tially resolved tail crops obtained from a quinine resolution of V were cut back to the free acid in the usual manner. The undistilled acid thus obtained was combined with anhydrous brucine (36 g.) in *ca.* 400 cc. of hot acetone. After standing overnight in the refrigerator, a crop of 25 g. was obtained, which, after one recrystallization from 300 cc. of acetone, gave VIIIa·H_2O, a monohydrate of the brucine salt of (+)-O-ethyl ethylphosphonothioic acid, 20 g., m.p. 163–165°.

Anal. Calcd. for $C_{27}H_{37}N_2O_6PS·H_2O$: C, 57.4; H, 6.9; neut. equiv., 566.6. Found: C, 57.2; H, 6.9; neut. equiv., 568, 569.

When dried under vacuum over phosphorus pentoxide at 100° for three hours, a sample of the monohydrate, above, lost 3.36% of its weight (calcd. 3.18% for one mole of water) and gave the anhydrous salt, m.p. 181–182°; equivalent weight calcd. for $C_{27}H_{37}N_2O_6PS$, 548.6; found 553, 550, 548. Samples of both the m.p. 163–165° and 181–182° brucine salts were cut back to the free acid in the usual manner, then converted to dicyclohexylamine salts. The dicyclohexylamine derivative thus obtained (unrecrystallized) from the 180–181° salt (in 81% yield) had m.p. 160–161°, $[\alpha]_D$ +6.90 ± 0.25° (α_{obsd} +0.357 ± 0.013°, methanol, 1 dm., *c* 5.182); that obtained (unrecrystallized) from the 163–165° form (in 73% yield) had m.p. 160–161°, $[\alpha]_D$ +6.94 ± 0.22° (α_{obsd} +0.341 ± 0.011°, methanol, 1 dm., *c* 4.922).

Dicyclohexylamine Salt of *d,l*-O-Ethyl Ethylphosphonothioic Acid.—The acid V (0.80 g., 0.0052 mole) in 5 ml. of petroleum ether was added to a solution of 1.05 g. (0.0058 mole) of dicyclohexylamine in 5 ml. of petroleum ether. The product, which immediately crystallized, was filtered to give 1.60 g. (92%) of salt, m.p. 166–167.5°. Recrystallization of a 0.5-g. portion from acetone–petroleum ether gave 0.30 g., m.p. 166–168°.

Anal. Calcd. for $C_{16}H_{34}NO_3P$: C, 57.28; H, 10.22. Found: C, 57.2; H, 10.1.

Dicyclohexylamine Salts of Va and Vb.—The dicyclohexylamine salt prepared directly from the undistilled Va residue obtained from IXa·H_2O (m.p. 151–153°) as described above had m.p. 159–160.5°, $[\alpha]_D$ −7.11 ± 0.23° (α_{obsd} −0.153 ± 0.005°, methanol, 1 dm., *c* 2.150). A similar salt prepared from the residue obtained as above from a sample of IXb (m.p. 163–166°) had m.p. 158–160°, $[\alpha]^{25}_D$ +6.85 ± 0.25° (α_{obsd} +0.221 ± 0.008°, methanol, 1 dm., *c* 3.230).

Anal. Calcd. for $C_{16}H_{34}O_3NP$: C, 57.28; H, 10.22. Found: (−)-antipode, C, 57.38; H, 10.00; (+) antipode, C, 57.30; H, 10.02.

Determination of the Neutralization Equivalents of the Quinine and Brucine Salts.—The equivalent weights of the diastereoisomeric salts obtained from the resolutions were determined by titration of the salts (0.2 g.) in 25 ml. of 3:2 methanol:water with standard 0.1 *N* aqueous base. The change in the apparent *p*H was recorded *via* a *p*H meter and the end-point was obtained from a graphical plot of the titration data.

ARMY CHEMICAL CENTER, MD.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGY, THE UNIVERSITY OF ROCHESTER SCHOOL OF MEDICINE AND DENTISTRY]

The Synthesis of 6-Ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine and 6-Methyl-7-ethyl-9-(1′-D-ribityl)-isoalloxazine[1]

BY JOHN P. LAMBOOY

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The unequivocal syntheses of these two flavins have been accomplished by the condensation of the appropriately substituted *o*-aminoazo compounds with barbituric acid. The same synthetic route has been employed for the synthesis of 6,7-diethyl-9-(1′-D-ribityl)-isoalloxazine while 6-ethyl-9-(1′-D-ribityl)-isoalloxazine has been prepared by an alternative procedure.

