THE CRYSTALLOGRAPHIC STUDY OF RIBOFLAVIN

HACHIRO SAKATE

Department of Biochemistry, Nagoya University School of Medicine
(Director: Prof. Kazuo Hotta)

Many researches and descriptions have been published in detail on the physiological significance and biological distribution of riboflavin In recent years continuous to these, new studies have been made in succession on the physical and chemical properties. In the meanwhile, publications on crystallographic researches of riboflavin itself have been very few, and many deal with the exterior shape of the crystal, and little achievment made regarding the minute observations, except for A. Watanabe[1] who described the polymorphism of riboflavin and S. Shimizu[2] who reported on the melting point and polymorphism, confirming by means of X-ray powder photograph, existence of polymorphism having different internal structures of the crystal and Means[3] who reported on the melting point for the same purpose.

Thus, there has been no study and report on the crystal system yet. The crystal of riboflavin therefore, is usually described as needle shape, plate shape, and long plate shape. Crystal habitus is able to be changed by solvent. This means that the habitus is dependent on the solvent which is used as a mother-liquid in case of recrystallization and it is easily considered that some may be crystallized as needle shape and others plate shape. The crystal habitus is generally meant to be change of the exterior shape of the crystal but not by what is formed internally. Polymorphism is meant by formation of a certain substance in an absolutely different internal structure under the different external conditions. That is to say, it makes different packing from a certain building substance unit.

In some instance, one special internal structure may be retained as it is within a limited scope of temperature. So, should the temperature be out of the limit, the building units will set on rapid reorganization and thus a different internal arrangement of atom, molecule, and ion comes into existence. This is how polymorphism is created. Therefore, polymorphism is greatly influenced by the external conditions at the time it is formed, namely, temperature and pressure. These external conditions, such as temperature, pressure, etc. in the case of crystallization, should be kept constant for the study of crystal systems as well as for the purpose of promoting purity of crystallized substances by recrystallization. When the conditions are optional, there may be a fear that the crystal system of riboflavin can not be obtained correctly due to confusion which may arise at the time of observation owing to existence or appearance of polymorphism.

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Our study of the subject problem was set to prove microscopically that crystals of the natural and synthetic riboflavin have the same sterometric structure; prove further that crystals of riboflavin and araboflavin are optically different; and thus contribute to a clarification of the physical and chemical nature of riboflavin. I prepared the substance recrystallized under the possible external conditions in our laboratory, made observations and measurement of various optical properties of the recrystallized substance, and, by determination of optical orientation, fixed the crystal system of the natural and synthetic riboflavin and araboflavin, along with observation of the shape of the crystal.

MATERIALS

I selected products of the Wakamoto Company as natural riboflavin those of Merck Company and Takeda Pharmacies Company as synthetic ones, and those of Fujisawa Pharmacies as araboflavin. Observations were made after having each recrystallized. The way of recrystallization was such that the three products as above, were dissolved by heating in distilled water to make a supersaturated solution. The solution was then put into a vacum desiccator of brown colour immediately after filtration of pure substances when still hot, and kept at a normal room temperature in a dark room for several days to make it condensed. After sufficient condensation, it was poured into a desicator of prosphorus pentoxide for perfect drying. Recrystallized riboflavin crystal thus made was observed. Temperature of the distilled water at the time of dissolution was about 80° C and the solution was filtrated after being shaken for 15 minutes. When 90% methanol and 99% ethanol were used as motherliquid, the dissolution was made with an reflux condenser. Time used for this dissolution was the same as in the case of water. (The temperature was around the boiling point.) Important thing to be noted in this procedure was to do in dark place and to cool it to the normal room temperature slowly and not speedy. Quite separately from these, riboflavin solution dissolved into each solvent was sucked up by the Komagome pipette. On a slideglass a drop of the solution was placed and was covered with a coverglass.

In this way, riboflavin crystallized around the coverglass was observed for shape.

