# Changes of Flavin Content of Wheat during the Course of Growth

By

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Many studies have been reported on the respiratory systems in wheat seed during the course of germination (1–5). It has been found that a part of the respiration was insensitive to cyanide and the cyanide sensitivity changed during the course of germination of seeds (5). The mechanism of respiration which is not inhibited by cyanide is not yet completely clear. The participation of flavoprotein system is considered as a possibility.

The purpose of the present investigations is to determine flavin contents of wheat during the course of the growth including germination, assuming that the determinations might make some contributions to the elucidation of the problem of cyanide insensitive respiration. Effect of vernalization treatment of the seedling on the content of flavin was also investigated, because it has been found that the treatment modified cyanide sensitivity of wheat respiration (5).

Before the determination of flavin content during the growth of wheat, the methods of determination of flavin in smaller amounts and also the methods of fractionation of various flavin compounds were examined.

## Materials and Methods

Winter wheat (*Triticum aestivum* L. var. akasabishirazu No. 1) was used throughout these studies. This strain needs cold treatment of about 50 days for the sowing in spring.

Enzyme preparations were applied to make free the bound forms of flavin. Trypsin, lipase, ficin and papain were commercial products. Pancreatin was prepared from pig's pancreas.

*Germination of Seeds*

Seeds were sterilized with 0.1% solution of "Uspulun" for an hour and then washed with tap water for half an hour. After this treatment, the

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seeds were allowed to germinate for 1, 2, 3, 4 and 5 days in a large petri dish in the dark at 24°C.

*Growing Leaves and Maturing Seeds*

Seeds were sown in the field. Growing leaves and maturing seeds were sampled for determination of flavin at various stages of growth.

*Determination of Total Flavin Contents*

Flavin in "Hot Water Extract" was assayed by fluorometric method of YAGI (6). Sample was previously treated in hot water at 80°C for 5 minutes, homogenized and extracted at 80°C for 15 minutes. After centrifugation, the resultant supernatant was adjusted to a suitable volume and flavin was determined fluorometrically.

Flavin in "Trypsin Sample" was made as the following. Tissues were treated in hot water at 80°C for 5 minutes, homogenized and diluted with deionized water to a suitable volume. The homogenate was incubated in the dark at 37°C for 2 hours with trypsin of 50 mg/g-fresh weight at pH 7.0. The reaction was stopped by 5% trichloroacetic acid. After centrifugation, the amount of flavin in the supernatant was measured by fluorometric method (6).

To prepare "Lipase-Trypsin Sample" the homogenized materials were pretreated with lipase of 100 mg/g-fresh weight at 37°C for 2 hours at pH 7.0 and were followed by trypsin treatment as above described.

*Fractionation of Various Flavin Compoumds*

Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin (FR) were separated by paper chromatography on "Hot Water Extract" (7). Each flavin fraction was cut out, eluted to a fixed amount of water and determined for flavin.

*Condition of Vernalization*

The sterilized seeds were germinated for 6 hours in a large petri dish in the dark at 24°C. This pregerminated seeds were vernalized at 4°C in the refrigerator during 60 days.

## Results

*Changes of Total Flavin Contents during the Course of Growth*

a. *Flavin Contents obtained by Various Treatments* There is a great deal of method to determine flavin content (6, 10–13). Amoung of them the method of "Hot Water Extract" by YAGI (6), namely extraction of flavin in water at 80°C for 15 minutes has been used by many authors to determine flavin contents in animal and plant tissues. Further, trypsin treatment to

make free a tightly bound flavin with protein has been reported by BOUKINE (14) and SINGER (15). However in plants, especially in seeds, lipase was effective to make free more fiavin at certain stage of growing. Table I shows flavin contents of wheat seeds by treatments with various enzymes. It was shown that flavin of seeds was made free more effectively by digestion with lipase or lipase and trypsin than with trypsin alone.