In 1936, Karrer and Quibell[2] reported the synthesis of riboflavin by the reduction of N-(D-ribityl)-2-phenylazo-4,5-dimethylaniline to the corresponding *o*-phenylenediamine which in turn was condensed with alloxan. It was subsequently suggested by the Merck group in the monumental paper by Tishler[3] and associates that the procedure employed by Karrer might have produced a mixture of two flavins, namely, riboflavin and isoriboflavin. They do not appear to have demonstrated this directly but the following observations lend strong support to their suggestion. Tishler and associates discovered that the preparation of the *o*-aminoazo compounds from 4,5-disubstituted ribitylanilines did not yield pure compounds but mixtures resulting from the introduction of the arylazo group into either the 2- or the 6-position. When these isomeric forms were separated and independently reduced to the *o*-phenylenediamines and then condensed with alloxan, the 2-arylazo isomer was converted to riboflavin while the 6-arylazo form became isoriboflavin.

In the same paper, Karrer and Quibell[2] reported the synthesis of 6-ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine and simply stated that at the level of 10 micrograms per day it was nearly as potent as riboflavin in stimulating the growth of riboflavin deficient rats. The procedure used for the synthesis of this compound was the same as that for the synthesis of riboflavin, namely, the reduction of what might have been a mixture of *o*-aminoazo compounds to a mixture of *o*-phenylenediamines. Subsequent condensation of the reduction product with alloxan might have produced a mixture of the desired 6-ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine and 5-methyl-6-ethyl-9-(1′-D-ribityl)-isoalloxazine.

An additional point of interest to us was that we had found that intermediate levels of 6,7-diethyl-9-(1′-D-ribityl)-isoalloxazine stimulated the growth of the riboflavin deficient rat.[4] This material could not, however, be considered an adequate substitute for riboflavin in the nutrition of the rat because the animals failed to survive. This raises

(1) This investigation was supported in part by research grant No. CY-2940 from the National Cancer Institute, United States Public Health Service.

(2) P. Karrer and T. H. Quibell, *Helv. Chim. Acta*, **19**, 1034 (1936).

(3) M. Tishler, K. Pfister, R. D. Babson, K. Ladenburg and A. J. Fleming, THIS JOURNAL, **69**, 1487 (1947).

(4) J. P. Lambooy and H. V. Aposhian, *J. Nutrition*, **47**, 539 (1952).

the question as to whether 6-ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine does in reality have riboflavin activity or if it merely stimulates the growth of the riboflavin deficient rat. The need to clarify this important point relative to biological activity made it essential that we synthesize this interesting compound and, so that we might further elucidate the relationship between structure and biological activity, we also synthesized the 6-methyl-7-ethyl-9-(1′-D-ribityl)-isoalloxazine. The need for liberal quantities of 6,7-diethyl-9-(1′-D-ribityl)-isoalloxazine[5] and 6-ethyl-9-(1′-D-ribityl)-isoalloxazine[6] prompted us to investigate more productive means for their preparation.

We prepared N-(D-ribityl)-2-phenylazo-4-ethyl-5-methylaniline of a purity (m.p. 157–158°) comparable to that used by Karrer and Quibell (m.p. 152°). This material was reduced to the *o*-phenylenediamine and condensed with alloxan and boric acid as described by these workers. The flavin preparation was purified and then chromatographed on paper in a water, butanol and acetic acid system to reveal the presence of two flavins. The contaminant moved more rapidly than the 6-ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine which is consistent with the observation[7] that isoriboflavin moves more rapidly than riboflavin. We were compelled, therefore, to make use of procedures which would yield the flavins in good quantity and of unquestioned purity. The elegant procedures perfected by the Merck group were found to satisfy these criteria in the synthesis of three of the four flavins reported here.

Experimental

All melting points were taken on calibrated thermometers. The decomposition points of the flavins were taken in a rapidly heated bath and are uncorrected.