METHOD

The shape and optical property of the crystal were observed with a biological microscope and polarizing microscope (Manifactured by E. Leitz and Leichert). As to light source, a sodium lamp was used as monochromatic light and complete misky bulb as white light. Interfacial angle was read from the turning degree of the stage of a polarizing microscope. Measurement of three principal refractive indexes of the crystal, as referred to in the following section, was according to the immersion method. As immersion fluids, cedar oil, cassia oil, α-monobromonophtnalene, and methyleneiodide (CH_2J_2) were used, which all were preferably and generally taken up for measurement of refractive index of mineral powder. These liquids were mixed in various ratios so as to have

difference in the refractive indexes of about 0.002. With several kinds thus prepared, comparative measurements were made one by one. For measurement of higher indexes, immersion liquids having higher indexes had to be used. The following mixed liquids which seemed good for the purpose of this experiment were prepared. These liquids do not dissolve crystals of riboflavin.

(a)	Sulphur	10	
	Iodoform	35	by weight mixed (Larsem Bergmann's liquid)
	SnI_4	31	ND ≒ 1,808
	SbI_4	8	(ND is refractive index against sodium light)
	AsI_3	16	
	CH_2I_2	100	
(b)	Yellow phosphorus	8	by weight mixed
	Sulphur	1	(Merwin's liquid)
	Methyleneiodide	1	ND ≒ 2.06

By mixing adequately in (a) and (b) other immersion liquids which had lower refractive indeces, liquid having any optional refractive index could be made. With preparation of as many different indices of liquor as possible from the lowest to the highest, the three principal refractive indices of riboflavin crystals were measured comparatively. The index of the immersion liquids, as far as Nd = 1.7 approximately, were read with the Abbe's refractometer. For determination of higher values of the index, special prism spectrometer was made to compute mathematically that of the immersion liquids filled in the prism by measuring of refracting angle and minimum derivation angle of the prism. It was very convenient for the reading to choose a prism where the refracting angle was around 30 degrees.

The indeces of immersion liquids as above mentioned, how correctly its indexes were measured at the first time, if they were kept for a long time, have possibilities to change in the values because of crystallization of a certain element of the mixed liquid. Therefore, care should be exercised to correct recalculation of the indexes at each time of experiment. It must be noted further that observation must be carried out promptly, because of evaporation of liquids, more or less, at the time of the measurement.

EXPERIMENTAL

1. Recrystallized Riboflavin Crystal Separated from Redistilled Water

The natural and synthetic riboflavin which have been separated from redistilled water by recrystallization, have the same characters and crystal habitus, most of them are gathered radially, as yellow coloured but transparent, and plate shaped hexahedral crystals. By observation with a microscope, they exist in general, adhered or irregularly clinged to each other at the long pinacoids, but if they exist separately, their long pinacoids, are undergrown, and rarely exist as single crystals, having a distinctly developing edge.

The crystal faces consist of (*hoo*), (*oko*), (*ool*) and (*hol*) and show low crystal evolution. The faces, which are parallel to the long axis grew well,

especially (*oko*).

All interfacial angles between the pinacoids and bases are 90° (Fig. 1 (*A*), Fig. 1 (*B*)).

Consider the crystallographic axes are "*a* axis"—from front to rear—that is parpendicular to (*hoo*), "*b* axis"—from right to left—perpendicular to (*oko*), and "*c* axis"—from top to bottom—perpendicular to (*ool*).

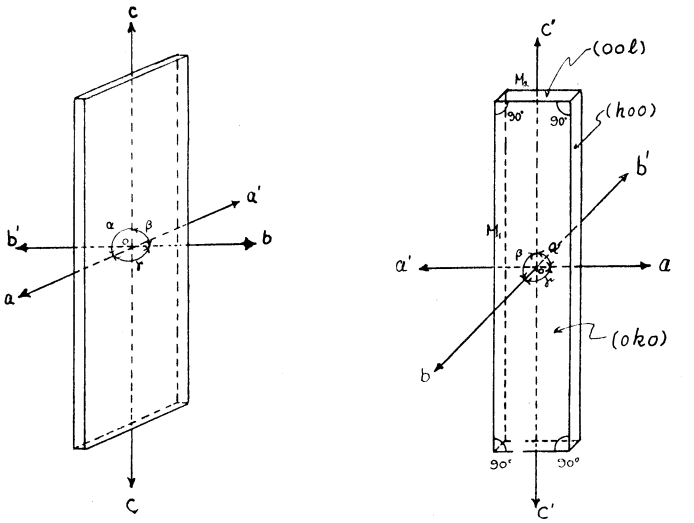


FIG. 1 (*A*)

