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◆ Review ◆

# Coordination of Basic and Development Researches on Vitamins**

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## 1. A Viewpoint for Basic and Development Researches

The food deficiency is becoming more evident throughout the world. The world population is now over 3,500 million, and the latest statistics by the United Nations project a population of 6,600 million at the end of this century as the most likely expectation.[1] Particularly, the expansion of the population is explosive in Asia. As shown in Scheme 1, the population in Asia, Africa and Latin America is summed up to more than 70% of the world population, and is foreseen to exceed 5,000 million by the beginning of the 21st century. However, the population distribution does not harmonize with the regional food productivity[2]. A wide gap existing between the population increase and the food supply has been developing markedly in Asia, Africa and Latin America. The developing countries in such regions have hardly attained self-sufficiency in their food supplies. Now, food production is self-sufficient only in Europe and North America. The Food and Agricuture Organization of the United Nations registers 10 to 15% of the world population is undernourished, and up to half as suffered from some degree of hunger or malnutrition, or both, even at the present time[3]. The food deficiency in those developing countries becomes terribly intensified by their population explosion. Those nations have often suffered from the diseases caused by foods of inadequate nutritional quality: lacking of protein, vitamins and minerals.

Protein malnutrition which affects primarily infants is the most widespread nutritional deficiency. Malnutrition causes retardation of the growth and development, both physical and mental, of infants. Among the malnourished children, mortality and morbidity are markedly high and a common infectious disease can be catastrophic[1].

On the other hand, even though Japan looks like an exception in Asia to such a food deficiency, she is only 70 to 75% self-sufficient in her food production. These conditions compel her to import foods; for an example, Table 1 shows that her almost all domestic demands for wheat, soy bean and cereals other than rice are obliged to be provided by the import. Such is the actual food situation in Japan. The author would like to give a warning to the people having an optimistic misleading notion about the food situation only on the basis of the continued large crop of rice during the recent four year.

Now, provision of adequate diets for the coming food deficiency throughout the world caused by a rapidly expanding population has come under more anxious scrutiny than ever before. Solution of this problem is the greatest challenge for the scientists of the world today. The author believes that, in coordinated scientific efforts to prevent the malnutrition, it has become more important than before that food and agricultural researches should be developed in close association with nutritional researches, and that these applied researches should be essentially based on theories of both chemistry and biology.

The fields of food and agricultural science deal with food productivity that is, the main urgent task assigned to them is to find how to intensify more effectively food production than before. Nutritional researches deal with nutrient availability in human body, namely, the fundamental task imposed on them is to elucidate more logical and not wasteful utilization, even if empirical, of the energy and the nutrients of

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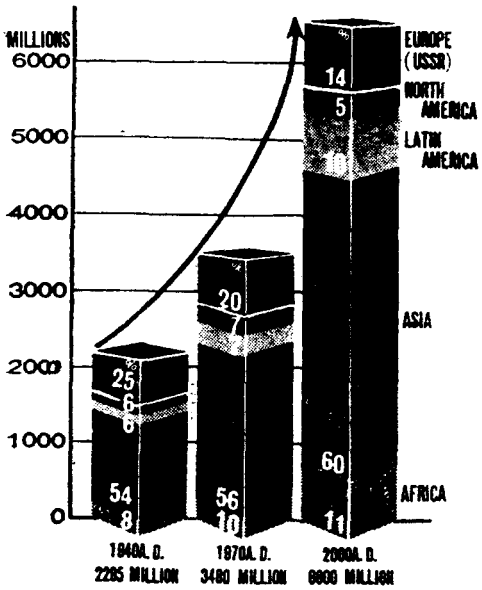
** 이 총설은 本學會 特別講演(1971. 8. 30)의 要旨를 再編輯 收錄한 것임.

food to maintain human health and performance, and, further, to make it possible to relate ingesta to all physiological functions. Determination of body's nutrient demand accomodated for changes of physiological and environmental conditions will pave the way for more efficient food supply. For one of such examples, incomplete foods in nutrient quality and unbalanced foods in nutrient assortment could be exactly and profitably remade to the food products agreeing with the fluctuation of body's need by combinations of foods or by enrichment of foods with the lacked nutrients.

Our knowledge at the present stage, however, does not get to provide us with the most valuable of practical tools founded on a unificative chmical on biological theory to control through diet all physiological functions of human body, which may be one of the ultimate tasks of nutritional science. Perhaps, this thought will never be essentially impossible to be substantiated, since a theoretical support for this can be found in thermodynamics on life. Living cells are thermodynamically open system: they exchange matters with their surroudings, but never totally in equilibrium, and thus the cells keep their systems in the steady states only by a continuous supply of matters or energy from their surroundings. From this thermodynamic point of view, we can grasp theoretically ***foodstuffs or nutrients as the active matters through which the external environments act directly on the internal environments of living organisms.*** It is the fundamental feature of living organisms that they can construct themselves from foodstuffs with nearly perfect fidelity, and can maintain themselves by using free energy from oxidation of foodstuffs, and that if the foodstuffs supply is cut off their systems run down led to the breakdown: the environment is absolutely essential to living organisms, not only as a source of raw materials but also as a source of free energy. Essential orderliness of living organisms is created and maintained only by the expense of their environment, which they cause to become thermodynamically more disordered and random. All equipments comprising living organisms operate on the principle of maximum economy to maintain their open systems under the steady states antagonizing against the world increment of entropy. They are endowed with the capacity for self-organization and self-regulation to maintain their steady states and to their energy-transforming efficiency.