TABLE I. *Flavin Contents of Wheat Seeds by Various Treatments.* 50 grains of wheat seed were used for this experiment. Samples were heated at 80°C for 5 minutes, homogenized and diluted with deionized water to 100 ml. These were treated with each enzyme in the dark, then flavin content was determined.

| Added Enzyme | (g) | pH | Temp. (°C) | Time (hrs) | Weight (g/50 grains) | Flavin Contents | |
|---|---|---|---|---|---|---|---|
| | | | | | | (μg/grain) | (μg/g-F.W.) |
| Trypsin | 0.1 | 7 | 37 | 2.5 | 2.13 | 0.035 | 0.65 |
| Trypsin+Lipase | 0.1+0.2 | 7 | 37 | 2.5 | 1.85 | 0.106 | 2.87 |
| Lipase | 0.2 | 7 | 37 | 2.5 | 1.74 | 0.095 | 2.71 |
| Pancreatin | 0.08 | 7 | 30 | 2.5 | 1.81 | 0.079 | 2.18 |
| Papain | 0.1 | 5 | 30 | 2.5 | 1.67 | 0.039 | 1.17 |
| Ficin | 1.0 | 5 | 30 | 2.5 | 1.76 | 0.037 | 1.06 |

b. *Changes of Total Flavin Contents during the Course of Germination*
Changes of total flavin contents determined by three kinds of treatments, "Hot Water Extract", "Trypsin Sample" and "Lipase-Trypsin Sample", during the course of germination were shown in Fig. 1. Flavin contents in whole grain increased as germination proceeded (Fig. 1, a). At an early stage of germination flavin in "Hot Water Extract" was the same with that in "Trypsin Sample". However, the content in "Lipase-Trypsin Sample" was about twice of these amount. This result shows that flavin which is bound with lipid or lipoprotein exists at an early stage of seed germination. And the contents of three types of flavin increased as aging proceeded. Further, at the latter stage of germination the contents of "Trypsin Sample" and "Lipase-Trypsin Sample" were higher than that of "Hot Water Extract". It is suggested from this results the increase of protein bound flavin. From the results of Fig. 1, b and c, it is also suggested that mainly lipid or lipoprotein bound flavin was contained in endosperm at an early stage of germination and became free as germination proceeded and in embryo flavin was biosynthesized actively.

Flavin contents per fresh weight during seed germination are shown

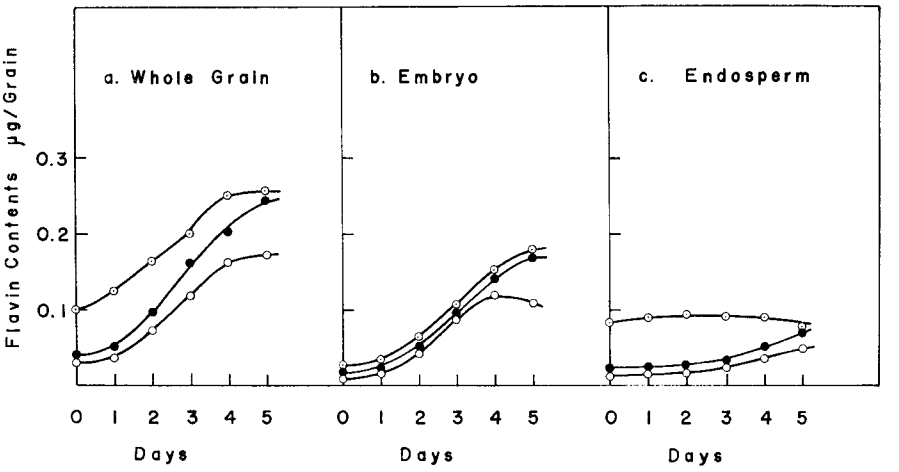


**Fig. 1.** *Flavin Contents per Grain during the Course of Germination of Seeds.* Flavin contents in endosperm were calculated from the contents in whole grain minus in embryo.
○ ; Hot Water Extract, ● ; Trypsin Sample,
⊙ ; Lipase-Trypsin Sample.

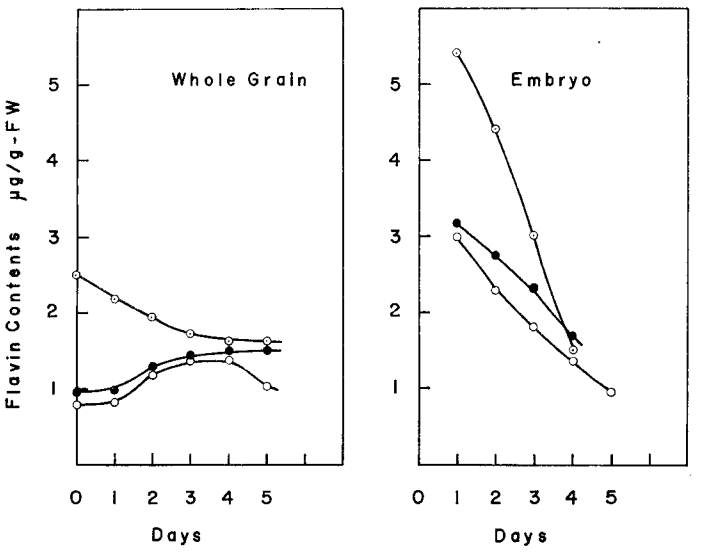


**Fig. 2.** *Flavin Contents per Fresh Weight during the Course of Germination of Seeds.*
○ ; Hot Water Extract, ● ; Trypsin Sample,
⊙ ; Lipase-Trypsin Sample.

