Hydrochloride Salts.—The monobasic esters were dissolved in ether and treated with dry HCl. The precipitated hydrochloride salts were filtered, washed with a small amount of ether, and recrystallized from absolute ethanol–ether. The dibasic esters were dissolved in 95% ethanol and treated with a calculated amount of 1 *N* HCl to form the monohydrochloride salts. The solvents were evaporated on a water bath *in vacuo* and the residue was dried (P_2O_5) in a desiccator before recrystallization from absolute ethanol–ether (see Table II).

Acknowledgment.—The author wishes to thank the University of Kentucky for a summer research fellowship during the summer of 1964 to initiate this work. Thanks are also extended to Mr. Hugh Frazier for assistance in the testing of the compounds and to Dr. Arthur C. Glasser for calling the method of testing to the author's attention.

Synthesis and Biological Activity of 7-Bromo-8-methyl-10-(1-D-ribityl)isoalloxazine, an Analog of Riboflavin[1,2]

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7-Bromo-8-methyl-10-(1-D-ribityl)isoalloxazine has been synthesized by the condensation of 2-bromo-4-nitro-5-chlorotoluene with D-ribamine to produce 4-bromo-5-methyl-2-nitro-N-D-ribitylaniline. The latter was reduced and the obtained *o*-phenylenediamine was condensed with alloxan monohydrate. This new flavin stimulates the growth of the riboflavin-deficient rat at all quantities used but, in spite of this activity, the higher quantities are lethal for the animal. Since riboflavin protects the animal against the toxicity of this flavin, the analog is a reversible antagonist of riboflavin in the rat. The flavin is also an antagonist of riboflavin in *Lactobacillus casei*, but it stimulates growth of the organism in the presence of small quantities of the vitamin and under specific conditions.

Weygand, Löwenfeld, and Möller[3] synthesized 7,8-dibromo-10-(1-D-ribityl)isoalloxazine (7,8-dibromoflavin) and compared its ability to inhibit the growth of *Streptobacterium plantarum* P32 with the activity of the previously known 7,8-dichloro-10-(1-D-ribityl)isoalloxazine (7,8-dichloroflavin) against this organism. The 7,8-dichloroflavin possessed an inhibition index (*II*)[4] of 398 while the 7,8-dibromoflavin, with an inhibition index of 1750, was far less potent against *S. plantarum* P32. We have prepared 7,8-dichloroflavin also, and our improved procedure yielded a product of unquestioned purity.[5]

The bacterium, *S. plantarum*, does not require an exogenous source of riboflavin, and we considered it important to test 7,8-dichloroflavin on two test systems which do require exogenous riboflavin. We found that 7,8-dichloroflavin was not an antagonist of riboflavin in *Lactobacillus casei*[6] (ATCC 7469),[7] and thus confirmed an earlier report by Snell, *et al.*,[8] and further demonstrated that this material was also completely devoid of biological activity in the rat.[6] We have synthesized 7-chloro-8-methyl-10-(1-D-ribityl)isoalloxazine[9] (7-chloro-8-methylflavin) and studied its biological activity in both the rat and *L. casei*.[10] It is a potent inhibitor of *L. casei* with an *II* of 76 (in this test system, it is exceeded in potency by only 7-methyl-8-chloro-10-(1-D-ribityl)-isoalloxazine).[11] The 7-chloro-8-methylflavin is the most potent antagonist of riboflavin in the rat to be described to date. This antagonism is expressed in terms of lethality because, paradoxically, the compound is an excellent stimulant for the growth (but not survival) of the riboflavin-deficient rat.[10]

Weygand, *et al.*,[3] found 7,8-dibromoflavin to be far less active (*II* = 1750) than 7,8-dichloroflavin (*II* = 440) as an antagonist of riboflavin for *S. plantarum*. We have found 7-chloro-8-methylflavin to be a potent inhibitor (*II* = 76),[10] while 7,8-dichloroflavin was inert[6] as an antagonist of riboflavin in *L. casei*. It was of interest to us to learn if the incorporation of one bromine atom would produce an analog whose activity was related to that of 7,8-dibromoflavin in a way which resembled the relationship we had discovered between the analogs containing one and two chlorine atoms.

