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Reaction between Riboflavin and Sulfuric Acid: Formation and Characterization of Riboflavin Polysulfates

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When riboflavin was dissolved in concd. H_2SO_4, it was quantitatively transformed into sulfated riboflavins without any polymerization or degradation. The sulfated riboflavins were separated by ion-exchange chromatography into four components which were identified to riboflavin mono-, di-, tri-, and tetrasulfates, respectively. The results of periodate oxidation revealed that the riboflavin mono- and disulfates were rather complex in their compositions.

The influence of reaction conditions on the sulfation of riboflavin was examined. It was found that both the temperature and time of reaction did not so much affect the sulfation, but the temperature of ether used for separating products from concd. H_2SO_4 did decisively.

In 1819, Braconnot reported the dissolution of cellulose "linen" into concd. sulfuric acid,[2] and later Carolles and Fehling made it clear that the products formed in the concd. sulfuric acid were highly degraded cellulose sulfates.[3-5] Similar chemical changes of starch in concd. sulfuric acid were reported by several investigators.[5-9] Recently, the transformation of polysaccharides in cold concd. sulfuric acid in relation to reaction conditions has been examined in detail.[10] On the other hand, it was found by the present authors that when glucose is dissolved in cold concd. sulfuric acid all the glucose was transformed into sulfated glucose monomer and oligomers.[11]

The present paper describes an examination concerning the reaction between riboflavin and concd. sulfuric acid, and the properties of reaction products.

Experimental

Materials——Riboflavin and other reagents were all special reagent grade. Concentrated sulfuric acid used throughout this investigation was a special reagent grade containing 96% of sulfuric acid by weight. Organic solvents were dried and redistilled for use. Triethylamine·SO_3 complex was prepared from SO_3 and the amine.[12] Type 20/32 of Visking tube (Visking Co. Ltd., U.S.A.) was used for dialysis.

Paper Electrophoresis——Paper electrophoreses for qualitative analysis were carried out on Toyo Roshi No. 51 filter paper (25×7—11 cm) with pyridinium acetate solution, pH 5.8 (pyridine–acetic acid–butanol–water=5:1:5:250 v/v), and subjected to a potential of 21 V/cm for 30 min. Samples were spotted on line

1) Location: *Shirokane 5-9-1, Minato-ku, Tokyo.*
2) H. Braconnot, *Ann. Chim. Phys.*, [2], **12**, 185 (1819).
3) B. de Carolles, *Ann.*, **52**, 412 (1844).
4) H. Fehling, *Ann.*, **53**, 135 (1845).
5) R. Marchand, *J. Prak. Chem.*, **35**, 199 (1845).
6) C. Blondeau, *Rev. Sci. Ind.* (Paris), **15**, 69 (1843).
7) B. de Carolles, *Rev. Sci. Ind.* (Paris), **15**, 83 (1843).
8) M. Hoening and S. Shubert, *Monatsh.*, **6**, 708 (1885).
9) J. von Kalinowsky, *J. Prakt. Chem.*, **35**, 193 (1845).
10) K. Nagasawa, Y. Tohira, Y. Inoue and N. Tanoura, *Carbohydrate Res.*, **18**, 95 (1971).
11) K. Nagasawa, Y. Inoue, N. Tanoura and S. Shinkai, The 89th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April 1969.
12) J. A. Moede and C. Curran, *J. Am. Chem. Soc.*, **71**, 852 (1949).

positioned at 4.5 cm near to the cathodic site from the center of the filter paper. The riboflavin derivatives were detected with ultraviolet (UV) light as yellow fluorescent spots.

For quantitative analysis, samples were streaked along the line positioned at 4.5 cm near to the cathodic site from the center of the filter paper (25 × 9 cm). The paper was subjected to a potential of 21 V/cm for 60 min with 0.05M ammonium acetate solution (pH 6.9). Each paper zone resolved was cut off and extracted with 0.1M phosphate buffer (pH 7.0) for 2 hr in the dark. Absorbance of the extract was measured at 450 mμ.

DEAE-Cellulose Column Chromatography——DEAE-cellulose was washed with 0.5N NaOH and water, successively. The washed DEAE-cellulose was packed into a glass column (1.5 × 20 cm) to form a column bed as indicated in the text. A sample solution (1—3 mg of sample/ml of water) decationized with Dowex 50W (H^+) resin was applied on the top of the column. Elution was stepwisely performed with 0.005→0.3M pyridinium formate (pH 4.4) at a flow rate 1.25 ml/min, and each 15 ml fraction was collected. The concentrations of riboflavin and its sulfated products in the fractions were determined with the absorbance at 450 mμ.