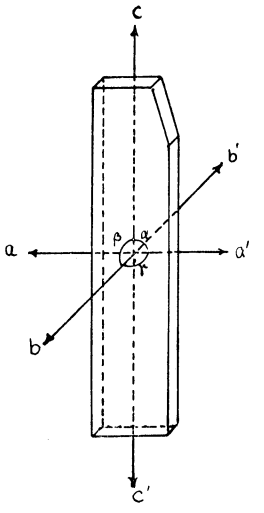


FIG. 1 (*B*)

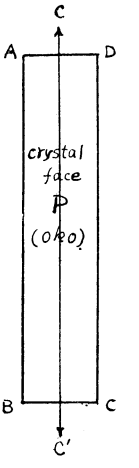


FIG. 2

Above figures are the linear projections showing general crystal habits of natural and synthetic riboflavin crystals. Araboflavin has the same crystal habits and characters as above figures.

Now we take face "*p*" for (*oko*) (in Fig. 2).

In this case $\angle A = \angle B = \angle C = \angle D = 90°$.

Each angle is an interfacial angle between (*ool*) and (*hoo*).

We have found no twin nor hemimorphy in further observations on these crystals.

Next we intend to investigate the optical crystallographic characters of natural and synthetic riboflavin crystals and araboflavin crystal by mean of a polarizing microscope.

Face (*hoo*) and (*oko*) or face "*p*" show straight extinction on the direction of the long axis by observation of a orthoscope. In this case, long axis ("*c* axis") is taken parallel to $\overrightarrow{AB}$ and $\overrightarrow{DC}$, which are the principle directions of crystal (Fig. 2), so it should be at straight extinction refering to edges *AB* and *DC*; and it is a matter of course that refering to $\overrightarrow{AD}$ and $\overrightarrow{BC}$, which are perpendicular to $\overrightarrow{AB}$ and $\overrightarrow{DC}$, their directions are at straight extinction. That is to say, "*c* axis" is at straight extinction.

Setting face "*P*" or (*hoo*) at the diagonal position by rotating the crystal to the right at 45° from the extinction position, the face "*P*" shows subtruction phenomenon when there is inserted the gypsum test plate Red 1 under illumination of a white light; then it shows additional phenomenon by rotating it to left 90° from above position (Fig. 3).

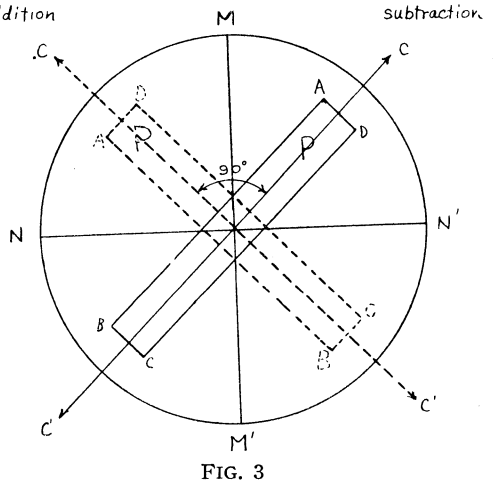


FIG. 3

The vibration direction of the faster light wave against a gypsum test plate Red 1 (X') and that of the slower one (Z') are shown in the following figure (Fig. 4), then at face "*P*", $\overrightarrow{AB}$ and $\overrightarrow{DC}$ are the vibration directions of

the faster light wave; $\overrightarrow{AD}$ and $\overrightarrow{BC}$ that of the slower (Hereafter admit X' as the vibration direction of the faster light wave and Z' as that of the slower).

Put n_1 and n_2 as the refractive index of X' and Z' respectively. As velocity of the light wave is inverse proportional to the refraction index, so $n_2 > n_1$, namely refractive index is less at the directions of $\overrightarrow{AB}$ and $\overrightarrow{DC}$, and greater at a perpendicular direction upwards or directions of $\overrightarrow{AD}$ and $\overrightarrow{BC}$.

The optical character of the elongation of crystal is negative according to the theory of piling a crystal plate over the other as above stated (This is quite different from positive or negative optical angle of a crystal and adaptable only when the thin plate is at straight extinction).

The result of observation of interference figures on "P" is as shown in Fig. 4 hereunder by setting the crystal for the conoscope under illumination of a white light. It is not suitable for observation of interference figures at all faces except "P",—for example (*hoo*)—, because they are too thin and the conoscope figures are not clear.