in Fig. 2. The content in whole grain in "Lipase-Trypsin Sample" decreased during germination, but that in "Trypsin Sample" and "Hot Water Extract" increased slightly. While, flavin obtained from these treatments tended to decrease in embryos.

Flavin contents per nitrogen during the course of germination in both whole grain and embryo increased greatly as germination proceeded.

c. *Changes of Total Flavin Contents during the Course of Maturation of Seeds* As seen is Fig. 3, flavin contents per grain increased by three kinds of treatment according to the course of maturation. At drying

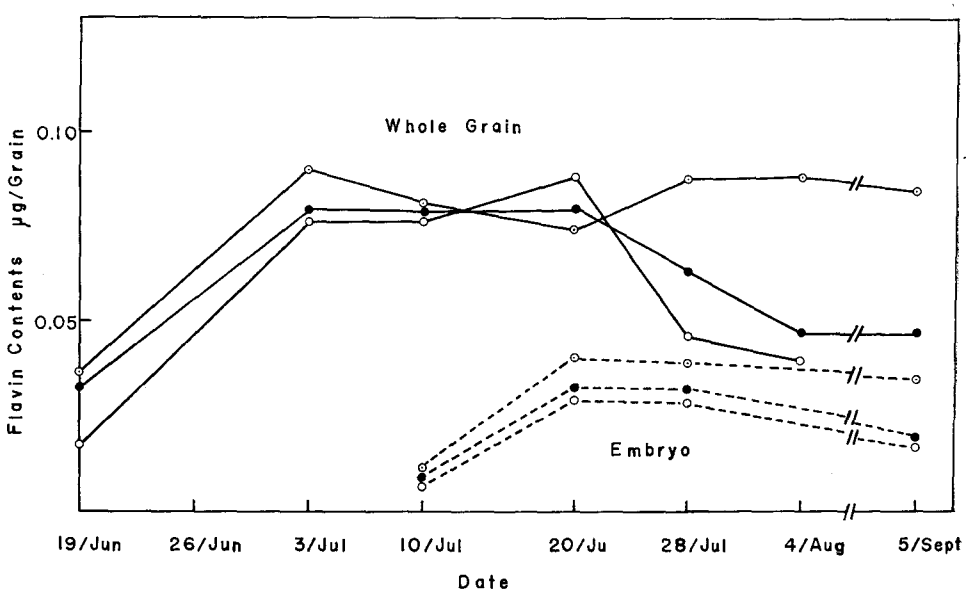


**Fig. 3.** *Flavin Contents per Grain during the Course of Maturation of Seeds.*
○; Hot Water Extract, ●; Trypsin Sample,
⊙; Lipase-Trypsin Sample.

stage of seed maturation flavin contents in "Hot Water Extract" and "Trypsin Sample" decreased in about a half. However, flavin contents in "Lipase-Trypsin Sample" kept a maximum. The same tendency was shown during maturation of embryo.

Flavin contents per fresh weight during the course of maturation of seeds seemed to decrease as the storage substances accumulated, as seen in Fig. 4. But, a marked increase of the contents was shown in embryos.

The same results were obtained on flavin contents per nitrogen.