Now, we will arrive at a self-evident recognition that the essential thesis previously proposed for human nutrition, that is, logical utilization of foodstuffs for human body, and, to say more positively, regulation of human body through foodstuffs, is profoundly linked with the most fundamental principles governing the living state of all organisms, and that there is no accurate resolution for the thesis without deep understanding of the principles. The emphasis is that dietics should join tightly with biology founded on chemistry and equip oneself with practical theories obtained from this basic area.

We have been considering the fundamental aspect and significance underlying in the relationship between the basic and applies sciences, and emphasizing their deep collaboration to contribute really to the promotion of the development of science and of prosperity and welfare of human being. Similarly this concept means that opportunism in applied researches supported only by a short-sighted view point for an immediate profit, which has often come out the history of application of science, not only hinders the science from its sound development but also reacts rather destructively on survival of all organisms including mankind himself. Collaboration, or rather reciplocal interaction, of the basic and development researches has been historically proved to be a positive facting on evolution of the science. Origin of the science has profound relations with knowledge for nature through production carried out by human being. In the very begining, the science is empirical and has a nature of application. However, the empirical and applied science has been transformed into the theoretical one by generalizing the results achieved from the field of itself, and at this time, has been able to have a potentiality to produce a basic science unified by a few essential concepts. Conversely, the applied science has been undergoing a profound transition and stimulated by development of the basic science born of the former. This relationship is reciplocal, not one-sided, and develops stepwise just like the spiral stairs.



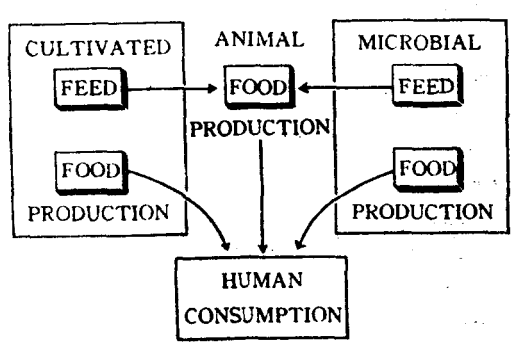

**Scheme 1. World human population from 1940 to 2000**

**Table 1. Demand and supply of foods in Japan (1968)**

| Commodity | Production (10,000 ton) | Import(%) (10,000 ton) | Total (10,000 ton) |
|---|---|---|---|
| Rice | 1,445 | 27(2) | 1,472 |
| Wheat | 101 | 400(80) | 501 |
| Other cereals | 19 | 770(98) | 798 |
| Tubers | 765 | 0(0) | 765 |
| Soybean | 17 | 242(93) | 259 |
| Other legumes | 37 | 23(38) | 60 |
| Meats | 119 | 22(16) | 141 |
| Eggs | 137 | 4(3) | 141 |
| Milk & dairy products | 414 | 63(13) | 477 |
| Fishes & shellfishes | 817 | 93(10) | 910 |
| Oils & fats | 93 | 35(27) | 128 |

## 2. Researches on Vitamins in Plants

The interests of the present author and his coworkers have been more oriented toward vitamins in plant tissues. Our basic and development researches on this problem have been essentially originated from and supported by a viewpoint of eternal cycle of matter in nature. The cycle consists of two parts, living things under highly-organized states of matter and inanimate matters under random states of matter. The autotrophic organisms, mainly green plants, hold an unique position in the cycle; they play an important role in linking the two parts in the cycle. Green plants can synthesize every organic materials their life requires from very simple inorganic materials by utilizing sunlight as their main source of energy. This photosynthetic process is the starting point in the cycle of matter through living organisms. Also, living things can interact individually with inanimate matters in their enviroment, for example, by gas exchange, decay of their bodies, etc. However, it is well known that living things are uniquely organized in the cycle of matter to be linked together into the food webs, so-called interlinked "food-chains". Each of the chains initiates with the autotrophic organisms, usually green plants, and passes through the herbivores to the carnivores. This social organization, which has indubitable value from the ecological standpoint, is very essentially based on nutritional requirements. Animals can not synthesize the essential substances such as amino acids, fatty acids and various vitamins needed to form and maintain their bodies. All these substances are produced by green plants. Therefore, the animals are directly or indirectly dependent on the green plants, not only for their indispensable factors but also for their supplies of energy-yielding foods such as fats and carbohydrates as well. We can observe how the heterotrophic organisms are constructed and maintained in the end by the organic materials originated from the autotrophic organisms. Of course, human being is not an exception to this rule(Scheme 2).




**Scheme 2. Cycle of food for human consumption**

Now, from the fundamental aspect as mentioned above in the interrelationship through nutrients between the autotrophic organisms and the heterotrophic organisms, we can realize a concept that logical food production and human nutrition does not materialize without deep understanding with the features of production and metabolism of organic compounds in green plants, and of the flow of these compounds through the food-webs. The first problem on green plants is, indeed, the most important rather from a viewpoint of food production. But, we still does not have complete knowledge enough to control the production of organic materials in plants. The main reason for this seems to be that, although there are some exception, it has been carried out too little with plants to elucidate the metabolic processes of organic compounds and the relation of these processes with the plant physiological functions. The similar is said with the researches on vitamins in plant tissues.