Observation result of interference figures on "P" at right diagonal position (MM' and NN' are the vibration directions of the upper and the nicol respectively).

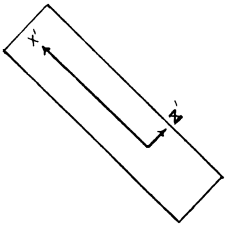


FIG. 4

Vibration direction of two light wave against gypsum test plate Red 1 at the position of left diagonal.

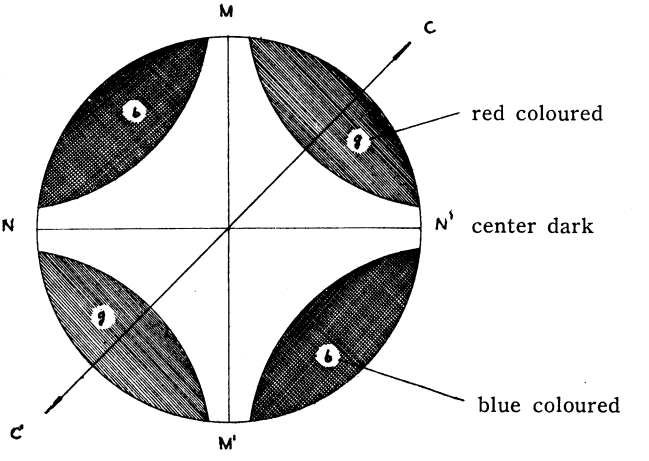


FIG. 5

Putting r for the retardation at the centre, g for the parts coloured red, and b for blue parts, the relation between r, g, and b is $g < r < b$, if this crystal is uniaxial, but at the thin face "P" difference of b and g are not clear (Fig. 5).

When "P" is at straight extinction as the orthoscope, the interference figure is a wide dark cross and occupies a wide field of vision; by rotating the stage slightly, the isogyre of the dark cross would be divided into two parts of a hyperbolic shape and soon vanish from vision to the opposite side of the quadrants. This shows the characteristics of flash figure.

This figure is observed at both faces, the one parallel to the optic axis (c)

of the uniaxial crystal and the other parallel to the optic axial plane of the biaxal crystal. Therefore it cannot be classified as to which is uniaxial crystal or biaxial.

If we could observe the interference figures on the other faces, we should be able to clear the characteristic by which we would judge the crystal as uniaxial or biaxial, but the faces are too small for observation of conoscope, except on "P".

Next, we judge whether riboflavin crystal is uniaxial or biaxial optically by measuring the refractive indices of the crystal according to the immersion method. We adopted for this experiment the central illumination method (otherwise called Becke's line method) among two immersion methods.

In the case of the uniaxial crystal, there are two rays (two polarized rays passing through the crystal), one is ordinary and the other is extraordinary. Put n_0 for infractive index of the ordinary ray and n_e for that of the extraordinary, then we have the following relation according to the law of optic crystallographic positive and negative.

In the case of positive $\varepsilon \geqq n_e \geqq n_0 = \omega$.

In the case of negative $\omega = n_0 \geqq n_e \geqq \varepsilon$.

(where ε and ω are the proper max. and min. values of refractive indexes of the crystal respectively.)

By observing the powder of riboflavin crystal (the finer the better) about the above relation, we learn that the two polarized lights, which pass through it, change the relation within a definite limit according to the directions of the lights. This shows the non-existence of light wave such as the ordinary ray, refractive index of which is constant for all directions. Therefore riboflavin belongs to the biaxial crystal, not to a uniaxial optically.

In the case of the biaxial crystal, two polarized lights are extraordinary. Put n_2 for refractive index of "Z" direction vibrate light and n_1 for that of "X" direction vibrate one; we know the following relations

$$n_2 > n_1$$

$$\gamma \geqq n_2 \geqq \beta \geqq n_1 \geqq \alpha$$

where γ = Maximum value of n_2

α = Minimum value or n_1

β = Refractive index for direction of optical axis

γ, β and α are the preper values for the crystal and each shows the principal refractive index refering to the principal velocity. We shall be able to compute the value of Ω (angle between optic elasticity axes "Z" and optic axes) by the following formula.

$$\tan \Omega = \frac{\gamma}{\alpha} \sqrt{\frac{(\beta+\alpha)(\beta-\alpha)}{(\gamma+\beta)(\gamma-\beta)}}$$

When the optical character of the crystal is positive the optical angle $2V$ is equal to 2Ω; if it is negative $2V$ is equal to $180° - 2\Omega$.