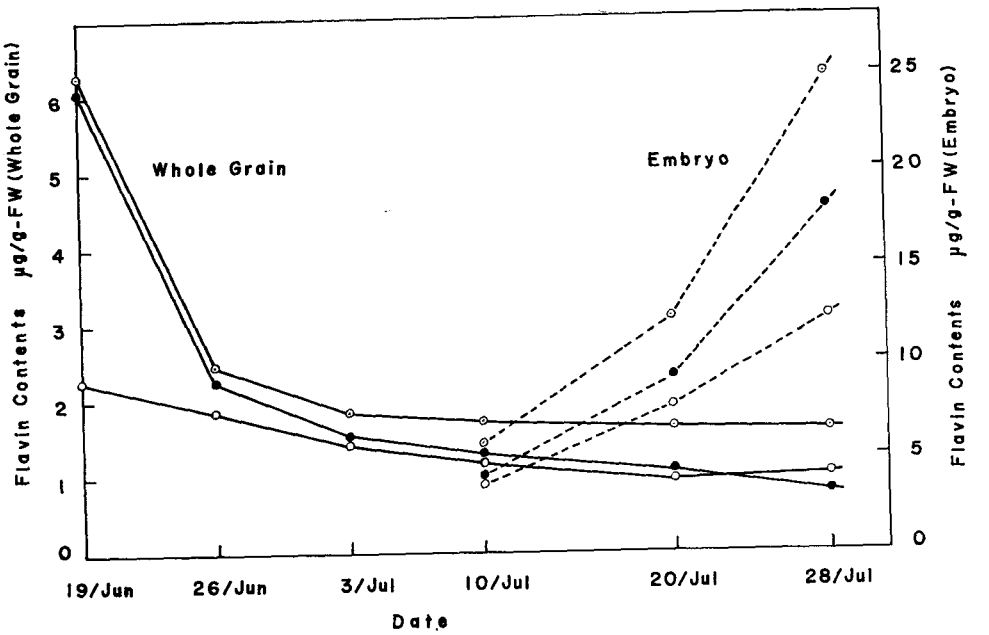


**Fig. 4.** *Flavin Contents per Fresh Weight during the Course of Maturation of Seeds.*
○; Hot Water Extract, ●; Trypsin Sample,
⊙; Lipase-Trypsin Sample.

d. *Changes of Total Flavin Contents during the Course of the Growth of Leaves* This strain of wheat has generally ten leaves. Seventh, nineth and tenth leaves were assayed to determine flavin contents.

Flavin contents in "Hot Water Extract" per fresh weight of leaves were shown in Fig. 5. In each leaf, as shown typically in the nineth leaf, flavin contents increased until a suitable growing stage and reached the maximun. This coincided to the maximun in fresh weight and dry weight of the leaf. Then, the flavin contents seemed to decrease. Comparing upper and lower leaves, the increase of flavin contents in upper leaves accompanied with decrease of that in lower leaves. Transportation of flavin among different parts of plant are suggested by these observations.

e. *Changes of Total Flavin Contents during the Vernalization Process* SISSAKIAN and FILIPOVICH (16) and TERAOKA et al. (5, 17) reported that at earlier vernalization stages respiratory mechanisms changed from cytochrome oxidase system to other terminal oxidase systems.

As seen in Table II, as the course of vernalization proceeded, flavin contents in "Hot Water Extract" increased in both whole grain and embryo.

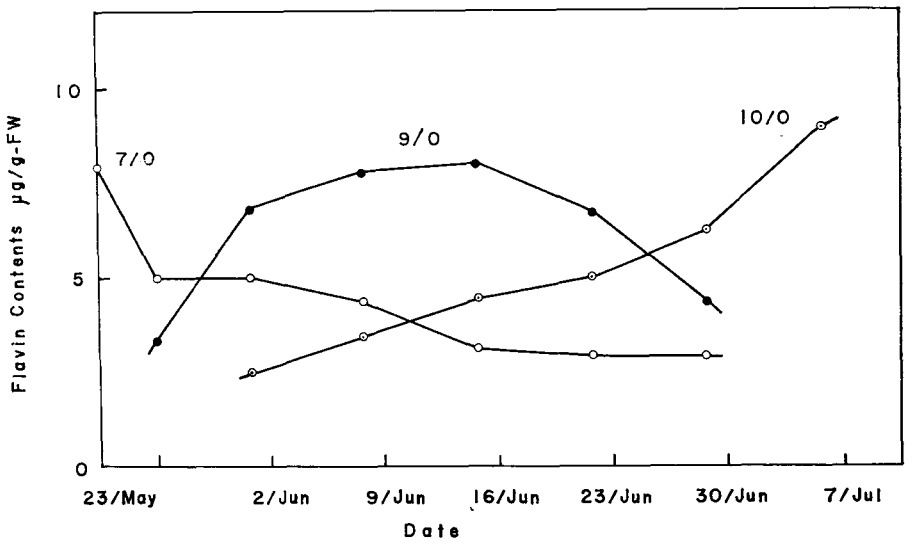


**Fig. 5.** *Flavin Contents per Fresh Weight during the Course of Growth of Leaves.*
7/0; The seventh leaf in the main stem.
9/0; The nineth leaf in the main stem.
10/0; The tenth leaf in the main stem.