3-Methyl-4-ethylaniline was prepared by the procedure outlined by Morgan and Pettet.[8] *m*-Toluidine (1750 g.) yielded after three fractional distillations, 376 g. (17%),[8a] b.p. 234–238°,[8b] of 3-methyl-4-ethylaniline. The aniline (312 g.) was converted into the acetanilide and following fractional crystallization yielded 241 g. (59%) of 3-methyl-4-ethylacetanilide, m.p. 91–92°[8c] and 47 g. of an unknown acetanilide, m.p. 75–77°. A portion of the 3-methyl-4-ethylacetanilide was stored in this form and constituted the starting material for the 6-ethyl-7-methylflavin.

3-Methyl-4-ethylacetanilide, 17.7 g. (0.1 mole), was nitrated by the procedure described for the nitration of 3,4-diethylacetanilide[5] and after recrystallization from ethanol yielded 13.1 g. (59%) of 4-ethyl-5-methyl-2-nitroacetanilide, m.p. 104–105°.[8d] The above nitroacetanilide (60 g.) was hydrolyzed by the procedure described for the hydrolysis of 4,5-diethyl-2-nitroacetanilide[5] and after recrystallization from 50% (v./v.) ethyl alcohol, yielded 46 g. (94%) of 2-nitro-4-ethyl-5-methylaniline, m.p. 91°.[8e] The above nitroaniline was deaminated by the following method. The nitroaniline, 18.0 g. (0.1 mole), 105 ml. concd. hydrochloric acid and 100 ml. of water was heated, with stirring, to 100°. Good stirring was maintained during cooling to keep the precipitate which forms in suspension. When the temperature had been reduced to −5°, 7.25 g. of sodium nitrite in 18 ml. of water was added dropwise, over 1 hr. While the temperature was maintained at −5° to 0°, 52 ml. (0.5 mole) of hypophosphorous acid was added over a period of 1.25 hr. Stirring was continued for an additional hr. at 0° and the reaction mixture stored in the refrigerator overnight. The product was extracted with ether, the ether washed with sodium hydroxide solution and then with water and dried. Following removal of the solvent the product was vacuum distilled to yield 14.5 g. (88%)[8f] of 3-ethyl-4-methylnitrobenzene, b.p. 130–131° (10 mm.), m.p. 23°.[8g] The above nitrobenzene, 37.9 g. (0.23 mole), was reduced over platinum oxide in ethanol at 3 atm. of hydrogen. After removal of the solvent the product was dissolved in 120 ml. of 6.5% hydrochloric acid and extracted with ether. The acid solution was made alkaline and the aniline isolated in the usual manner and vacuum distilled to yield 29.7 g. (96%)[8h] of 3-ethyl-4-methylaniline, b.p. 107–108° (10 mm.). The above aniline was converted almost quantitatively to the 3-ethyl-4-methylacetanilide. Following recrystallization from 50% (v./v.) ethyl alcohol the product melted at 89–90°.[8i] This material was stored in this form and constituted the starting material for the 6-methyl-7-ethylflavin.

The preparation of the 3,4-diethylaniline has been reported before.[5]

N-(D-Ribosyl)-3,4-(substituted)-anilines.—Ten grams of the appropriate acetanilide (3-methyl-4-ethylacetanilide, 3-ethyl-4-methylacetanilide or 3,4-diethylacetanilide) was hydrolyzed by refluxing with 50 ml. of concentrated hydrochloric acid for 1 hr. The product was diluted with 100 ml. of water, made alkaline with 20% sodium hydroxide and isolated in the usual manner. Following vacuum distillation the yields varied: 3-methyl-4-ethylaniline, 95–96%, b.p. 110° (10 mm.), 123° (20 mm.); 3-ethyl-4-methylaniline, 95–97%, b.p. 106 to 108° (10 mm.); 3,4-diethylaniline, 95–100%, b.p. 131° (18 mm.), 133° (19 mm.), 135° (20 mm.). To the freshly distilled aniline was added the stoichiometric amount of crystalline D-ribose in 100 ml. of absolute ethyl alcohol. The mixture was left at room temperature for 3 hr. and then placed in the refrigerator. (All additions were made to the reaction flask through a filter so that the products could be analyzed without recrystallization.) The ribosides were filtered and washed on the filter with absolute ethyl alcohol and absolute ether.

N-(D-Ribosyl)-3-methyl-4-ethylaniline yields varied from 84 to 91%; m.p. varied from 121–122° to 123–124°; analyzed sample, m.p. 122–123°, white needles.

Anal. Calcd. for $C_{14}H_{21}NO_4$: C, 62.9; H, 7.9; N, 5.2. Found: C, 63.2; H, 8.0; N, 5.3.