The present article will be roughly divided into two parts of the basic and development researches on vitamins. The first part will include biosynthesis in plant of thiamine, riboflavin and pteridines involving folate, and possible regulatory mechanisms of these synthetic processes, mainly discussing on relationship between the biosynthetic mechanism and ATP functions as the major carrior of chemical energy in the cells. Such relationship between the synthetic processes and energy-yielding systems is the most fundamental in physiological functions of plants, and is essentially differed from the heterotrophic organisms. It is because the heterotrophic organisms can utilize the energy of highly-reduced, energy-rich organic molecules, while the autotrophic organisms can utilize directly sunlight as their main source of energy. We shall then proceed to consider the physiological significance of ascorbic acid in plants. It is necessary to point out that there is no available knowledge about biochemical significance in plant and animal tissues of this vitamin, although its deficiency has been well known to cause scurvy in the human, the monkey and the guinea pig. The latter half of the present article will be treated with the development of researches on vitamins, particularly describing the developmental transition of the basic researches into the development researches. Among these, are there the researches on enrichment of rice with vitamin $B_1$ and lysine, on preservation by nucleotides of a chemical seasornor in raw food products, on prevention by vitamin C of the haize development in beer, and on an application of vitamin $B_1$ for the crop yield of rice. In the last, away from the researches on vitamins, we will present a typical successful exmple of the coordination of the basic and development researches, namely, two development researches founded on the basic research on catalase: a new storage of cereial grains and a new utilization of microbial protein as foodstuff.

### 3. Biogenesis of Thiamine

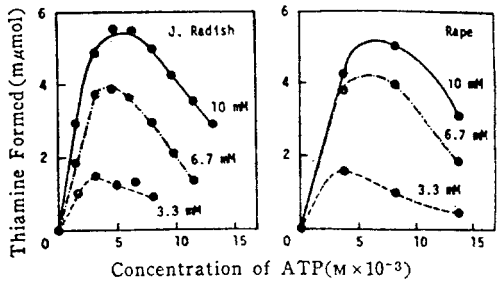
The biosynthesis of thiamine has been extensively studied in microbial system from baker's yeast and *Escherichia coli* etc., [6-8] whereas little is carried out in plant systems. There is a gap to be filled in the knowledge of thiamine synthesis in plants and a possible alteration of it's pathway from that of microbial system may exist.

The authors demonstrated by using isolated roots, segmented green leaves and seedings that thiamine was yielded from 2-methyl-4-amino-5-hydroxymethyl pyrimidine (OMP) and 4-methyl-5-*B*-hydroxy-ethyl thiazole (Th) ***in vivo***.[9,10] This finding was well consistent with an observation that nutritional requirement of isolated roots for thiamine was replaced by OMP and Th. Furthermore, the authors found in green leaves the existence of an enzyme system which catalyzed formation of thiamine monophosphate from OMP and Th in the presence of ATP and $Mg^{++}$.[11]

This chapter deals mainly with activation mechanism by ATP of OMP and Th followed by this condensation to thiamine monophosphate, and with discussion on the regulatory mechanism how ATP level is maintained under the relation with thiamine monophosphate synthesis in the tissues.

The enzyme preparation is obtained from green leaves of *Japanese radish* and *rape*. As shown in Fig.1, enzymatic formation of thiamine from OMP and Th is influenced by ATP concentration present. Maximal synthesis of thiamine is observed at 3 to 6 mM of ATP levels beyond this point are rather inhibitory on the synthesis. Also, the rate of the synthesis is markedly accelerated by increasing $Mg^{2+}$ concentration, and the optimum amounts of ATP

required is dependent on the $Mg^{2+}$ level. These results suggest that biosynthetic pathway of thiamine involves at least two enzyme systems differed in their responsibility for ATP, one requiring ATP, and the other blocked by ATP but this inhibition being reversed by $Mg^{++}$ increment.




The optimum concentration of ATP and its dependence on the concentration of $Mg^{++}$. The concentration of $MgCl_2$ was $3.3\times10^{-3}$M (- - -), $6.7\times10^{-3}$M (-·-·), and $10^{-2}$M (——)

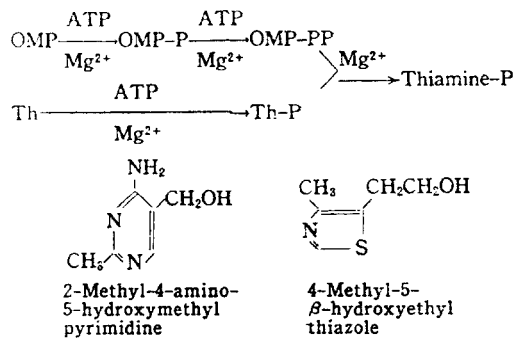
**Fig. 1. Biosynthesis of thiamine**

**Table 2. Thiamine biosynthesis with thiazole or thiazole phosphate as substrate**
(Thiamine formed)

| Reaction system | OMP | OMP-P | OMP-PP |
|---|---|---|---|
| | mμmol | mμmol | mμmol |
| Th | 0 | 0 | 0 |
| Th, ATP | 0 | 0 | 5.2 |
| Th, $Mg^{2+}$ | 0 | 0 | 0.3 |
| Th, ATP, $Mg^{2}$ | 4.8 | 6.5 | 27.0 |
| Th-P | 0 | 0 | 43.0 |
| Th-P, ATP | 0 | 0 | 12.8 |
| Th-P, $Mg^{2+}$ | 0 | 1.2 | 44.4 |
| Th-P, ATP, $Mg^{2+}$ | 5.3 | 7.6 | 36.2 |