Left figure shows the relation between the optic elasticity axes "X", "Z" and optic axes AA', BB' (Fig. 5).

Positive...
$2V = 2\Omega \quad (2\Omega < 90°)$
Negative...
$2V = 180° - 2\Omega \quad (2\Omega > 90°)$

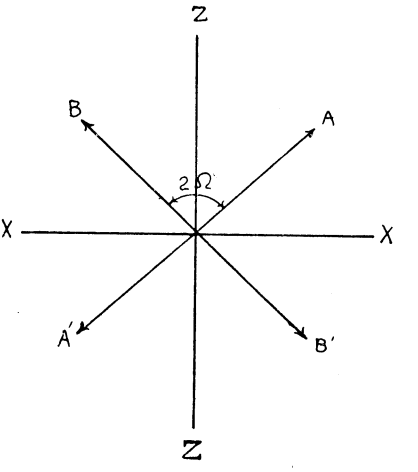


FIG. 6

After obtaining immersion liquids, whose values are equal to α, β, and γ or riboflavin crystal by measurement of plenty of powder crystals of riboflavin according to Becke's method, we measured the indexes by Abbe's refractometer or a special prism spectrometer. The principal refractive indexes of natural and synthetic riboflavin crystals and araboflavin crystal are shown as follows:

1. Natural riboflavin crystal (The source of light:—sodium Temperature:—measured at 12° C)

$\alpha = 1.6275 \pm 0.0010$
$\beta = 1.8333 \pm 0.0020$
$\gamma = 1.8951 \pm 0.0010$
$2\Omega = 127° 56'$
$2V = 52° 04'$
Optical character: negative
Elongation character: negative

2. Synthetic riboflavin crystal (The same light and temperature as above)

$\alpha = 1.6281 \pm 0.0010$
$\beta = 1.8337 \pm 0.0020$
$\gamma = 1.8955 \pm 0.0010$
$2\Omega = 127° 54'$
$2V = 52° 06'$
Optical character: negative
Elongation character: negative

3. Araboflavin (The same light and temperature as 1.)

$\alpha = 1.6317 \pm 0.0010$
$\beta = 1.8397 \pm 0.0020$
$\gamma = 1.8966 \pm 0.0010$
$2\Omega = 129° 57'$
$2V = 50° 03'$
Optical character: negative
Elongation character: negative

As above data, natural and synthetic riboflavin crystals and araboflavin crystal belong to the negative biaxial crystal. Next we should decide whether they belong to monoclinic, trictrinic or orthorhombic (rhombic) system of biaxial crystals.

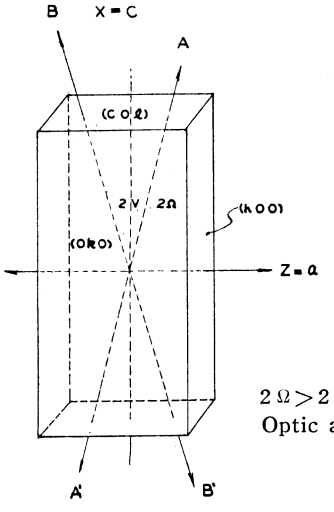
The face (*oko*) or "*P*" in Fig. 1 is obviously parallel to the optic axes, namely perpendicular to the optic elasticity axis "*Y*". The optic elasiticity axes "*X*" and "*Z*" coincide with the vibration directions of the upper and lower nicol at the extinction position against the orthoscope. $\overrightarrow{AB}$ and $\overrightarrow{CD}$, being the principal directions of "*P*" whose the extinction angle is zero (straight extinction); therefore axis "*C*", being taken parallel to *AB* and *CD*, should be "*X*" or "*Y*". Setting "*C*" at the diagonal position by rotating it to right 45° from the extinction position as above stated, the crystal shows subtraction phenomenon when the gypsum test plate Red 1 is inserted. This means that the direction of $\overrightarrow{AB}$ and $\overrightarrow{CD}$ is "*X'*", and the direction perpendicular to "*X'*" is "*Z'*". (In this case edge *AD* and *BC* show the principal direction of the crystal which is perpendicular to $\overrightarrow{AB}$ and $\overrightarrow{CD}$.)