N-(D-Ribosyl)-3-ethyl-4-methylaniline yields varied from 88 to 95%; m.p. varied from 127–128° to 134–135°; analyzed sample, m.p. 131–132° white needles.

Anal. Calcd. for $C_{14}H_{21}NO_4$: C, 62.9; H, 7.9; N, 5.2. Found: C, 63.2; H, 8.0; N, 5.6.

N-(D-Ribosyl)-3,4-diethylaniline yields varied from 71 to 82%; m.p. varied from 116–119° for the 82% yield to 126–127° for the 71% yield; analyzed sample, m.p. 122–124°, white needles.

Anal. Calcd. for $C_{15}H_{23}NO_4$: C, 64.0; H, 8.2; N, 5.0. Found: C, 64.1; H, 8.4; N, 5.1.

In all but one case the diethylaniline preparation took an abnormal course. When placed in the refrigerator the product assumed a "gel" structure which could be converted to a crystalline form by leaving the material at room temperature for about 12 hr. It was then returned to the refrigerator.

N-(D-Ribityl)-3,4-(substituted)-anilines.—The N-(D-ribosyl)-3-methyl-4-ethylaniline was found not to be reduced consistently successfully with Raney nickel. It was found that palladium-on-calcium carbonate[9] could be used successfully. Routinely, equal weights of compound and catalyst were suspended in absolute ethyl alcohol (about 10–15 ml. per gram of compound) and shaken for 6 to 8 hr. for the ethyl–methyl substituted ribosides and 24 hr. for the diethyl riboside at 70° and 3 atm. of hydrogen. The N-(D-ribityl)-3-methyl-4-ethylaniline was isolated as the free aniline (m.p. 109–110°) or as the hydrochloride salt in yields which, in the latter case, varied from 80 to 99%, m.p. 120 to 124°[10] and these were used in the next reaction. It was

(5) J. P. Lambooy, THIS JOURNAL, **72**, 5225 (1950).

(6) H. V. Aposhian and J. P. Lambooy, *ibid.*, **76**, 1307 (1954).

(7) G. L. Kilgour, S. P. Felton and F. M. Huennekens, *ibid.*, **79**, 2254 (1957).

(8) G. T. Morgan and A. E. J. Pettet, *J. Chem. Soc.*, 418 (1934); (a) reported yield, 9%; (b) reported b.p. 236°; (c) reported m.p. 90°; (d) reported m.p. 103°; (e) reported m.p. 90°; (f) reported 47% yield by alcohol deamination; (g) reported b.p. 271° and m.p. 23°; (h) reported 70% yield by iron and hydrochloric acid reduction, b.p. 234–235°; (i) reported m.p. 88°.

(9) R. Kuhn and R. Ströble, *Ber.*, **70**, 773 (1937).

(10) Karrer and Quibell, ref. 2, used the hydrochloride, but obtained a yield of only 35% and report no m.p.

found, however, that far less work was involved if following the reduction and removal of the catalyst and solvent the residual ribitylaniline was assumed to have been produced quantitatively and immediately dissolved in dilute hydrochloric acid or acetic acid as described below.

N-(D-Ribityl)-2-arylazo-4,5-(substituted)-anilines.—The procedure used for the methyl-ethyl substituted anilines was basically that described by Shunk[11] and associates. The only difference in the procedure was that the ribitylanilines were not isolated. The appropriate amount of hydrochloric acid and water was added to the residue after the evaporation of the solvent used for the reduction. The diazotized aniline solution was then added to this as described. The products were recrystallized from 60% ethyl alcohol.

N-(D-Ribityl)-2-phenylazo-4-ethyl-5-methylaniline.—The yields varied from 74 to 86% of orange-red prisms, m.p. 158 to 163°.[12] When an isolated sample of the hydrochloride salt of the aniline was diazotized, the yield was 74%, m.p. 158–161°. When an isolated sample of the free aniline was used the yield was 97%, m.p. 155–156°. This yield is misleading because large losses accompany the isolation of the free aniline.

N-(D-Ribityl)-2-phenylazo-4-methyl-5-ethylaniline.—The yields varied from 69 to 89%. The melting point was very erratic and a single recrystallization appears to give a product of adequate quality for subsequent use, m.p. 102 to 108°. Successive recrystallizations produced the following melting points: 101–103°, 101–103°, 122–129°, 108–110° and 95–101°. It appears that these changes may be due to fluctuating concentrations of the *o*-aminoazo isomers; brownish-orange prisms.

