THE SOLUBILITY INCREASING SUBSTANCES AND THE MECHANISM OF SOLUBILIZING ACTION OF RIBOFLAVIN

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Riboflavin is little soluble by its nature, so there is need to increase the solubility for various purposes. For this purpose riboflavin is turned to a water soluble derivative or some solubility increasing substances are added. The latter method has been utilized most widely as it increases the solubility without harming the biochemical action of riboflavin. A number of solubility increasing agents have been proposed for this purpose. However, it is little known by what mechanism these agents increase the solubility of riboflavin.

My work involves studies of the effects of various substances including well-known ones, on the solubility and other physicochemical properties of riboflavin, and I obtained several findings regarding the mechanism of solubilizing action of solubility increasing substances.

MATERIALS

Riboflavin used for the experiment were 97.5–98.5% pure with a melting point of 280°< and made riboflavin spot only utilizing paper partition chromatography (PPC).

Lumiflavin used here was purified photolysis product of riboflavin, riboflavin-5′-phosphate (FMN-Na) was Roche's; riboflavin-4′, 5′-diphosphate[1], riboflavin-trisulfate,[2] riboflavin-borax,[3] and monomethylol-riboflavin[4] were synthesized respectively by the methods indicated in the reference and comfirmed further to be free from any impurity utilizing PPC.

"Chemically pure" materials were used for solubility increasing substances.

METHODS

Determination of solubility: Riboflavin pulverized in an agatemortar was placed in a shaking machine for 10 hours at room temperature together with the solvent, collected from the solution by filtration, and then determined quantitatively riboflavin content.

Determination of polarity: 2 ccm of 0.1 N alcoholic sodium hydroxide solution was added to 25 mg of riboflavin and sodium salt of solubility increasing substance. So as to make the whole quantity 100 ccm, water free from carbon dioxide was added. The measurement was carried out within 30 minutes after pouring the solution thus prepared, in a tube of 10 cm long. The tube was covered by black paper to avoid the effect of light.

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PPC: As for developer, *n*-butanol (*n*-BuOH), acetic acid (HAc) and water (H_2O) were mixed in a proportion of 4 : 1 : 5, and Toyo's filter paper No. 131 was used. The room temperature was 20–23° C.

EXPERIMENTAL

I. Relation between Chemical Structure and Solubilizing Action of Solubility Increasing Substances

TABLE 1. Solubility Increasing Action of Substances Having —OH, —NH_2, —COOH, —SO_3H Groups against Riboflavin

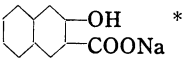
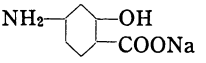
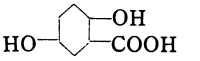
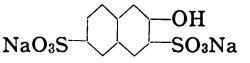
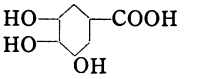
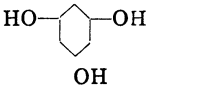
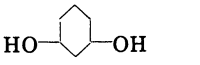
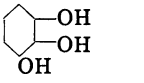
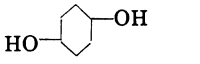
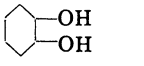
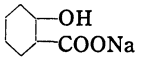
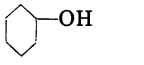
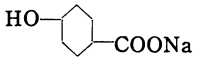
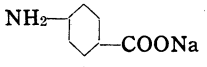
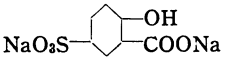
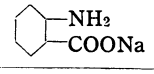
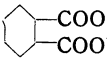
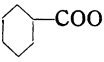
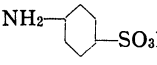
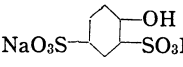
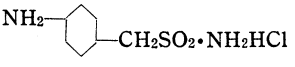
	Solubility increasing substance		Solubility of riboflavin (mg/ccm)	Rf	Fluovescence of riboflavin solution
1		* β-hydroxynaphthoic acid	40≦	0.33(B_2)	−
2		* β-aminosalicylic acid	11	〃	−
3		gentisic acid	6.3	〃	−
4		2-hydroxy-naphthaline-3,6-disulfonic acid	5.7	〃	−
5		gallic acid	4.5	〃	−
6		resorcinol	4.0	〃	−
7		phloroglucinol	3.4	〃	−
8		pyrogallol	3.2	〃	−
9		hydroquinone	3.1	〃	−
10		pyrocatechol	2.7	〃	−
11		salicylic acid	2.3	〃	−
12		* phenol	1.7	〃	−
13		* *p*-hydroxybenzoic acid	1.3	〃	−
14		* *p*-aminobenzoic acid	1.1	〃	+
15		* sulfosalicylic acid	1.0	〃	−
16		* anthranilic acid	1.0	〃	+