TABLE II. *Changes of Total Flavin Contents during Vernalization Process.* L-T; Lipase-Trypsin Sample, W; Hot Water Extract. μg/Grain

| Days | Whole Grain | | Embryo | |
|---|---|---|---|---|
| | L-T | W | L-T | W |
| 6 | 0.082 | 0.037 | 0.032 | 0.022 |
| 26 | 0.169 | 0.076 | | 0.072 |
| 57 | 0.279 | 0.130 | | 0,104 |

μg/Grain

*Changes of Various Flavin Compounds during the Course of Growth*

a. *Changes of Various Flavin Compounds during the Course of Germination of seeds* Flavin in plant tissues exist in three types generally; flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin (FR). In addition, although many fluorescent substances and the precursors of flavin are contained, they are only in a very small quantity in wheat, comparing with the amount of three types of flavin. Each of flavin in "Hot

Water Extract" was separated by paper chromatography and measured fluorometrically.

As seen in Fig. 6, contents of FAD and FMN per grain increased slightly as germinating stages proceeded and that per fresh weight decreased greatly, while, the contents per nitrogen remained rather constant. The content of FR increased in every determinations. On the course of the separation of flavin compounds, the substances which showed green and blue fluorescence and had Rf-value of 0.14, 0.37 and 0.47 appeared clearly from third day to fifth day of germination, but at the first day of germination, these substances were indistinct.

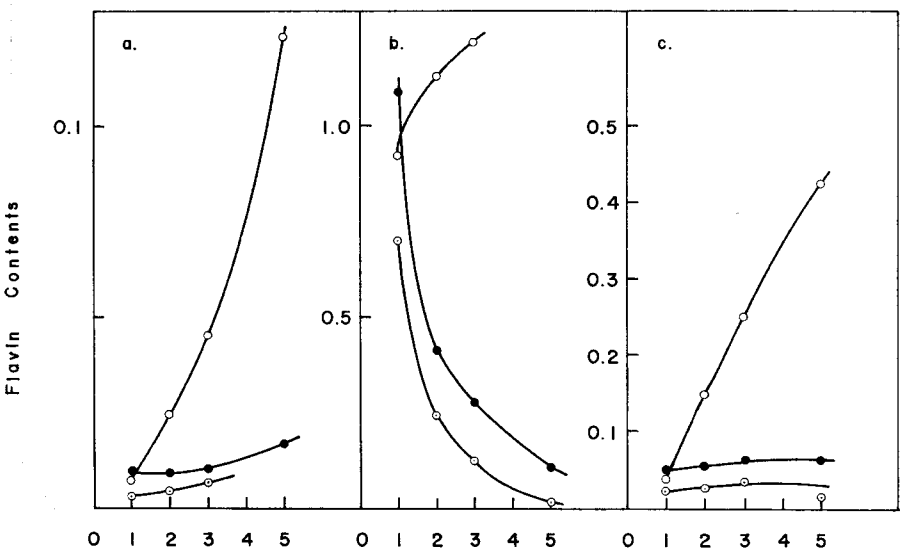


**Fig. 6.** *Fractionation of Flavin Compounds during the Course of Germination of Embryo.*
a; μg/Grain, b; μg/g-Fresh Weight,
c; μg/mg-Nitrogen.
○; FR, ●; FMN, ⊙; FAD.

b. *Changes of Various Flavin Compounds during the Course of Maturation of Seeds* As shown in Fig. 7, analogous results on the contents of FAD, FMN and FR were obtained during maturation. Each of them increased with maturation and decreased at drying stage and then remained constant.

c. *Changes of Various Flavin Compounds during the Course of Growth of Leaves* In the growing leaves, contents of FAD, FMN and FR paralleled