Procedure for the Reaction between Riboflavin and Concd. H_2SO_4 (Exp. 5 in Table I)——Finely powdered riboflavin was dried over P_2O_5 at 80° *in vacuo* for 2 hr. The dried riboflavin (1 g) was added to 7 ml of concd. H_2SO_4 at 0° within 5 min to form a homogeneous solution, which was kept at the same temperature for 30 min under stirring. To separate products, the reaction mixture was gradually poured into 100 ml of ether, keeping the mixture at 20° under vigorous stirring. The precipitate formed was collected on a glass filter, washed several times with ether, and dissolved immediately in 30 ml of ice–water, neutralized with powdery $BaCO_3$ and saturated $Ba(OH)_2$ solution, successively. After removal of the precipitate, the supernatant obtained was concentrated to *ca.* 5 ml *in vacuo* and centrifuged to remove some impurities. The supernatant was poured into 80 ml of EtOH and the product precipitated was collected, washed with EtOH and ether, successively. The product was dried over P_2O_5 at 80° for 2 hr and analyzed for its composition by the quantitative paper electrophoresis. Yield, 1.40 g.

Sulfation of Riboflavin with $(C_2H_5)_3N \cdot SO_3$ Complex (Exp. 10 in Table I)——The powdered dried riboflavin (1.13 g, 3 mmoles) was dissolved in 60 ml of dimethyl sulfoxide at 85° and added with $(C_2H_5)_3N \cdot SO_3$ complex (0.57 g, 3 mmoles) with stirring. Two additional $(C_2H_5)_3N \cdot SO_3$ (0.57 g each) were added to the reaction mixture 30 and 60 min after the start. After reacting for 2 hr, the reaction mixture was evaporated at 72°, 8 mmHg and the residue obtained was dissolved in 20 ml of water, mixed with saturated $Ba(OH)_2$ solution (9 mmles). The precipitate formed was supernatant was evaporated to *ca.* 10 ml *in vacuo* to remove triethylamine. After centrifugation, the supernatant was poured into 100 ml of EtOH, then added with 30 ml of ether. The precipitate formed was collected, washed with EtOH and ether, successively, and dried over P_2O_5 at 80° for 2 hr. The product was analyzed for its composition by the quantitative paper electrophoresis. Yield, 1.08 g.

Isolation of Sodium Salts of Riboflavin Monosulfate (FMS) and Isomeric Riboflavin Disulfates (FDS-I and II)——The sample of Exp. 9 (Ba salt, 150 mg) was chromatographed on a DEAE-cellulose column (3 × 12 cm) with the same elution systems as shown in Fig. 3 and 4. The fractions of FMS, FDS-I, and FDS-II were pooled separately and each of them was repeatedly evaporated to dryness with addition of water below 40° in the dark. The residue freed from pyridinium formate was dissolved in 5 ml of water and its pH was adjusted to 9.5 with 1N NaOH. To the solution, 10 times volume of EtOH and 5 times volume of ether were successively added to precipitate sodium salt of the riboflavin sulfate separated. The yellow precipitate formed was collected, washed with EtOH and ether, and dried over P_2O_5 *in vacuo* at room temperature. Yields: FMS (Na) 40 mg; FDS-I (Na) 20 mg; FDS-II (Na) 25 mg.

Determination of Molar Ratio of Sulfate to Riboflavin in Sulfated Riboflavins——The sodium salt of a sulfated riboflavin (2—4 mg) was weighed and dissolved in 1 ml of water. An aliquot (0.1 ml) of this solution was diluted with 0.1M phosphate buffer (pH 7.0) to an appropriate volume and the absorbance of the solution diluted was measured at 450 mμ. The molar amount of riboflavin moiety in a sulfated riboflavin was determined on the basis of the molar absorbance of riboflavin at 450 mμ, 11.5×10^3.