Chemistry.—2-Bromo-5-chloro-4-nitrotoluene was condensed with D-ribamine to produce 4-bromo-5-methyl-2-nitro-N-D-ribitylaniline. This compound was reduced to 2-amino-4-bromo-5-methyl-N-D-ribitylaniline which was immediately condensed with alloxan monohydrate in the presence of boric acid to produce 7-bromo-8-methyl-10-(1-D-ribityl)isoalloxazine.

Biological Activity.—7-Bromo-8-methyl-10-(1-D-ribityl)isoalloxazine is a strong antagonist of riboflavin in *L. casei;* the *II* was found to be 137. It appears to be a slightly better stimulant for the growth of the riboflavin-deficient rat and, also, it appears to be less toxic than the previously studied 7-chloro-8-methyl-10-(1-D-ribityl)isoalloxazine. The antagonism of the an-

(1) This work was supported in part by Grant CY-2940 from the National Cancer Institute, U. S. Public Health Service.

(2) The numbering system used for the flavins named in this article conforms to that of *Chemical Abstracts*. All papers constituting the literature citations have made use of the older ring-numbering system. The older numbering system will conform to the *Chemical Abstracts* system by increasing each position number by one.

(3) F. Weygand, R. Löwenfeld, and E. Möller, *Chem. Ber.*, **84**, 106 (1951).

(4) Inhibition index (*II*) = micrograms of analog at half maximum growth/0.3 μg of riboflavin times the molecular weight of riboflavin/molecular weight of analog.

(5) The melting point of our material remained 288–290° dec through repeated recrystallizations; R. Kuhn, R. Weygand, and E. F. Möller, *Ber.*, **76**, 1044 (1943), reported mp 273–275° dec.

(6) J. P. Lambooy, R. A. Scala, and E. E. Haley, *J. Nutr.*, **74**, 466 (1961).

(7) American Type Culture Collection, Rockville, Md. 20852.

(8) E. E. Snell, O. A. Klatt, H. W. Bruins, and W. W. Gravens, *Proc. Soc. Exptl. Biol. Med.*, **82**, 853 (1953).

(9) E. E. Haley and J. P. Lambooy, *J. Am. Chem. Soc.*, **76**, 5093 (1954).

(10) E. E. Haley and J. P. Lambooy, *J. Nutr.*, **72** 169 (1960).

(11) Synthesis, ref 9; biological activity, ref 6.

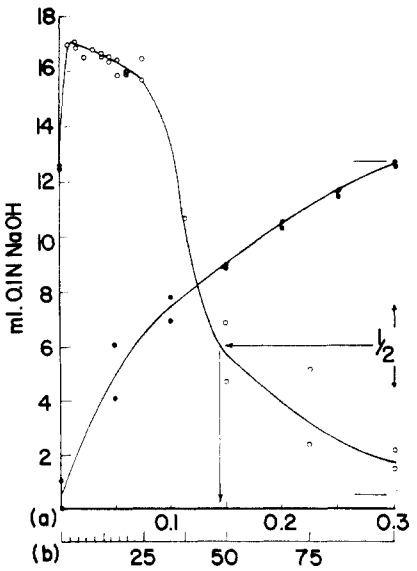

Figure 1.—Lactic acid production by *L. casei* grown in a culture medium containing riboflavin (—●—) and in a culture medium containing mixtures of riboflavin and 7-bromo-8-methylflavin (—○—): (a) riboflavin, μg/10 ml of culture medium; (b) 7-bromo-8-methylflavin, μg/10 ml of culture medium with each tube containing 0.3 μg of riboflavin.

alog can be reversed by the simultaneous administration of riboflavin.

Experimental Section[12]

Practical grade 2-methyl-4-nitroaniline[13] was purified by recrystallization from ethanol to obtain a product melting at 133–134° (lit.[14] mp 127–134°); the N-acetyl derivative of this material melted at 203–204° (lit.[15] mp 198°). The purified 2-methyl-4-nitroaniline was converted to 2-bromo-5-nitrotoluene by a combination of described procedures[16] to yield material with mp 76° (lit.[16] mp 76°) and bp 130° (10 mm).[17] The 2-bromo-5-nitrotoluene (90 g) was converted to 4-bromo-3-methylaniline by the addition of successive small quantities of 40 mesh iron (until a total of 125 g had been added) to the refluxing mixture of the compound in 1050 ml of concentrated HCl and 1500 ml of water. The product was steam distilled from the mixture after treatment with 700 g of NaOH, to yield 65.5 g (86%) of the corresponding aniline, mp[18] 81–82° (lit.[19] mp 78–82°). 4-Bromo-3-methylaniline was acetylated with acetic anhydride to yield material of mp 101–103° (lit.[20] mp 101–102°). The 4-bromo-3-methylacetanilide was nitrated by a procedure described[21] to yield 66–67% of desired material melting at 121–123° (lit.[20] mp 125–126°) after recrystallization from ethanol, and a small amount of material of mp 126–136°. The 2-nitro-4-bromo-5-methylacetanilide was hydrolyzed as described[21] to yield 2-nitro-4-bromo-5-methylaniline, mp 182° (lit.[22] mp 179–181°).