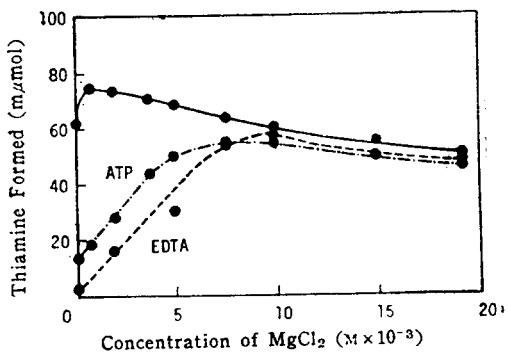
Reaction mixture contained in 3 ml: Substrates; each 0.2 μmol; ATP, 20 μmol; $MgCl_2$, 30 μmol; ascorbic acid, 30 μmol; enzyme, 0.5 ml.
Reaction conditions: 40°C, 90 min, pH 7.5 (0.1 M phosphate buffer)

As shown in Table 2, the phosphorylated substrates such as OMP monophosphate, OMP-pyrophosphate and Th-monophosphate are effectively utilized for enzymatic production of thiamine. ATP and $Mg^{++}$ are required for the conversion only when OMP, OMP-monophosphate and Th are used as substrates, but not when OMP-pyrophosphate and Th-monophosphate are used. This finding indicates definitely that OMP and Th are phosphorylated by ATP in the presence of $Mg^{++}$ prior to their binding, and the immediate substrates are OMP-pyrophosphate and Th-monophosphate. Biosynthetic mechanism of thiamine in plant system deduced from these result are illustrated in Scheme 3, which agrees essentially with that for microbes.




**Scheme 3. Pathways of thiamine biosynthesis**

As shown in Fig. 2, thiamine formation from OMP-pyrophosphate and Th-monophosphate is potently inhibited by ATP, but the inhibition is removed by raise of $Mg^{2+}$ concentration. Besides, EDTA retarded.




Effect of $MgCl_2$ on the recovery of the TMP-synthetase activity inhibited with ATP and EDTA —— Control; -·- $7.5\times10^{-3}$M ATP; - - - $7.5\times10^{-3}$M EDTA

**Fig. 2. Biosynthesis of thiamine**

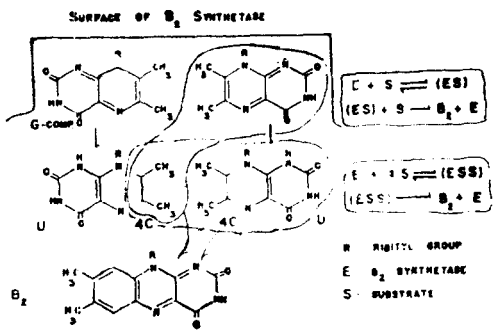
*1) OMP=2-methyl-4-amino-5-hydroxymethylpyrimidine
*2) Th=4-methyl-5-β-hydroxyethylthiazolium chloride
*3) TDP=thiamine diphosphate

the reaction, but its action is also removed by $Mg^{2+}$. This finding represent that target site attached by ATP in thiamine synthetic pathway is a reaction catalyzed by thiamine monophosphate pyrophosphorylase. It is fairly conceivable that the physiological significance of this inhibition by ATP may be related to the metabolic regulation of thiamine synthesis in plants. Thiamine pyrophophate is formed from hydrolysis of thiamine monophosphate followed by pyrophosphorylation of thiamine by ATP. Also thiamine pyrophosphate has been known to be contained as a coenzyme in some key enzymes comprising photosynthetic, glycolytic pathways and tricarboxylic acid cycle, which function as ATP-yielding machineries in living organisms. From these facts, it is reasonable to consider that the inhibition by ATP of thiamine synthesis is one of the homeostatic mechanisms of ATP-generation at a cofactor level: ATP at higher level would depress thiamine pyrophosphate level by acting as an inhibitor for thiamine monophosphate pyrophosphorylase. Recently, Yamazaki and Hayaishi[12] reported that thiamine pyrophosphatase from bovine liver was activated by ATP as an allosteric effector, and suggested that this enzyme might have a role in a control mechanism of ATP generation through TCA cycle. These relationships can be shown in Scheme 4.

### 4. Biosynthesis of Riboflavin

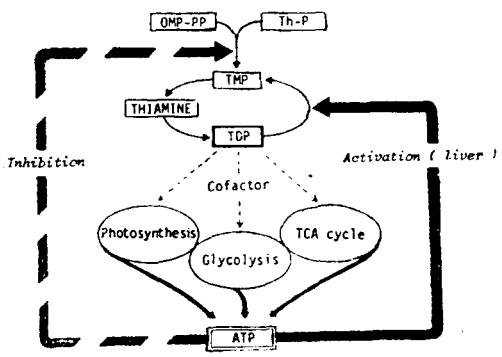
Although it has been recognized that riboflavin is formed from simple precursors in plants and certain microbes, virtually nothing was known about its exact biosynthetic mechanism until recent years. After works made by Maclaren[13] on a fungi, it has been known that a "purin-pathway" does exist in the biosynthetic process in flavinogenic microorganisms such as ***Eremothecium ashbyii*** and ***Ashbya gossypii***. Studies on the vitamin synthesis has been significantly aided by isolation from these microbes of 6,7-dimethyl-8-ribityllumazine and 6-methyl-7-hydroxy-8-ribityllumazine.[14,15] The former lumazine was established as an immediate precursor to riboflavin in these microbes. Practically, however, no information was available for the vitamin synthesis until the present authors initiated their works on this problem.