TABLE 1. (*Continued*)

	Solubility increasing substance	Solubility of riboflavin (mg/ccm)	Rf	Fluovescence of B_2 solution
17	 * phthalic acid	0.8	0.33(B_2)	+
18	 * benzoic acid	0.8	〃	+
19	 * *o*-ethoxybenzoic acid	0.6	〃	+
20	 * sulfanilic acid	0.5	〃	+
21	 * phenol-2,4-disulfonic acid	0.3	〃	−
22	 paramenyl	0.3	〃	+
23	Control	0.2	〃	+

* Used as its salt.

Table 1 shows solubility increasing abilities of substances having phenolic hydroxyl group (OH), amino group (NH_2), carboxyl group (COOH), and sulfonic group (SO_3H) against riboflavin.

As the table shows, OH and NH_2 are the groups which directly help to increase solubility of riboflavin. It is apt to increase generally the solubilizing action as the number of these groups increases. It is recognized that OH group is more active than NH_2 group in solubilizing action. From the fact that *p*-hydroxybenzoic acid and salicylic acid are more active than *p*-aminobenzoic acid and anthranilic acid respectively, OH group loses its activity when it turns to ether. COOH group (or its salt) is not a direct functional group because there is no difference in effect between benzoic acid and phthalic acid. We can judge that substances increase solubilizing action when COOH group is introduced from the fact that salicylic acid, gentisic acid and gallic acid show more effective solubilizing action phenol, hydroquinone and pyrogallol respectively. On the contrary sulfanilic acid, sulfosallcylic acid, and phenol-2, 4-disulfonic acid have less activity in solubilizing action than their respective corresponding carboxylic acid as salicylic acid and *p*-aminosalicylic acid; this fact shows that SO_2H group (or its salts) acts inhibitively. The solubilizing actions of substances are closely related to the positions of groups in them, that is to say, *p*-hydroxybenzoic acid effects less than phenol does, and COOH group at ortho-position increases the action of OH group without exception. As β-hydroxynaphthoic acid and 2-hydroxynaphthaline-3, 6-disulfonic acid have more active solubilizing action than the respective corresponding benzene derivatives salicylic acid and phenol-2, 4-disulfonic acid, it is presumed that the nucleus influences greatly the action. It became clear that substances having OH group quench the fluorescence of riboflavin and none of Rf spot but riboflavin spot was obtained by means of PPC.

II. Bonding between Solubility Increasing Substance and Riboflavin

If there arises some bonding between solubility increasing substance and riboflavin, abnormality in freezing point depression of the solution should be shown. FMN-Na, and Table 2 gives these results. From this table we may presume that salts of β-hydroxynaphthoic acid or salicylic acid and resorcinol make bonding between riboflavin and FMN-Na in the solutions, and nicotinic acid amide and salt of benzoic acid makes no bonding.

TABLE 2. Depression Grade of Freezing Point (Δt° C) of Solubility Increasing Substances under Co-Existence with Riboflavin of FMN-Na Solutions

Δt° C→ Solvent ↓		Solvent (I)	Solvent+B_2 (II)	II−I	Solvent+FMN-Na (III)	III−I
	5%	0.678	B_2 20 mg/ccm 0.607	−0.071	0.678	−0.004
	10%	1.958	5 mg/ccm 1.945	−0.013	2.008	+0.050
	20%	1.397	20 mg/ccm 1.324	−0.073	1.477	+0.080
	20%	1.555	4 mg/ccm 1.555	0.000	1.806	+0.256
	20%	2.220	4 mg/ccm 2.266	+0.046	2.413	+0.193
H_2O		0.000	10 mg/ccm 0.050*	+0.050	0.220	+0.220

*: Figure got by computation.

