compounds of Magnesium and Phosphorus in Relation to Chlorophyll Formation

BY JAMES H. C. SMITH

Introduction

When etiolated seedlings are placed in the light, one of the most conspicuous changes observed is the formation of chlorophyll. During the formation of chlorophyll some transformation of magnesium compounds must necessarily take place. Whether this is a transformation of preformed organic magnesium compounds closely related to chlorophyll or a utilization of magnesium from simpler sources is uncertain. A distinction between these two types of reaction in the formation of chlorophyll would contribute to our knowledge of this synthesis and possibly lead to a clearer understanding of the photosynthetic process itself. In this research, some progress has been made toward distinguishing between these two types of reaction by comparing the amounts of magnesium attributable to chlorophyll with the total amounts of ether-soluble magnesium present at different stages of greening.

Other syntheses also occur in etiolated leaves upon illumination. In the present experiments on the greening of etiolated barley leaves, two such syntheses have been followed: the formation of total ether-soluble material, and of ether-soluble phosphorus compounds.

Inasmuch as albino leaves are abnormal in regard to the formation of chlorophyll, albino corn leaves have been examined to determine whether they are deficient with respect to other ether-soluble compounds.

Methods

Leaf Material.—The barley leaf material was obtained from seedlings grown in darkness. For convenience, the seedlings were grown in small flats of soil—25 g. of seed sowed to the flat. This amount of seed yielded over 50 g. of leaf material. About nine days after planting, the flats of seedlings were removed from the dark cabinet and illuminated for various periods of time. A 100-watt Mazda lamp, hung 40 cm. above the flat, furnished the illumination. After greening for the desired period, the leaves were harvested, cut into short lengths, and 50 g. weighed out for analysis.

A series of experiments was also run on excised leaves. The leaves were cut from the roots and the cut ends placed in vials of tap water. After the leaves had been illuminated in the manner described and for the period desired, they were cut into short lengths and 50 g. taken for analysis.

In both series of experiments, additional samples were removed for dry-weight determinations.

Analytical Procedures.—Extraction of the cut leaves was carried out by grinding them with sand under a solvent in a large mortar. Six changes of solvent were used as follows: 200 ml. of 90% acetone, 100 ml. of diethyl ether, 100 ml. of 80% acetone, 100 ml. of ether, 100 ml. of 80% acetone and 100 ml. of ether. The combined extracts were filtered and washed four times with 200 ml. of water. The ether solution was filtered into a 200-ml. volumetric flask and diluted to volume with ether.

The absorption spectrum of the ether solution was measured spectrophotometrically[1] and the chlorophyll content of the solution was determined by use of the equation published by Comar and Zscheile[2]: Total chlorophyll (mg./liter) = $7.12 \log_{10}(I_0/I)_{6600} + 16.8 \log_{10}(I_0/I)_{6425}$. From the weight of chlorophyll determined spectrophotometrically the weight of magnesium attributable to the

(1) Smith, THIS JOURNAL, **58**, 247 (1936).

(2) Comar and Zscheile, *Plant Physiol.*, **17**, 198 (1942).