During our studies on distribution of flavine in plant and animal tissues,[16] a green fluorecent substance besides riboflavin derivatives was isolated from young wheat leaves, and a blue fluorescent compound from spinach leaves. Their chemical structure was identified as 6,7-dimethyl-8-ribityllumazine[16] and mono-*p*-coumaryl-*meso*-tartaric acid.[18]




Scheme 5. Reaction with riboflavin synthetase

The authors demonstrated that extracts from chinese cabbage, wheat leaves, lettuce, radish leaves and spinach catalyze the conversion of 6,7-dimethyl-8-ribityllumazine to riboflavin, and this reaction does not require any substrates or cofactor except the substrate.[19] In the conversion, two molecules of the lumazine are consumed to yield one molecule of riboflavin. This stoichiometry suggest that the lumazine itself acts both as donor of 4-carbon unit and acceptor of the carbon to complets the o-xylene ring of the vitamin.[20,21] This mechanism is shown in Scheme 5. The some mechanism has been proved to operate in




Scheme 4. Possible control mechanism by thiamine coenzyme level in energy metabolism

microbial systems about at the same time as in plant system.[22] Although such a curious mechanism has aroused interest of numerous investigators, few attempt to isolate and purify the enzyme, riboflavin synthetase, has been made except by the authors with spinach,[23] and by Plant and his co-workers with baker's yeast and *A. gossypii*.[24] An example of the enzyme purification is given in Table 3. The stoichiometric relation in the enzyme reaction has been found to remain unchanged after the enzyme is purified highly.[25]

**Table 3. Purification of spinach riboflavin synthetase (presented to 5 kg spinach leaves)**

| Stage | Total protein (mg) | Activity | | Recovery of activity(%) | Degree of purification |
|---|---|---|---|---|---|
| | | Total (units) | Specific (units/mg prot) | | |
| 1 | 43,235 | 9,517.8 | 0,022 | 100 | 1 |
| 2 | 18,117 | 7,058.8 | 0.39 | 74.4 | 17.7 |
| 3 | 10,518 | 5,833.6 | 0.55 | 61.3 | 25.0 |
| 4 | 7,182 | 5,353.8 | 0.75 | 56.3 | 34.1 |
| 5 | 5,297 | 4,140.4 | 0.78 | 43.5 | 35.5 |
| 6 | 522 | 2,635.4 | 5.05 | 27.7 | 229.5 |
| 7 | 117 | 1,760.8 | 15.05 | 18.5 | 684.1 |

1. Extraction with phosphate buffer (pH 7.0)
2. Ammonium sulfate fractionation (0~38%)
3. Protamine sulfate treatment
4. Ammonium sulfate fractionation (23~40%)
5. CM-cellulose treatment
6. First DEAE-cellulose column chromatography
7. Second DEAE-cellulose column chromatography

Also, 6,7-dimethyl-8-ribityllumazine can be metabolized in green leaves to 6-methyl-7-hydroxy-8-ribityllumazine.[17] This reaction is characterized as a dehydrogenic demethylation of the substrate by the quinones, which can be regenerated by coupling with polyphenol oxidase system in green leaves.[25,26] It is fairly conceivable that the levels of riboflavin synthetase and this oxidase together with the physiological condition of the tissues determine the amounts of the dimethyllumazine to be shared between two metabolic pathways. The authors have shown that the activity of the hydroxylumazine formation is by far higher in green leaves than that of the vitamin yield, and that aerobic condition favours to form the hydroxylumazine, whereas anaerobic condition such that the reaction system contains cysteine and ascorbate as antioxidants leads to produce exclusively the vitamin (as shown in Fig. 3).[20] This finding suggestes that ascorbate-ascorbate oxidase system in plant tissues may play an important role on the metabolism of the dimethyllumazine, since the levels of ascorbate and this enzyme are very high in plant system, and balance between the vitamin and its oxidised form may be controlled by the oxidase system.[28] It should be noted also that the hydroxylumazine acts as a potent competitive inhibitor on riboflavin synthetase, suggesting that the rate of the vitamin production is strictly competed by the formation of the hydroxylumazine and hence

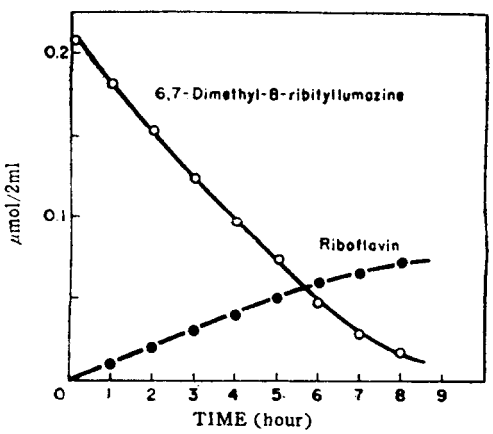
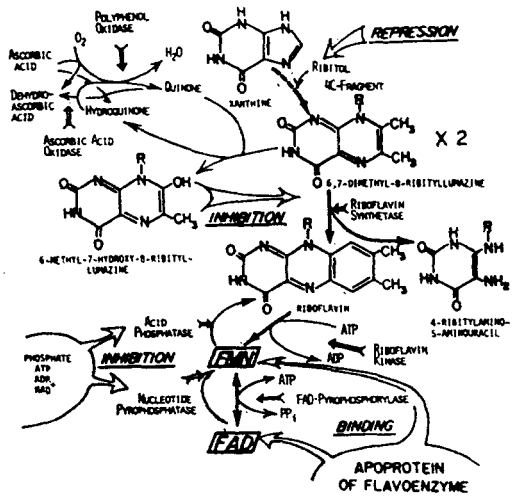



**Fig. 3. Enzymatic consumption of 6,7-dimethyl-8-ribityllumazine and production of riboflavin in the presence of cysteine and ascorbic acid**

affected by the systems concerned with the formation of this lumazine. These relationships can be shown in Scheme 6.