To another aliquot (0.5 ml) of the test solution, 0.5 ml of 2N HCl was added and the solution was heated in a sealed tube at 110° for 5 hr. The amount of inorganic sulfate liberated was determined by a modified procedure of Dodgson's turbidimetric method.[13]

Periodate Oxidation of Sulfated Riboflavins——Into a graduated flask (10 ml), the sodium salt of a sulfated riboflavin (10 μmoles) was weighed. Water (5 ml), 0.05M $NaIO_4$ (2 ml), and an additional volume of water were successively added to the flask up to 10 ml volume. The solution was kept at 15° in the dark, and an aliquot (1 ml) of the solution was removed at intervals and assayed on IO_4-consumption by Fleury–Lange's method.[14]

Examination of Dialyzability of Sulfated Products of Riboflavin——The samples of Exp. 9 and 10 (5.0 mg each) were dissolved in 5 ml of water and dialyzed against 40 ml of water, separately, at room temperature.

13) In this experiment, turbidity was measured at 550 mμ in place of 500 mμ used in the original method which is K. S. Dodgson, *Biochem. J.*, **78**, 312 (1961).

14) P. F. Fleury and J. Lange, *J. Pharm. Chim.*, **17**, 107 (1933).

At intervals as indicated in Fig. 6, the dialyzed portions outside cellophane tube were removed and replaced with 40 ml each of water. Each of the dialyzed portions was corrected its volume to 40 ml every time and the absorbance was measured at 450 mμ, respectively. After dialyzing for 116 hr, the cumulated absorbances of the dialyzed portions of both the samples were 2.57 (recovery, 93.4%) and 2.50 (recovery, 92.5%), respectively.

Result and Discussion

When dried and powdered riboflavin is added to concd. H_2SO_4 kept at a constant temperature under stirring, the mixture becomes a homogeneous solution within 5—15 min. After keeping at an aimed temperature during the reaction, the reaction mixture is gradually poured into excess of ether to precipitate product. The precipitate separated is dissolved in water and neutralized with $Ba(OH)_2$. As described above, the product obtained by the reaction at 0° for 30 min followed by precipitation with ether at 20° were resolved on paper electrophoresis into four yellow fluorescent spots which are +1.90, +3.05, +3.80 and +4.25 in their relative mobilities to riboflavin, respectively (Exp. 5 in Fig. 1 and Table I).

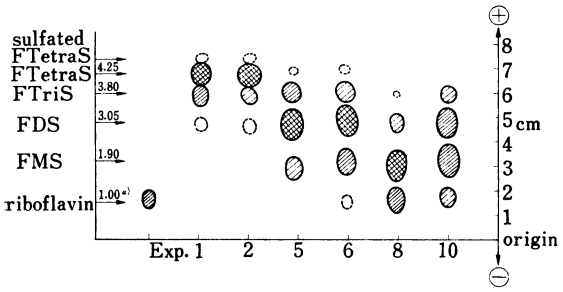

Fig. 1. Paper Electrophoretic Pattern of Sulfated Products of Riboflavin obtained by Different Reaction Conditions

Exp. 2,6,8 and 10 correspond to those shown in Table I. Paper electrophoresis was carried out according to the procedure for qualitative analysis (see Experimental).
a) The values indicated signify relative mobilities of sulfated riboflavins to riboflavin.

To obtain riboflavin sulfate esters, riboflavin was reacted in DMSO with $(C_2H_5)_3N \cdot SO_3$ complex which has been known as a general and mild sulfating reagent[15] (Exp. 10 in Fig. 1 and Table I). Paper electrophoresis of the product obtained revealed the formation of three sulfated riboflavins (+1.90, +3.05 and +3.80) which coincide with those of the product obtained by concd. H_2SO_4 method (Exp. 5). It was also observed that the spots of +1.90 and authentic riboflavin 5'-sulfate[16] overlapped each other. The product of Exp. 5 freed from inorganic sulfate was hydrolyzed in 1N HCl at 110° for 5 hr, and paper electrophoresis of the hydrolysate showed formation of the spots of riboflavin and inorganic sulfate with the disappearance of the spots of +1.90, +3.05, +3.80 and +4.25. The absorption spectrum of the same product represented λ maxima at 225, 270, 375 and 445 mμ which coincide with those of riboflavin. These preliminary results described above suggested that the product obtained by concd. H_2SO_4 method had to be a mixture of riboflavins esterified by sulfate groups at their ribitol moieties.