(12) All melting points were determined in Pyrex capillary tubes and observed on thermometers calibrated against U.S.P. reference melting point standards. Decomposition points were obtained by immersing the capillary into a rapidly heated bath at 150° with rapid heating to the decomposition point.

(13) Whether pure or impure, this material was found to produce severe contact dermatitis in the case of one of us (J. P. L.) but need not do so in all individuals.

(14) F. Beilstein and A. Kuhlberg, *Ann.*, **158**, 345 (1871), reported mp 127–128°; A. Shimomura and J. B. Cohen, *J. Chem. Soc.*, **119**, 745 (1921), reported mp 134°.

(15) M. T. Bogert and E. P. Cook, *J. Am. Chem. Soc.*, **28**, 1451 (1906).

(16) C. S. Gibson and J. D. A. Johnson, *J. Chem. Soc.*, 1229 (1929).

(17) The readmission of air into the distillation flask containing the hot residue resulted in a mild explosion producing great quantities of smoke.

(18) Attempts to accomplish this reduction by means of PtO_2 in alcohol led to excessive hydrogen uptake and a high-melting, unknown material.

(19) H. C. Nevile and A. Winther, *Ber.*, **13**, 969 (1880), reported mp 78°; E. Bamberger, *Ber.*, **57**, 2088 (1924), reported mp 82°.

(20) J. B. Cohen and P. K. Dutt, *J. Chem. Soc.*, **105**, 515 (1914).

(21) J. P. Lambooy, *J. Am. Chem. Soc.*, **72**, 5275 (1950).

(22) J. B. Cohen and C. J. Smithells, *J. Chem. Soc.*, **105**, 1908 (1914).

2-Bromo-5-chloro-4-nitrotoluene.—2-Nitro-4-bromo-5-methylaniline (8.6 g, 0.037 mole), dissolved in 100 ml of hot glacial acetic acid, was kept hot and added slowly to a stirred solution of 4.2 g (0.06 mole) of $NaNO_2$ in 45 ml of concentrated H_2SO_4. The nitrite–sulfuric acid solution was kept in an ice bath while the hot acetic acid solution was added, but the rate of addition was such that the temperature was maintained below 40°. The last of the nitroaniline was added by using three 10-ml portions of glacial acetic acid. The reaction mixture was stirred for 30 min while the temperature was kept at about 35°. Cuprous chloride (8.6 g, 0.0866 mole) was added to 125 ml of concentrated HCl and the solution cooled to 5–10°. The diazonium solution was added to the cuprous chloride solution with vigorous stirring and with the temperature kept below 25°. The reaction mixture was heated to 60° with stirring until no more nitrogen was evolved; 500 ml of water was added and the mixture was cooled. The product was filtered and washed repeatedly with water, dried (yield 8.8 g), and recrystallized from 50 ml of 80% alcohol. The yield was 7.2 g (77%) of light yellow product, mp 65–66°.

Anal. Calcd for $C_7H_5BrClNO_2$: C, 33.6; H, 2.0; Br, 31.9; N, 5.6. Found: C, 33.5; H, 1.8; Br, 31.9; N, 5.6.

4-Bromo-5-methyl-2-nitro-N-D-ribitylaniline.—2-Bromo-5-chloro-4-nitrotoluene (5.0 g, 0.02 mole) and 10 g of D-ribamine were refluxed in 150 ml of pyridine for 6 hr and processed as described for 2-nitro-4-chloro-5-methyl-N-D-ribitylaniline.[9] The product was obtained as orange-red needles (2.8 g, 45%) which melted at 187–188°.