**Scheme 6. Pathway and regulating system in the biosynthesis of riboflavin and related compounds**

As given in Table 4. liver homogenates from human, monkey, guinea pig and ox have an enzyme activity to yield riboflavin from 6, 7-dimethyl-8-ribityl-lumazine, but not the homogenate of rat.[26]

**Table 4. Conversion of G-compound to riboflavin by liver homogenate**

| Animals | $B_2$ formed (in 2 ml) |
|---|---|
| Human (30, ♂) | 0.80 μg |
| Human (2, ♀) | 0.50 |
| Monkey | 1.60 |
| Guinea pig | 0.15 |
| Ox | 3.50 |
| Rat | 0 |

Homogenate 1.0 ml, 0.05M phosphate buffer (pH 7.0) 0.95 ml, G-compound 0.05 ml (32.6 μg). in 2.0 ml were incubated at 30°C for 12hrs.

Synthetic mechanism of 6, 7-dimethyl-8-ribityllumazine from any purine derivative has never been elucidated. The major difficulty that, as yet, no enzyme system able to catalyze the lumazine formation has been obtained in cell-free extracts. The latter part of this chapter will deal with discussion of a regulatory mechanism how the relative constancy of flavin coenzymes in their contents is maintained in plant tissues.

Activities of the enzymes in spinach leaves, which take part in synthesis of riboflavin coenzymes and degradation of these compounds, are summarized in Table 5 together with some properties of the respective

**Table 5. Activities of the enzymes for the biosynthesis and hydrolysis of flavin compounds in spinach leaves**

6, 7-Dimethyl-8-ribityllumazine $\xrightarrow{(1)}$ Riboflavin $\underset{(3)}{\overset{(2)}{\rightleftarrows}}$ FMN $\underset{(5)}{\overset{(4)}{\rightleftarrows}}$ FAD

(1) Riboflavin synthetase (riboflavin formed)
(2) Riboflavin kinase (FMN formed)
(3) Acid phosphatase (FMN hydrolyzed)
(4) FAD pyrophosphorylase (FAD formed)
(5) Nucleotide pyrophophatase (FAD hydrolyzed)

| Enzyme | Activity* (mμmol/hr/g leaves) | Optimum pH | *Km* (m) | Reversibility |
|---|---|---|---|---|
| (1) | 3.4 | 7.5-8.2 | $4.5\times10^{-5}$ | Irreversible |
| (2) | 17.5 | 8.4-9.1 | $1.3\times10^{-5}$ | 〃 |
| (3) | 18,400.0 | 5.5 | $2.0\times10^{-4}$ | 〃 |
| (4) | 0.4 | 8.0 | $6.4\times10^{-5}$ | Reversible |
| (5) | 560.0 | 5.5 | $4.0\times10^{-5}$ | Irreversible |

*The enzyme activity was assayed under the optimal condition of each enzyme reaction except that the reaction mixture was incubated at 30°C.

enzymes.[27] Both hydrolytic enzymes, acid phosphatase and nucleotide pyrophosphatase are present in the leaves with considerably higher activities than the biosynthetic enzymes, riboflavin synthetase, riboflavin kinase and FAD pyrophosphorylase. On contrary to this fact, the levels of flavin coenzymes in living organisms have been well known to be mained at a certain level, and thus it is quite feasible to assume a some regulation mechanism which may serve to explain the observed discrepancy. ATP is essential for synthesis of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are catalyzed by riboflavin kinase and FAD pyrophosphorylase, respectively. As shown in Table 6, it is found that hydrolysis of FMN by acid phosphatase to riboflavin and inorganic phosphate is remarkably blocked by inorganic phosphate itself and also by a number of nucleotides such as ATP, ADP and AMP. The rate of FAD hydrolysis by nucleotide pyrophosphatase is also depressed in competitive fashion by the nucleotides having an adenosine pyrophosphate moiety in their molecule, such as ATP, ADP and NAD. It is very likely that ATP favors to accumulate FMN and FAD by preventing their hydrolysis and serving as a reactant in the synthetic processes. The level of ATP in the cells, as affected by metabolism of the cellular components, plays an important role in controlling the flavin coenzymes levels *in vivo*.

It is well known that flavin coenzymes exist in the forms bound to proteins. It is worthwile thus to know whether the flavins of bound forms serve as substrates especially for hydrolytic enzymes. The authors[28] found that neither activity of the glucose oxidase from *Aspergillus notatum* is decreased nor free FMN is liberated from FAD bound to the enzyme after incubation with nucleotide pyrophosphatase of spinach. The observed fact that FAD bound closely to the native proteins is not attacked at all by the hydrolytic enzyme, provides a reasonable basis for an assumption that levels of flavin coenzymes are regulated both by the amount of apoprotein of flavin enzymes present and available in the cells and by the magnitude of dissociation constant of the holoenzymes.