To separate and characterize each component of the sulfated riboflavins obtained by concd. H_2SO_4 method, a several chromatographical procedures were tested. The cellulose powder column[17] using benzylalcohol–EtOH–water (3:1:1, v/v) was not an answer to the present purpose. The DEAE-cellulose column chromatography with 0.01→0.3M LiCl in 0.01N HCl was an effective one, but it was unsuitable for a preparative purpose. It was found that the DEAE-cellulose column chromatography with 0.05→0.3M pyridinium formate (pH 4.4) was suitable for the separation in both analytical and preparative scales.

As can be seen from Fig. 2, the sample applied on this column was resolved into its four sulfated constituents giving each single spot of +1.90, +3.05, +3.80 and +4.25 on paper

15) E. E. Gilbert, *Chem. Rev.*, **62**, 553 (1962).
16) K. Nagasawa and H. Yoshidome, *Chem. Pharm. Bull.* (Tokyo), **18**, 2023 (1970).
17) R. Takahashi, K. Yagi and F. Egami, *Nippon Kagaku Zasshi*, **78**, 1287 (1957).

TABLE I. Influence of Reaction Conditions on the Sulfation of Riboflavin with Concd. H_2SO_4

Exp. no.	Reaction conditions: Temp. (°C)	Time (hr)	Temp. of ether(°C)	Yield and composition of sulfated riboflavins (%): Unreacted riboflavin	FMS	FDS	FTriS	FTetraS	Sulfated FTetraS	Yield[a] (g)
1[b]	0	0	0	0	0	2.9	32.5	63.7	0.9	2.11
2	0	0	−40	0	0	1.5	17.1	80.4	1.0	2.10
3	0	2	0	0	0	2.9	34.0	66.2	0.5	2.16
4	0	2	−40	0	0	1.7	23.3	75.4	0.6	2.35
5[c]	0	0.5	20	0	8.8	53.9	35.5	1.9	0	1.40
6	0	0.5	34.5	1.6	31.9	48.7	17.2	0.6	0	1.06
7	30	0.5	20	0.9	8.6	35.8	45.1	4.5	0	2.02
8[d]	30	0.5	40	30.2	59.4	10.0	0.4	0	0	1.16
9[e]	0→40	0.5	0	6.9	43.0	41.7	8.4	0	0	1.05
10[f]	85	2	—	7.3	44.6	39.6	8.5	0	0	1.08

a) The yield of each experiment is given in the amount of the product obtained from 1g of riboflavin.
b) Exp. 1—4 consumed 20 min for dissolving riboflavin into concd. H_2SO_4. Both the reactions of Exp. 1 and 2 were stopped by mixing with excess ether after all the riboflavin was dissolved.
c) Exp. 5—9 consumed 5 min for dissolving riboflavin into concd. H_2SO_4.
d) In the case of Exp. 8, excess ether was poured into the reaction mixture keeping the temperature indicated.
e) In the case of Exp. 9, riboflavin was dissolved into concd. H_2SO_4 at 0°, and the reaction temperature was elevated and kept at 40° thereafter.
f) This experiment was carried out for a comparison between the products obtained by different sulfating procedures. Details of the reaction were described in experimental.

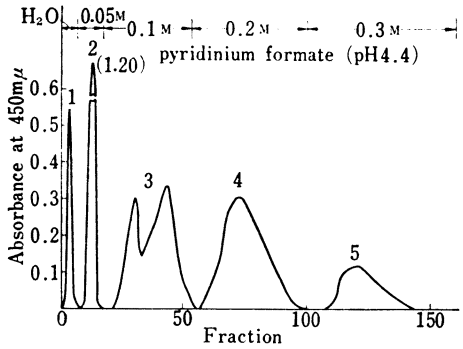

Fig. 2. Separation of Riboflavin and Its Sulfated Products by DEAE-Cellulose Column Chromatography

peak 1=riboflavin; peak 2=riboflavin monosulfate (FMS); peak3=riboflavin disulfate (FDS); peak 4=riboflavin trisulfate (FTriS); peak 5=riboflavin tetrasulfate (FTetraS)
The sample (15 mg) which is a mixture consisted of each product of Exp. 4, 5 and 8 in a ratio 1:2:2, respectively. column bed: 1.5×10 cm The value in parentheses indicates the absorbance of peak 2.