Anal. Calcd. for $C_{20}H_{27}N_3O_4$: C, 64.3; H, 7.3; N, 11.3. Found: C, 64.6; H, 7.2; N, 11.5.

N-(D-Ribityl)-2-*p*-tolylazo-4,5-diethylaniline.—When the procedure described immediately above was used it produced a red oil which would crystallize when free of solvent on long standing but could not be conveniently recrystallized. The procedure finally used was that described for the preparation of 1-(tetraacetyl-D-ribitylamino)-2-*p*-tolylazo-4,5-dimethylbenzene by Tishler, *et al.*[3] The only modification was that 15 to 20% of the calculated required glacial acetic acid was used to dissolve the residual N-(D-ribityl)-4,5-diethylaniline following reduction. This was then added to the diazotized *p*-toluidine solution. The yield of crystalline product is only fair and large losses accompany recrystallization. The crude product is first recrystallized from 90% methanol, then *n*-butyl alcohol and finally 60% ethyl alcohol for yields of from 27 to 37%, m.p. 145–146°, orange prisms.

Anal. Calcd. for $C_{22}H_{31}N_3O_4$: C, 65.8; H, 7.8; N, 10.5. Found: C, 65.8; H, 7.8; N, 10.5.

Some flavin can be obtained from the aminoazo compound lost by recrystallization. One batch of riboside (21.5 g.) was reduced and converted to the aminoazo compound as described above. Following crystallization from 90% methyl alcohol 17.4 g. of aminoazo compound was obtained. This recrystallized from *n*-butyl alcohol produced 11.4 g. of aminoazo compound of suitable purity. The butanol solution was evaporated to yield 7.0 g. of residue. This was treated as if it were all aminoazo compound in the flavin synthesis to produce, following hydrochloric acid recrystallization, 1.34 g. of flavin, m.p. 280–284°.

6,7-Substituted-9-(1′-D-ribityl)-isoalloxazines.—The three flavins were prepared by the procedure described by Shunk,[11] *et al.*, for the preparation of 5′-desoxyriboflavin.

6-Ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine yields varied from 58 to 59%. All preparations and the analyzed sample melted at 277–279° dec.,[13] yellow needles.

Anal. Calcd. for $C_{18}H_{22}N_4O_6$: C, 55.4; H, 5.7; N, 14.4. Found: C, 55.6; H, 5.7; N, 14.2.

6-Methyl-7-ethyl-9-(1′-D-ribityl)-isoalloxazine yields varied from 52–60%; all preparations, m.p. 273–279° dec.; the analyzed sample, m.p. 282–284° dec., yellow needles.

Anal. Calcd. for $C_{18}H_{22}N_4O_6$: C, 55.4; H, 5.7; N, 14.4. Found: C, 55.7, 55.2; H, 6.0, 5.5; N, 14.5, 14.6.

6,7-Diethyl-9-(1′-D-ribityl)-isoalloxazine yields varied from 54–60%. All preparations melted between 278 and 282° dec.[14] This material is identical with the earlier preparation[5] in all respects.

N-(D-Ribosyl)-2-nitro-4-ethyl-5-methylaniline.—An attempt was made to prepare the 6-ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine by essentially the same procedure as that used for the earlier preparation of the diethylriboflavin.[5] This material was prepared from 2-nitro-4-ethyl-5-methylaniline and D-ribose by the use of the procedure described for the preparation of 4,5-diethyl-2-nitroaniline N-D-ribopyranoside.[5] It was recrystallized from ethanol to yield (60%), m.p. 184–185° dec.; yellow needles.

Anal. Calcd. for $C_{14}H_{20}N_2O_6$: C, 53.8; H, 6.5; N, 9.0. Found: C, 54.0; H, 6.6; N, 9.0.

This material was subjected to reduction making use of the catalyst referred to above,[9] at 70° and 3 atmospheres for 24 hr. When the reduction product was condensed with alloxan the yield of flavin was considerably less than that obtained above. To check the procedure and the catalyst, 4,5-diethyl-2-nitroaniline was converted to N-(D-ribosyl)-2-nitro-4,5-diethylaniline in 75% yield, m.p. 179–181°[15] dec. This riboside (2.61 g.) was reduced with palladium-on-calcium carbonate catalyst at 70° and 3 atmospheres for 24 hr. When the reduction product was condensed with alloxan, 6,7-diethyl-9-(1′-D-ribityl)-isoalloxazine was obtained in 31% (0.99 g.) yield of material, m.p. 285–287° dec.