Anal. Calcd for $C_{12}H_{17}BrN_2O_6$: C, 39.4; H, 4.7; Br, 21.9; N, 7.7. Found: C, 39.1; H, 4.4; Br, 22.1; N, 7.5.

7-Bromo-8-methyl-10-(1-D-ribityl)isoalloxazine.—4-Bromo-5-methyl-2-nitro-N-D-ribitylaniline (2.11 g, 0.006 mole) was dissolved in 39 ml of glacial acetic acid and 8.1 ml of water and reduced in the presence of 100 mg of PtO_2 at 4.2 kg/cm² for 4 hr. The reaction mixture was filtered directly into a hot solution of 1.2 g (0.0075 mole) of alloxan monohydrate and 2.2 g (0.035 mole) of boric acid in 115 ml of glacial acetic acid. The combined solution was heated at 50° for 1 hr and then placed in the dark for 4 days. The solution was evaporated and 300-ml portions of ethanol were evaporated from the residue in sequence. The residue was suspended in 120 ml of water and filtered to yield 1.80 g of flavin melting at 269–272° dec. The product was dissolved in 40 ml of concentrated HCl, treated with charcoal, and filtered hot. To the filtrate was added 80 ml of water. Cooling the solution produced 1.36 g (50%) of the desired flavin melting at 276–278° dec.

Anal. Calcd for $C_{16}H_{17}BrN_4O_6$: C, 43.6; H, 3.9; Br, 18.1; N, 12.7. Found: C, 43.7; H, 4.1; Br, 18.0; N, 12.6.

Ultraviolet Absorption Spectrum.—The spectrum was determined with a Beckman spectrophotometer, Model DU. The compound was dissolved in water and measurements made on a solution containing 5.00 mg/l. The values for ε for the maxima and minima were: maxima, 270 mμ (ε 47,800), 360 (12,600), 450 (14,700); minima, 245 mμ (ε 25,100), 305 (7300), 390 (7300).

Chromatography.—The flavins were chromatographed by the ascending technique on Whatman No. 1 paper using the upper phase of a water–*n*-butyl alcohol (Mallinckrodt AR)–acetic acid (5:4:1) system. When riboflavin and 7-bromo-8-methyl-10-(1-D-ribityl)isoalloxazine were done simultaneously, the R_f of the former was 0.30 and of the latter 0.43.

Biological Activity. A. For *L. casei*.—The quantity of lactic acid produced by *L. casei* 7469[7] was measured by titration with 0.1 *N* NaOH. The observed values were plotted against the flavin concentrations. One set of duplicate tubes (standard curve) was prepared by the routine procedure using graded increments of riboflavin[23] from 0 to 0.3 μg/tube. Another set of duplicate tubes (inhibition curve) was prepared to contain in all cases 0.3 μg of riboflavin/tube plus graded increments of the analog from 0 to 100 μg/tube. The tubes were incubated for 72 hr at 37°. The *II*[1] was determined from the ratio of the mixture of the two flavins which supported the production of one-half the amount of lactic acid formed in the presence of 0.3 μg of riboflavin alone.

Figure 1 shows that the ratio of the mixture of analog to riboflavin which supported 50% of the acid production to that produced by the same quantity of riboflavin alone was 48.3:0.3 μg. The *II* was found to be 137. The production of more

(23) U.S.P. Reference Standards, New York, N. Y.

acid than could be produced by the utilization of riboflavin alone in tubes containing from 2.5–35 μg of the analog in addition to the 0.30 μg of riboflavin, is due to the appearance and culturing of a mutant form of *L. casei* which is able to utilize the analog for its flavin requirements.[24]

B. For the Rat.—Weanling male rats of the Wistar strain[25] were used. The conditions under which the animals were maintained and the riboflavin-deficient diet used have been described before.[26] When the animals became satisfactorily riboflavin deficient,[26] the flavin supplements were administered by stomach tube as a solution or suspension in 0.5 ml of 6% gum acacia solution each day immediately before they were fed. The deficient control animals were given the corresponding vehicle without the flavin. All tests were continued for 28 days from the time the animals became deficient.