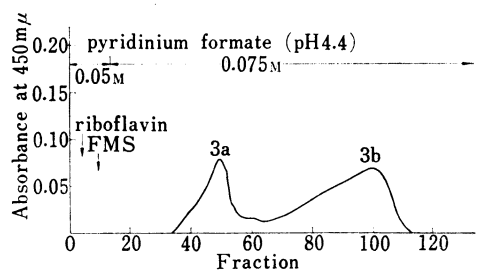

Fig. 3. Separation of Isomeric Riboflavin Disulfates by DEAE-Cellulose Column Chromatography

peaks 3a and b=isomeric riboflavin disulfates (FDS-I and II)
A sample (6.0 mg) of Exp. 9 was chromatographed on a column (bed size: 1.5×14 cm).

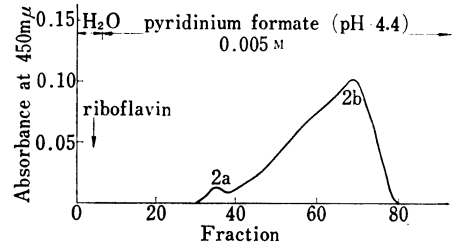

Fig. 4. Separation of Isomeric Riboflavin Monosulfates by DEAE-Cellulose Column Chromatography

peaks 2a and b=isomeric riboflavin monosulfates
A sample (4.0mg) of Exp. 9 was chromatographed on a column (bed size: 1.5×14 cm).

electrophoresis, respectively. Rechromatography with 0.075M pyridinium formate resolved the fraction 3 (Fig. 2) into the fractions 3a and b (Fig. 3) giving the same mobility, +3.05, on paper electrophoresis. The fraction 2 in Fig. 2 was also resolved into the fractions 2a and b as shown in Fig. 4 by eluting with 0.005M pyridinium formate.

To determine a molar ratio of sulfate to riboflavin, each of the fractions separated was hydrolyzed in 1N HCl at 110° for 5 hr and analyzed on inorganic sulfate and riboflavin. As shown in Table II, it was proved that each of the fractions 2—5 in Fig. 2 corresponds to riboflavin mono-, di-, tri-, and tetrasulfate, and each of the fractions 3a and b in Fig. 3 to an isomeric riboflavin disulfate, FDS-I and II, respectively. The spectral data given in Table II indicated that these sulfated riboflavins are esterified by sulfate group(s) at their ribitol moieties. Although no examination about the trace constituent termed "Sulfated FTetraS" was carried out in detail, it was assumed to be a pentasulfated riboflavin substituted in its isoalloxathin ring by the fifth sulfate group from its hydrolytic behaviour.

TABLE II. Composition, Electrophoretic Mobility and Absorption Maxima of Riboflavin Sulfates Separated by DEAE-Cellulose Column Chromatography

Sulfated riboflavins	Ratio of sulfate to riboflavin	Paper electrophoresis	Absorption λ_{max}(mμ)			
FMS	0.95	+1.95[a)]	223	267	374	445
FDS-I	1.99	+3.05	223	267	375	446
FDS-II	1.93	+3.05	223	267	375	446
FTriS	3.02	+3.80	223	267.5	375	447
FTetraS	4.13	+4.25	223	267.5	376	447

EMS=riboflavin monosulfate corresponding to peak 2b in Fig. 4
EDS-I and II=isomeric riboflavin disulfates corresponding to peaks 3a and b in Fig. 3
FTriS=riboflavin trisulfate corresponding to peak 4 in Fig. 2
FTetraS=riboflavin tetrasulfate corresponding to peak 5 in Fig. 2
a) The values indicated signify relative mobilities of sulfated riboflavins to riboflavin and a sign + signifies that these compounds migrate to anodic site.

Purified sodium salt of FMS, FDS-I, and FDS-II were prepared and oxidized with periodate, and their oxidized products were examined on paper electrophoresis. As can be seen in Fig. 5, 1 mole of FMS consumed 1.50 mole of periodate to afford a fluorescent fragment with no sulfate group together with a minute amount of a fluorescent fragment with a sulfate

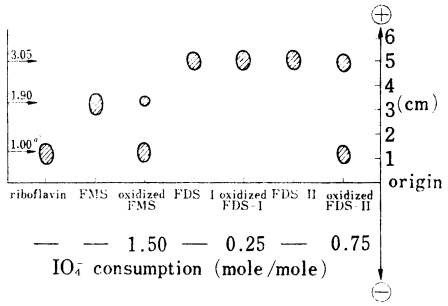

Fig. 5. Paper Electrophoretic Pattern of Riboflavin Sulfates and Their Periodate Oxidation Products

Paper electrophoresis was carried out according to the procedure for qualitative analysis (see Experimental). All the spots in this figure are yellow fluorescent in transmitted UV light.
a) The values indicated signify the relative mobilities of riboflavin sulfates and their oxidation products.