6-Ethyl-9-(1′-D-ribityl)-isoalloxazine.—The relatively low yields of this material which were obtained on a previous occasion[6] and the relative productiveness of the procedures employed for the synthesis of other flavins,[16] prompted us to try the latter method for the synthesis of this flavin.

2-Nitro-4-ethylchlorobenzene.—2-Nitro-4-ethylaniline[17] (10 to 20 g.) was converted to 2-nitro-4-ethylchlorobenzene by essentially the procedure used for the synthesis of 2,5-dichloro-4-nitrotoluene[16] except that after the addition of water at the end of the reaction, the product was removed from the reaction mixture by steam distillation and isolated in the usual manner in yields of from 74 to 82%. The product is a yellow oil, b.p. 133–135° (10 mm.), 151–153° (20 mm.), $n^{25}D$ 1.5456.

Anal. Calcd. for $C_8H_8ClNO_2$: C, 51.8; H, 4.3; N, 7.6. Found: C, 52.0; H, 4.5; N, 7.8.

N-(D-Ribityl)-2-nitro-4-ethylaniline.—The above 2-nitro-4-ethylchlorobenzene (5 to 22 g.) was treated with D-ribamine as described for the synthesis of 2-nitro-4-chloro-5-methyl-N-D-ribitylaniline.[16] The yield was always poor, and varying the heating period, the volume of pyridine or changing the solvent had no beneficial effect. Following the removal of the solvent by decantation, the pyridine was evaporated under vacuum. The residue was steam distilled to remove unreacted starting material and the water suspension of the residue placed in the refrigerator. The crystalline precipitate, m.p. 134–136°, was recrystallized from methanol and 50% (v./v.) ethyl alcohol to yield between 12 and 15% of the theoretical amount of the desired product, m.p. 139°.

Anal. Calcd. for $C_{13}H_{20}N_2O_6$: C, 52.0; H, 6.7; N, 9.3. Found: C, 52.2; H, 6.9; N, 9.5

We were equally unsuccessful in bringing about a satisfactory reaction between 2-nitro-4-methylchlorobenzene and D-ribamine.

6-Ethyl-9-(1′-D-ribityl)-isoalloxazine.—The N-(D-ribityl)-2-nitro-4-ethylaniline (2.00 g.) was reduced as described for the reduction of 2-nitro-4-chloro-5-methyl-N-D-ribitylaniline.[16] Following the condensation with alloxan as described and permitting the material to stand 3 days, the reaction mixture was evaporated to dryness and redissolved

(11) C. H. Shunk, J. B. Lavigne and K. Folkers, THIS JOURNAL, **77**, 2210 (1955).

(12) Karrer and Quibell, ref. 2, obtained 53% yield, m.p. 152°.

(13) Reference 2 reported m.p. 238–240° (fast heating).

(14) The decomposition point of this material was previously (ref. 5) reported as 255–256° dec. The decomposition points reported for the flavins described here were done in a rapidly heated bath. When the decomposition point of some of the original analyzed sample described in ref. 5 was determined in the rapidly heated bath it was found to be 277–280° dec.

(15) Previously, ref. 5, this compound was found to melt between 171 and 177° dec.

(16) E. E. Haley and J. P. Lambooy, THIS JOURNAL, **76**, 5093 (1954).

(17) J. P. Lambooy and E. E. Haley, *ibid.*, **74**, 1087 (1952).

in 60 ml. of hot water. Cooling produced 1.50 to 1.74 g. (60–68%) of the somewhat impure flavin, m.p. 285–286° dec.