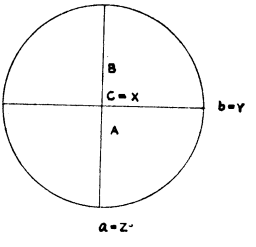
This fact is clear from the difference of indexes of refraction by observation "*P*" according to the immersion method. Optical orientations of riboflavin crystal are as follows, providing the crystallographic axes are *a*, *b*, and *c* as shown at Fig. 1.

$$b = Y$$
$$c = X \quad (Z > Y > X)$$
$$a = Z$$

This fact proves that natural and synthetic riboflavin crystals and araboflavin crystal belong to the rhombic system.

Axis "*Z*" is the obtuse bisectrix (bisector) (secondary bisector) of the obtuse optic angle, and "*X*" is the acute bisector (primary bisector) of the acute optic angle (2 *V*) (Fig. 7).





Optical orientation in the case of $a=Z$, $b=Y$, $c=X$.

$2\Omega > 2V$
Optic axial plare: (*oko*).

FIG. 7

The pleochroism of crystal is observed with the crystal of natural and synthetic riboflavin crystals and araboflavin crystal in common. The colour of thin

crystal plates such as the face (*oko*) and (*hoo*), changes according to the rotation of the stage, when these faces were observed under white light with lower nicol only.

With (*oko*), for example, it is colourless or light yellow when "*X'*" is set parallel to the vibration direction of the lower Nicol; while it is thick yellow when "*Z'*" is set in the same direction (Fig. 8).

This phenomenon may be observed in the other planes. The three axial colours would be classified if we could observe the colours on pinacoids which have grown well, but we could not observe them because the planes had grown litte except for the face (*oko*).

At any rate we noticed that there is pleochroism in riboflavin crystals.

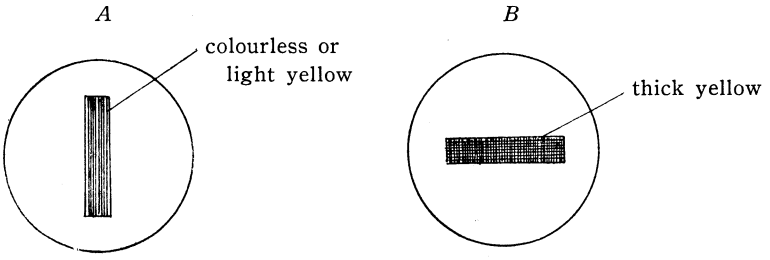


FIG. 8. *A*: Setting "*X'*" parallel to the vibration direction of lower Nicol. *B*: Setting "*Z'*" parallel to the vibration direction of lower Nicol.

2. Recrystallized Riboflavin Crystal Isolated from 90% Methanol

The bio-microscopic and optical findings, which were observed on riboflavin crystals separated from 90% methanol, are entirely the same as those from redistilled water, except for some habitus and their face developeing. Their existence are adhered or clinged to each other in fan-shape or radially and very few separated singly; there are, in addition, more crystals that grew longer in the direction of axis "*C*", showed the needle-shaped crystal form of druse in many instances. Hence their sizes are different, and researches into the shape of crystals were made in vain. It was learnt that fundamental optical constants and optical character, of riboflavin crystals and araboflavine crystals such as refractive indexes, optic angle, optical positive and negative, etc., are quite the same as those recrystallized from water.

3. Crystals separated from 90% Ethyl alcohol

They are entirely the same as those separated from 90% methanol. By lowering the percentages of alcohol, such as 80%, 70%, 60% etc., habitus nor nature of crystals changed remarkably. We obtained recrystalization of low evolution and never saw big crystals having higher isodiametric evolution.

4. Crystals Separated from Pyridine

Generally, a long time was needed to obtain crystals, and besides only a few were crystallized. The shapes were varied,—skeletal, acicular, tabular, etc. —and overlapped each other. These made necessary researches in crystallography. There were however, no differences in the fundamental optical constant and optical characters, as in the case of water.