Other factor enumerated to affect the level of riboflavin coenzymes is pH value in the intact cells. As shown in Table 5, the synthetic processes of the coenzymes predominantly favor to go on in neutral to alkaline regions, whereas this is not the case in acidic regions.

**Table 6. Inhibition of FMN and FAD hydrolysis**

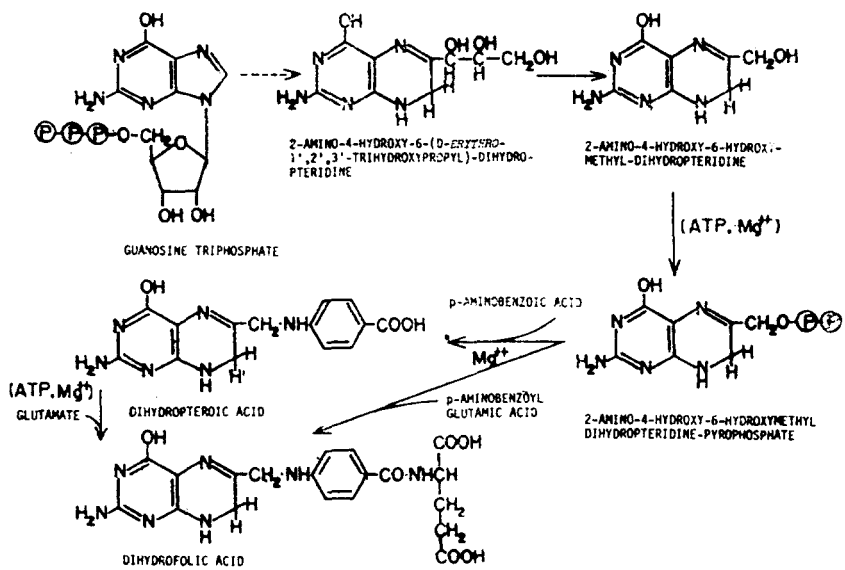
| Inhibitor | | Acid phosphatase | Nucleotide pyrophosphatase |
|---|---|---|---|
| Orthophosphate | $10^{-3}$m | 52.0% | 20.2% |
| Pyrophosphate | 〃 | 52.0 | 55.6 |
| ATP | 〃 | 75.0 | 89.9 |
| ADP | 〃 | 66.0 | 86.2 |
| AMP | 〃 | 50.0 | 43.3 |
| NAD | 〃 | 0 | 80.0 |
| NADP | 〃 | 0 | 73.2 |

*4) FMN=flavin mononucleotide
*5) ADP=adenosine diphosphate
*6) AMP=adenosine monophosphate
*7) FAD=flavin adenine dinucleotide

### 5. Biosynthesis of Pteridines Including Folate

The pteridine compounds have been known to be widely distributed over living organisms. The pteridines occurred in nature can be classified mainly into two groups: 2-amino-4-hydroxypteridines and 2,4-dihydroxypteridines (lumazines). The typical representatives of the first group are riboflavin and its closely related lumazines, and those of the second are antianemia factors, folate derivatives, and unconjugated pteridines. Although completely not clarified as mentioned before, biosynthetic pathway of lumazines might be tightly linked in the earlier step(s) with that of 2-amino-4-hydroxypteridines. It has been demonstrated that a preformed purine can be converted into riboflavin through 6,7-dimeth1-8-ribityllumazine with loss of only carbon 8 of the purine ring. Other purine carbons and the ring nitrogens appear finally in the pyrimidine moiety of this vitamin. The same finding has been presented with unconjugated 2-amino-4-hydroxypteridines and pteridine portion of folate. Indeed, Brown et al. with *E. coli* system,[29] Shiota et al. with *Lactobacillus plantalum*,[30] and the present authors with plant[31] system showed enzymatic synthesis of dihydrofolate from guanosine nucleotides, in which an early step is loss of carbon 8 of GTP as formate.

Outline of biosynthetic mechanism from guanosine nucleotides to dihydrofolate in plant has been established by the authors. Studies on the effectiveness of various synthetic pteridines in the enzyme reaction have led to a proposal that the over-all pathway involves as intermediates, 2-amino-4-hydroxy-6-(*D*-erythro-1′, 2′,3′-trihydroxypropyl)-7, 8-dihydropteridine, 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine and the pyrophosphate ester of the latter compound.[31] The powerful support for this mechanism has been offered by the inhibition tests with three types of inhibitors specific to act on the respective different enzymatic steps.[32] Formation of pteridine skeleton from GTP consists of the following steps: (a) cleavage of the imidazole ring of GTP with loss of carbon 8 to give 4-(5′-triphosphoribosyl)amino-2, 5-diamino-6-hydroxypyrimidine, (b) Amadori rearrangement of the ribose residue to yield a triphosphorylated derivative of the corresponding 1′-(substituted)-1′-deoxy-*erythro*-pentulose and (c) cyclization of this 2′-ketose to form 2-amino-4-hydroxy-6-(D-*erythro*-3′-triphosphoryl-1′, 2′-dihydroxypropyl)-7, 8-dihydropteridine. After dephosphorylation of this pteridine, the trihydroxpropyldihydropteridine yielded is attacked by an aldolase to form 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine. This pteridine is pyrophosphorylated by ATP in the presence of $Mg^{2+}$ and couples with *p*-aminobenzoate to give dihydropteroic acid, a direct precursor of dihydrofolate. These mechanisms are shown in Scheme 7, which agrees well with one for microbial systems.