This slightly impure material (4.66 g.) was divided into three lots, each of which was dissolved in hot water and poured into a column packed with a mixture of 150 g. of Magnesol and 150 g. of Hi-Flo Super-Cel. The column was washed with 10% acetone in water and the flavin eluted with 50% acetone in water. The eluate was evaporated to dryness and the residue recrystallized by dissolving in hot water. The yield was 3.09 g. of flavin, m.p. 288–289[18] dec. This material is identical in all respects to that prepared by the previous procedure.[6]

Chromatography.—The flavins were chromatographed by the descending method on Whatman #1 paper using the upper phase of a water (5)–*n*-butyl alcohol (4)–acetic acid (1) system. The flavins were found to have the following R_f values: riboflavin, 0.35; 6-methyl-7-ethylflavin, 0.48; 6-ethylflavin, 0.50; 6-ethyl-7-methylflavin, 0.51, and 6,7-diethylflavin, 0.62.[19]

Ultraviolet Absorption Spectra.—All ultraviolet absorption spectra were determined with a Beckman quartz spectrophotometer, model DU. The compounds were dissolved in water and measurements made on solutions whose concentrations were 5.00 mg./l. for riboflavin and 5.02 mg./l. for the 6-ethyl-7-methylflavin and 6-methyl-7-ethylflavin.

The spectra were almost superimposable. The values for ϵ for the maxima and minima were found to be as follows: riboflavin, maxima: (ϵ) 267 mμ (32,100), 373 mμ (10,400), 447 mμ (12,300); minima: 306 mμ (1050), 402 mμ (6800); 6-ethyl-7-methyl-9-(1′-D-ribityl)-isoalloxazine, maxima: 268 mμ (32,500), 374 mμ (10,300), 447 mμ (12,200); minima: 306 mμ (1,090), 402 mμ (6,800); 6-methyl-7-ethyl-9-(1′-D-ribityl)-isoalloxazine, maxima: 268 mμ (32,100), 375 mμ (10,500), 448 mμ (12,400); minima: 307 mμ (1,090), 402 mμ (6,900).

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(18) Reference 6 reported m.p. 284–285.

(19) The prevailing atmospheric temperature was between 85 and 90°F. during these runs. On an earlier occasion H. V. Aposhian and J. P. Lambooy, THIS JOURNAL, **77**, 6368 (1955), by means of the ascending method, found the R_f values of three of these flavins to be: riboflavin, 0.30; 6-ethylflavin, 0.43; and 6,7-diethylflavin, 0.54.

[CONTRIBUTION FROM THE INSTITUTE OF MICROBIOLOGY, RUTGERS, THE STATE UNIVERSITY AND THE DEPARTMENT OF MEDICINE, THE COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY AND THE PRESBYTERIAN HOSPITAL]

Immunological Specificities Involving Multiple Units of Galactose. III.[1]

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Further cross reactions of D-galactose-containing polysaccharides with Type XIV antipneumococcal serum are explored. Periodate-oxidized specific polysaccharide of Type XIV pneumococcus precipitates less than one-half of the antibody in the antiserum, confirming the importance of the eliminated non-reducing end groups of D-galactose in the over-all Type XIV pneumococcal specificity. It is also inferred that such groups are present in the specific polysaccharide of the anthrax bacillus, in a cold-soluble fraction of agar, and probably in the mucilage of okra pods. Serological confirmation of the presence of non-reducing end groups of D-galactose also is given for corn fiber hemicellulose.

In the first two papers of this series[4,5] it was shown that the cross-reactivities in Type XIV antipneumococcal sera of a number of galactose-containing polysaccharides of known constitution could be used to obtain advance information on the linkages of the galactose which occur in the capsular, immunologically type-specific polysaccharide (S XIV) characteristic of the virulent, or mucoid, form of pneumococcus Type XIV.[6,7] A study of the hydrolytic products of the methylated derivative of S XIV has already confirmed the prediction of non-reducing end groups of D-galactose and identified as 1,3-linked at least a portion of the galactose predicted as bound in 1,3-, 1,6- or 1,3,6-linkages.[8] Moreover, the uronic acid originally reported in a galactan isolated from beef lung[9] was shown to be D-glucuronic acid by the unexpected reactivity of the galactan in Type II antipneumococcal serum, a finding later confirmed by chromatography.[4] The glucuronic acid could also be shown to be a component of an impurity in, or degradation product of, the principal polysaccharide by the far greater content of uronic acid in the carbohydrate recovered from the specific precipitate in the Type II antiserum than in the polysaccharide derived from the Type XIV precipitate or in the original galactan. An extension of these findings to other galactose-containing polysaccharides seemed indicated by these promising beginnings, and the present paper records some additional results.

Experimental

A preparation of the specific polysaccharide of *B. anthracis*,[10] isolated from cultures grown in the guinea pig,[11] was kindly supplied by Dr. H. Smith, while two other poly-

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