SUMMARY AND DISCUSSION

1. Solvents suitable for recrystallization of riboflavin were in the order of distilled water, 90% methanol, 90% ethanol, and pyridine. With pyridine, recrystallization was most difficult.

2. Shape of the crystals separated from redistilled water was long plate or long needle. Their existence was generally separate and some were solid and too adhesive on long pinacoid. The crystals were hexahedron of plate shape.

3. Interfacial angle of each pinacoid and base in case where redistilled water was used, was apparently 90°. The crystal faces consisted of (*oko*), (*ool*) and (*hoo*), among which (*oko*) grew best. Correct measurement of the angle in cases of 90% methanol and 90% ethanol was impossible. According to conoscope observation, however, (*oko*) was found to be the best.

4. Crystal Axis

Axis "*a*" straight line perpendicular to (*hoo*). (Edge parallel to edge M_2).

Axis "*b*" straight line perpendicular to (*oko*). (Edge perpendicular to edges M_1 and M_2).

Axis "*c*" straight line perpendicular to (*ool*). (Edge parallel to edge M_1).

5. Optical Nature

(1) Extinction Position

On (*oro*), (*hoo*), and (*ool*), all show the straight extinction remarkably against the inside direction of crystals (these are shown by the edge which evoluted remarkably on crystal).

(2) Optical Orientation

$$\begin{aligned} a &= Z \\ b &= Y \quad (Z > Y > X) \\ c &= X \end{aligned}$$

X, Y, Z are optic elasticity axes, a, b, c are crystallographic axes

X axis = Primary bisector,
Z axis = Secondary bisector.

(3) Optical Character; optically negative

(4) Three Principal Refractive Indexes. (Sodium light 12° C)

	α	β	γ	2Ω	$2V$
Natural riboflavin	1.6275	1.8333	1.8951	127° 56′	52° 04′
Synthetic riboflavin	1.6281	1.8337	1.8955	127° 54′	52° 06′
Arabofravin	1.6317	1.8397	1.8966	129° 57′	50° 03′

(5) There was pleochroism of crystals common to them. On the whole, they were colourless or extremely light maize in the vibration direction of faster rays and some were dark yellow in the slower rays.

6. No twins and hemimorphy were recognized.

7. It was found from the above mentioned results that riboflavin crystals (both synthetic and natural) and araboflavin crystal belonged to the rhombic system and were optically negative crystals.

When these riboflavin crystals are compared with araboflavin crystal, as far as the optic angle and their shapes were concerned, they were equal. They were equal as regards crystal system and pleochroism. However, there was slight difference noted between their three principal refractive indexes. This difference arises as a difference of the optic axial angle. The difference of the optic axial angles of natural and synthetic riboflavin was only about 2′ which is likely to have arisen from observation and remained within the allowable limit of error. Accordingly, it is very clear from the optical constant that natural and synthetic riboflavin have the same crystal constitution. The three principal indexes of araflavin, on the other hand, showed a remarkable difference from those of riboflavin. It was a difference of about 2° at the optic axial angle on many powder crystals; this is appearently a difference of optical constant, and was above the allowable limit. It is presumable from these that the constitution of araboflavin is closely similar to those of riboflavin but that there are some differences between them.

CONCLUSION

1. Shape of crystals of riboflavin, natural and synthetic, and araboflavin recrystallized from redistilled water are hexahedral and plate-shaped, and the interfacial angles of each pinacoid and base are 90°. The surface consisted of (*ool*), (*oko*), (*hoo*), and (*hol*) and evolution of crystals was very low.

2. All these three are rhombic and are optically negative. Elongation characters are also negative, pleochroism of crystals is remarkable.

3. Although researches into those recrystallized from methanol, ethanol, and pyridine are not so perfect as the case of redistilled water, it is presumable from the extinctive position and from the negative character of elongation that they belong to rhombic.

4. Natural and synthetic riboflavin crystals are the same in respect to the fundamental optical constant. This allows a conclusion that their construction is the same accordingly. Araboflavine belongs to the same crystal system but has some slight difference in the fundamental optical constant. Therefore, its construction is very similar, but there still exist some differences.

In conclusion the writer would like to express his sincerest thanks for thorought instruction and suggestions given to him by Professor Hotta, the writer's teacher, Professor Yoshio Kondo of chemistry at the Cultural Dept., and assistant Professor Hirokichi Ishioka of the same Dept.

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