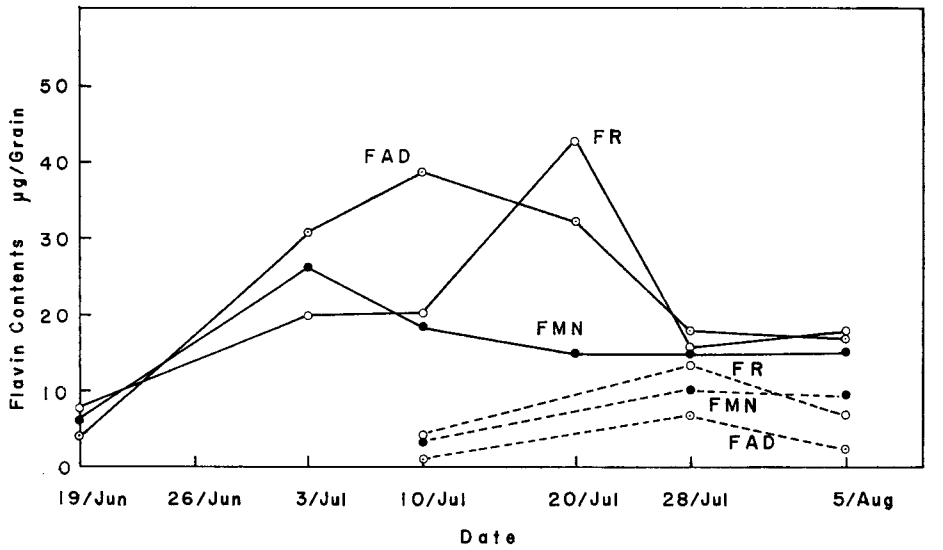


**Fig. 7.** *Fractionation of Flavin Compounds during the Course of Maturation of Seeds.*
Closed line; whole grain, dotted line; embryo.
○; FR, ●; FMN, ⊙; FAD.

to that of "Hot Water Extract". Each of them increased until a suitable growing stage and then tended to decrease.

## Discussion

BOUKINE (14) reported formerly that flavin contents in plants and animals increased greatly by trypsin treatment and decided from these observations the presence of flavin bounded tightly with protein in plants and animals. SINGER and KEARNEY (15) who investigated succinic dehydrogenase used trypsin as a method to separate flavin and protein.

In our experiments, the solubilization of bound flavin by lipase, papain, ficin and pancreatin were examined on wheat seeds. In the case of dried seeds lipase or lipase and trypsin treatment was the best to solubilize flavin, as seen in Table I.

As maturation of wheat seeds proceeded, flavin contents increased gradually. The flavin was almost soluble in hot water. Then, at drying stage of seeds, the soluble flavin decreased to about a half in a short time and then remained constant. The flavin seems to convert from the soluble form to the lipid or lipoprotein bound form during drying stage.

In germination, seeds begin to biosynthesize flavin remarkably in embryo.

Our data coincide with the reports which show that flavin contents increase by synthesis during the course of germination (18–21). The flavin was almost in soluble form in embryo. While, in endosperm flavin was in the form bound with lipid or lipoprotein and the bound form converted to soluble form gradually. The transportation of the flavin from endosperm to embryo during seed germination seemed impossible, because flavin content in endosperm did not decrease. However, to solve this problem further experiments are necessary.

The flavin in "Hot Water Extract" of embryo was mainly FAD, FMN and FR and the increase of FR during germination was remarkable. As GALSON (22) and KONDO (19) have reported, it may be correlated to photoreaction in plants.

Flavin in "Trypsin Sample" and in "Lipase-Trypsin Sample" was studied by paper chromatography. It contained a little of FAD, FMN and FR. Besides these flavin compound some unknown substances with Rf-value of 0.38 and 0.51 and with blue and green fluorescence by trypsin treatment and Rf 0.57 substance with yellow fluorescence by lipase and trypsin treatment were detected.

On the respiration syatems of vernalization process, SISSAKYAN et al. (16) and USAMI et al. (5) reported that respiratory changes from cytochrome oxidase system to the other terminal oxidase systems occured in an early stage of vernalization. In our experiments, the growth in 57th day of vernalization corresponded to that in second or third day of germination in normal temperature. It seemed that changes of flavin contents during the course of vernalization were not essentially different to that during the course of germination. There is a part of respiration which is not inhibited by cyanide during the course of germination of wheat seed. As a possibility of cyanide resistant respiration, the participation of flavoprotein may be considered.

## Summary

1. The flavin content of seed increased during germination and the increased flavin was mostly riboflavin. In embryo, flavin was found to be biosynthesized very actively and flavin in endosperm changed from bound form with protein and lipid to free form during its germination process. It was assumed that the flavin functioning in the growth process in embryo depended on the newly synthesized one.

2. During the maturation of seeds, the flavin content in seed increased. Entering to the drying stage, the water soluble flavin declined in quantity

to about a half and changed qualitatively to bound form which was made free by lipase treatment.

3. The growing leaves were rich in flavin containing several times of the seed. The flavin content in leaf increased until a certain period of growth and then decreased.

4. Changes of flavin content in the vernalization process were analogous to that in the late germination stage.

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