III. Bonding between Solubility Increasing Substance and Ribityl Group of Riboflavin

Table 3 gives optical rotation of water-soluble derivatives of riboflavin. We can recognize from this table that the bonding at ribityl group's position of riboflavin changes remarkably its optical rotation and Rf values of those substances are smaller than that of riboflavin.

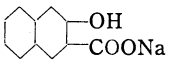
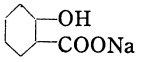
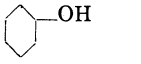
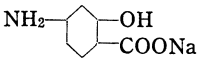
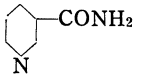
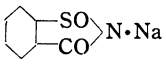
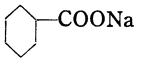
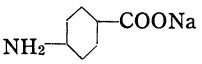
TABLE 3. Optical Rotation and Rf Value of Riboflavin Derivatives

B_2 derivatives	$[\alpha]_D^{15.5°}$	Rf
Monomelylol-B_2	+ 69°	0.20
B_2-Trisulfate (impure)	—	0.02, 0.04, 0.08, 0.116, 0.23
B_2-4′, 5′-diphosphate	—	0.12
B_2-5′-phosphate	− 28°	0.11
B_2-borax	+360°	0.33
Control (B_2)	−134°	0.33

Table 4 gives the optical rotation of riboflavin solution co-existing with solubility increasing substance. It can be recognized clearly from this table that co-existance of solubility increasing substance in riboflavin solution changes optical rotation of riboflavin solution, and this is not the effect of its salt, because riboflavin solution with sodium chloride have no change of rotation. But

this degree of changing is not so remarkable except for β-hydroxynaphthoic acid, and it is not judged that the bonding at the position of ribityl group is responsible for the main mechanism of solubilizing action.

TABLE 4. Effect on the Optical Rotation by Solubility Increasing Substane of Riboflavin

Solubility increase-substance of B_2	Added amount of solubility increasing substance to 0.25% of B_2, 0.02% of N-NaOH, and 18% of ethanol	$[\alpha]_D^{16.5°}$
	0.266 M/l	− 15°
	0.362	− 66°
	0.530	− 65°
	0.330	− 70°
	0.410	− 88°
	0.244	− 94°
	0.410	− 96°
	0.365	−112°
NaCl	0.50	−108°
〃	0.10	−127°
Control	—	−132°

IV. Bonding of Solubility Increasing Substance with Isoalloxazine Nucleus

β-hydroxynaphthoic acid (salt) and nicotinic acid amide well solubilize both riboflavin and lumiflavin as shown in Table 5. This fact shows that increasing of solubility effects mainly not the ribityl group but the isoalloxazine nucleus. The insolubility of 3-methyl-lumiflavin is caused by the effect of imino group at

TABLE 5. Solubilizing Action of Solubility Increasing Substance on Riboflavin, Lumiflavin, and 3-Methyllumiflavin

Solubility increasing substance		B_2		Lf		Methyl-Lf	
		Solubility	Ratio	Solubility	Ratio	Solubility	Ratio
	1%	10.7 mg/ccm	54	5.5 mg/ccm	21	0.70 mg/ccm	7.0
	5%	52.0 〃	251	18.7 〃	72	—	—
	5%	0.92 〃	4.6	1.25 〃	4.8	0.44 〃	4.4
Control (H_2O)		0.20 〃	1.0	0.26 〃	1.0	1.10 〃	1.0

the 3-position. In case of β-hydroxynaphthoic acid, at least, the bondings mainly of imino group at the 3-position and partly of carbonyl group at the 2- and 4-positions increase the solubility of riboflavin; and these are the main mechanisms of the solubilizing action of substances having OH group.

V. Effect of Solubility Increasing Substance on Absorption Spectrum of Riboflavin

Among the many substances which increase the solubility of riboflavin such as β-hydroxynapthoic acid and *p*-amino-salicylic acid, none alters the color of riboflavin solution to an orange-red. This phenomena almost vanished at the low concentration of the solution in general but β-hydroxynaphthoic acid causes changes in the absorption spectrum of riboflavin solution at low concentrations in a peculiar way.