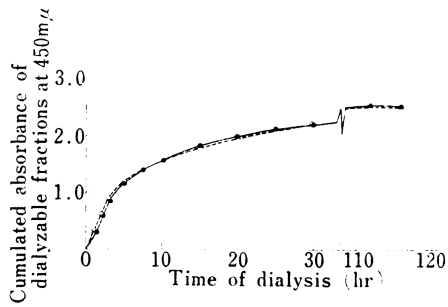

Fig. 6. Comparison between Dialyzabilities of Sulfated Products of Riboflavin obtained by Two Different Reactions

—●—: a sample (5.0 mg) of Exp. 9 (concd. H_2SO_4 method)
—○—: a sample (5.0mg) of Exp. 10(triethylamine-SO_3 method)

group. This result indicates that FMS is an isomeric mixture composed mainly of riboflavin 4'- and 5'-sulfate admixed with a small amount of riboflavin 2'- and/or 3'-sulfate. Judging from the data of oxidized FDS-I and II, it was deduced that FDS-I consisted of 25% of riboflavin 2',3'-disulfate which can consume 1 mole of periodate/mole of FDS, with not yielding any neutral fluorescent fragment, and 75% of the isomeric FDS such as 2',4'-, 3',4'- and 3',5'-disulfate which are resistant to periodate oxidation; FDS-II consists of 75% of the isomeric FDS such as 2',3'- and 4', 5'-disulfate which are able to consume 1 mole of periodate/mole of FDS with no formation of monosulfated fluorescent substance and 25% of the isomeric FDS such as 2',4'- 3',4'- and 3',5'-disulfate. The results of periodate oxidation described above indicated that FMS and FDS formed by this reaction were fairly complex in their compositions.

As reported in the previous work,[11] we have observed an occurrence of concomitant polymerization on the sulfation of D-glucose with concd. H_2SO_4. Accordingly, it is necessary to clarify whether polymerization of riboflavin occurs or not during the sulfation with concd. H_2SO_4. A comparison was made between the rates of dialyzability of two preparations, Exp. 9 and 10 in Table I which were obtained by concd. H_2SO_4 method and $(C_2H_5)_3N \cdot SO_3$ method,[18] respectively, and very close to each other in their compositions. It can be seen from Fig. 6 that the rate of dialyzability of both preparations are nearly equal, and the recovery of their dialyzable fractions are 93.4% for Exp. 9 and 92.5% for Exp. 10, 116 hr after the start, respectively. This result indicated that no polymerization of riboflavin occurred while being reacted with concd. H_2SO_4.

The influence of reaction conditions on the sulfation of riboflavin was examined and the results obtained were summarized in Table I. The results of Exp. 1 and 2 showed that an intensive sulfation occurred at the time of dissolution of riboflavin into concd. H_2SO_4, and elongation of the reaction time did not influence the sulfation at all (Exp. 1 and 3, 2 and 4). The results of Exp. 5 and 7 also indicated that the sulfation was affected positively with the reaction temperature, but the extent of it was not so decisive. As reported previously,[19] one of the authors found that on the reaction of chondroitin sulfate with concd. H_2SO_4, the temperature of ether used for separating products from the excess concd. H_2SO_4 influenced decisively on the degree of sulfation of products. It was observed in the present experiments that the product of Exp. 2 precipitated by ether at $-40°$ was composed of FTetraS (80.4%) and the other three sulfates (19.6%), but the product of Exp. 6 precipitated at $+34.5°$ was composed of FTetraS (0.6%), riboflavin (1.6%), and the other three sulfates (97.8%). This results, together with those of Exp. 1,5,7, and 8, revealed that the temperature of ether (used for separating products) exerted the same effect on the sulfation of riboflavin with that observed in the previous work.

Acknowledgment The authors are indebted to Mrs. A. Yuki for her technical assistance.

18) It has been known that the reagent, $(C_2H_5)_3N \cdot SO_3$, causes no polymerization during the sulfation with it.
19) K. Nagasawa, Y. Inoue and S. Wakabayashi, The 40th Annual Meeting of Biochemical Society of Japan, Osaka, April 1967; K. Nagasawa and Y. Inoue *Carbohydrate Res.*, in press.