Results

Table I shows that the administration of 5 μg/day of 7-bromo-8-methylflavin results in a stimulation of growth (group 2). The administration of 25 μg/day (group 4) results in growth which is approximately equal to that shown by the animals receiving 10 μg/day of riboflavin (group 3). All animals survived the administration of 50 μg/day (group 6). This is to be compared with the survival of only 40% of animals given 50 μg/day of the 7-chloro-8-methylflavin.[10] At 250 μg/day, the lethal property of the analog is revealed by death of one-third of the animals receiving it (group 7). That the compound is lethal is unequivocally demonstrated by the administration of 500 μg/day (group 8), which results in the death of two-thirds of the animals. Since the lethality of the analog is reversed by the simultaneous administration of riboflavin (group 10), it may be concluded that the analog is functioning as a reversible antagonist of riboflavin in the rat. The 40 μg/day of riboflavin does not completely reverse the antagonistic action of 500 μg/day of the analog. The ratio of analog to riboflavin of 5:1 is less disadvantageous to the animal than a ratio of 12:1 (group 10). The additive effect of the two flavins when small quantities are administered is shown by the growth response to a mixture of 50 μg of the analog and 10 μg of riboflavin/day.

TABLE I

GROWTH OF RATS RECEIVING ANALOG, RIBOFLAVIN, OR ANALOG PLUS RIBOFLAVIN

Group	Daily supplement	Wt gain, g[a]	Survivors
1	H_2O	5 ± 2	8/8
2	5 μg of analog	16 ± 2	8/8
3	10 μg of riboflavin	55 ± 4	8/8
4	25 μg of analog	62 ± 3	9/9
5	15 μg of riboflavin	76 ± 2	7/7
6	50 μg of analog	89 ± 3	9/9
7	250 μg of analog	103 ± 6	6/9
8	500 μg of analog	...[b]	3/9
9	50 μg of analog + 10 μg of riboflavin	117 ± 7	8/8
10	500 μg of analog + 40 μg of riboflavin	105 ± 5	8/8

[a] Net weight gain of survivors for the 4-week test period plus or minus an estimate of the standard error of the mean. If these 28-day means are plotted and a line drawn to the origin, an excellent reproduction of the plot of the average rate of growth of the various groups is obtained. [b] These animals showed the same rate of growth for the first 15 days (average weight of group 8 = 63 g) as shown by the animals in group 10 (average weight = 65 g), although two animals had already died. No weight is given because two-thirds of the animals had died. The three remaining animals showed a net weight gain by the 28th day of 63, 90, and 105 g.

(24) For further details of this type of occurrence, see ref 10.
(25) CFN rats, Carworth Farms, New City, N. Y.
(26) J. P. Lambooy and H. V. Aposhian, *J. Nutr.*, **47**, 539 (1952).

Synthesis and Pharmacological Activity of 4-D-Glutamine-oxytocin,[1] 5-D-Asparagine-oxytocin, and 4-D-Glutamine-5-D-asparagine-oxytocin

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5-D-Asparagine- and 4-D-glutamine-5-D-asparagine-oxytocin have been synthesized. Pharmacological testing showed that 4-D-glutamine- and 5-D-asparagine-oxytocin possess very low specific oxytocic and vasodepressor activities, and 4-D-glutamine-5-D-asparagine-oxytocin had no activity in these tests; however, by cumulative dose–response studies for oxytocic activity, it was found that 4-D-glutamine- and 5-D-asparagine-oxytocin had similar "intrinsic" activity to oxytocin

With a view to study the structure–activity relationship of oxytocin and related peptides, the synthesis of 5-D-asparagine- and 4-D-glutamine-5-D-asparagine-oxytocin and the pharmacological testing of these and 4-D-glutamine-oxytocin, synthesized earlier,[1] has been carried out.

p-Nitrophenyl benzyloxycarbonyl-D-asparaginate (I) was prepared from benzyloxycarbonyl-D-asparagine and *p*-nitrophenol by treatment with dicyclohexylcarbodiimide. I on condensation with S-benzylcysteinylprolylleucylglycinamide[2] yielded benzyloxycarbonyl-D-asparaginyl-S-benzylcysteinylprolylleucylglycinamide (II). II on treatment with HBr–AcOH followed by condensation with *p*-nitrophenyl benzyloxycarbonylglutaminate gave benzyloxycarbonylglutaminyl-

(1) A. S. Dutta and N. Anand, *Indian J. Chem.*, **3**, 232 (1965).
(2) M. Bodanzsky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).