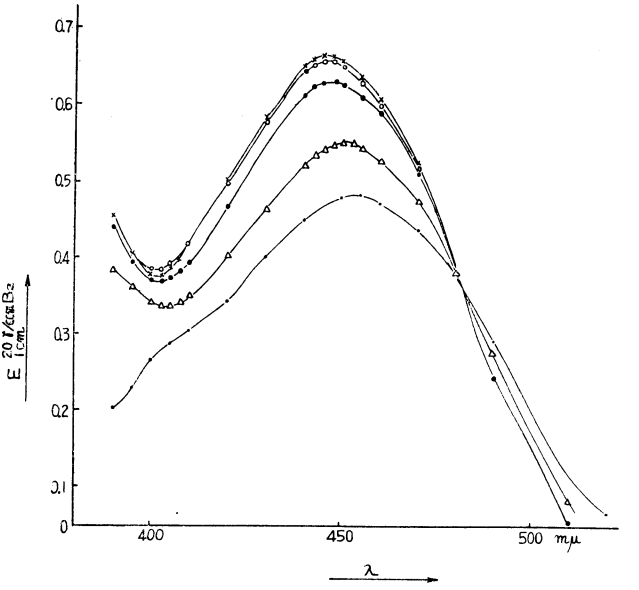


FIG. 1. Absorption spectrum of riboflavin solution at co-existence with solubility increasing substance

× —— ×	B_2, B_2 + Nicotinic acid amide	(1 : 100 M)
	B_2 + Salicylic acid	(1 : 100 M)
○ —— ○	B_2 + *p*-aminosalicylic acid	(1 : 100 M)
● —— ●	B_2 + hydroxynaphthoic acid	(1 : 10 M)
△ —— △	〃 + 〃	(1 : 100 M)
• —— •	〃 + 〃	(1 : 1 000 M)

DISCUSSION AND CONCLUSION

As for solubilizing agents of riboflavin, the following have been proposed; they are benzoic acid and its salts of hydroxy- and sulfimid-derivatives;[5-14] salt and acid amide of pyridine carboxylic acid;[15,16] 2,4-dimethoxy-benzyl alcohol;[17] pyridylcarbinol;[18] vanillin;[19] tryptophan;[20] acetyltryptophan;[21] and salt of β

hydroxynaphthoic acid[22] etc. I also noticed that water-soluble salts of hippuric acid, phenyl acetic acid, cinnamic acid and these hydroxy- and sulfon-derivatives solubulize riboflavin well; on the contrary, salts of almost all aliphatic carboxylic acid solubilize it scarcely. It is known too, that mono- and poly-valent alcohols and amino acid of most alphatic compounds have no solubility-increasing ability.

It is thus seen that in general almost all substances of solubility increasing ability are water-soluble cyclic compounds having one or more OH, NH_2, and COOH groups; and that many aliphatic compounds are not solubility increasing.

From the structures of solubility increasing substances, OH group and NH_2 group are conceived to be the functional groups for solubilizing action; the more of these groups the substance has, the greater the tendency to solubilizing action; where OH group effects more actively than NH_2 group does on it. COOH group increases the action of the functional groups, while SO_3H group quenches it. The position of the group and the nucleus effect greatly the solubilizing action.

It is interesting that substances having OH group exist as bonding with riboflavin and the solubility increasing substances having no OH group exist as not bonding with riboflavin in their solutions: in cases of the former, riboflavin exists in the state of hydrogen bonding with OH of solubility increasing substance which has COOH group at the terminal position, and this bonding substance is more solubilizing in water: this is taken to be a mechanism of solubility increasing; in cases of the latter, it is considered that a mechanism such as depression of surface tension is effected. It can not be judged in common by mean of the absorption spectrum and PPC, except in the case of *p*-hydroxynaphthoic acid if riboflavin combines with solubility increasing substance or not.

The bonding between solubility increasing substance and riboflavin may be judged to occur mainly not at the ribityl group but at the imino group (3-position), from the observations of changes in optical rotation, PPC and the action on riboflavin, lumiflavin, and methyllumiflavin. Moreover it might be capable of bonding with carbonyl group at 2, 4-position.

Further studies will be made on OH group regarding relation between quenching effect on the fluorescence and solubility increasing action of riboflavin.

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