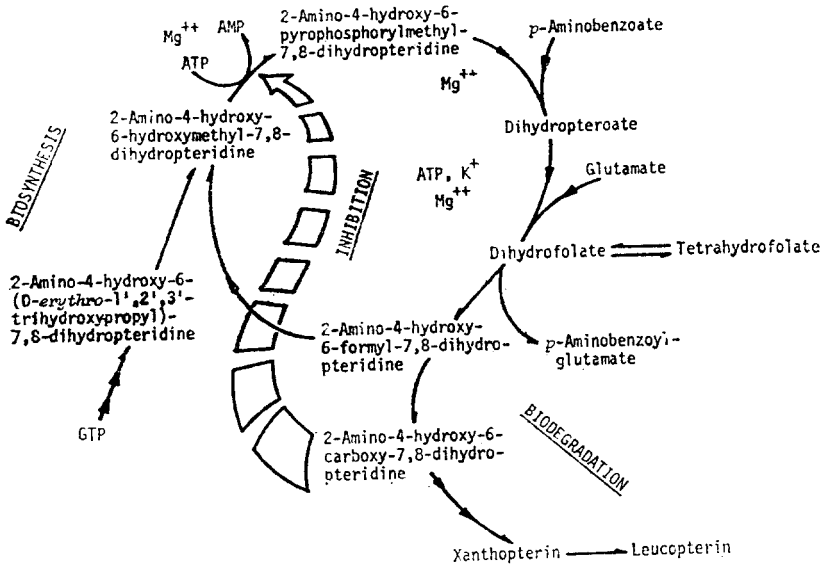

**Scheme 7. The pathway of dihydropteroic acid biosynthesis**

The authors found that 2-amino-4-hydroxy-6-carboxy-7, 8-dihydropteridine inhibits dihydrofolate synthesis from GMP by specific attacking an enzymatic reaction whereby 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine is pyrophosphorylated.[33] The oxidized form of the inhibitor is ineffective on this reaction. Also, the carboxydihydropteridine can block strongly *E. coli* growth.[34] These findings suggest that the inhibitor blocks the bacterial growth by antagonizing utilization of the hydroxymethyldihydropteridine for dihydrofolate synthesis.

Enzymatic transformation of 2-amino-4-hydroxy-6-formyl-7, 8-dihydropteridine to 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine, an established intermediate in folate synthesis, has been shown by the present author with *E. coli* system.[35] This reaction requires NADPH as an essential cofactor. As described before, however, the formyldihydropteridine is not a member of the intermediates in the main dihydrofolate synthesis from GTP. Biological significance of the transformation must be clarified. An hypothesis to account for the discrepancy has been

proposed by the authors:[36] the formyldihydropteridine, but not its oxidized form, might be a direct product from dihydrofolate in its catabolism, and could be metabolized into the hydroxymethyldihydropteridine and the carboxydihydropteridine. The former reaction might provide a salvage route for reutilization of the catabolic product, whereby living organisms can be protected against a futile wastage of their metabolites. The latter might have another kind of biological significance from a viewpoint of regulation of folate level *in vivo*, because the carboxydihydropteridine can act as a potent antagonist against dihydrofolate synthesis *in vivo* and *in vitro*. Overproduction of folate coenzymes exceeding their metabolic needs might cause to promote their degradation, allowing to elevate the level *in vivo* of the inhibitor. The rate of dihydrofolate synthesis can be reduced at the pyrophosphorylating step of the hydroxymethyldihydropteridine to maintain the physiological levels of the coenzymes in the cells. This mechanism can be characterized as a kind of feed-back inhibitions by the product of folate catabolism. These relationships are shown in Scheme 8.

The authors found that inhibition of *E. coli* growth by 2-amino-4-hydroxy-6-carboxydihydropteridine can be effectively reversed by 2-amino-4-hydroxy-6-formyldihydropteridine, but the growth is blocked by the formyldihydropteridine in the absence of the carboxydihydropteridine.[34] This contradictory finding is well explained by the proposed hypothesis as




Scheme 8. Pathway and regulatory system in the biosynthesis and biodegradation of dihydrofolate

mentioned before. From this finding, the salvage route able to convert the formyldihydropteridine to the hydroxymethyldihydropteridine can function dominantly when drop in folate coenzyme level *in vivo* caused by inhibition of the main folate synthetic pathway. The microbe under the normal growth can get chiefly folates by the pathway from GTP, and the formyldihydropteridine supplied may be metabolized to the carboxydihydropteridine in the cells. This conversion is likely to proceed under the reduced level of the pteridine, but not under their oxidized ones, since the oxidized form of the carboxydihydropteridine fails to affect *E. coli* growth. Very recently, Mathis and Brown[37] reported that the formyldihydropteridine inhibits strongly dihydroneopterin aldolase in dihydrofolate synthesis. This finding may give explanation from another point of view for the dualism in the action of the formyldihydropteridine on dihydrofolate synthesis.

### 6. Vitamin C in Plants

A dietary dificiency of vitamin C, ascorbic acid, has been well known to cause scurvy only in the human, the guinea pig, and the monkey; other mammals are capable of synthesizing their needs. Also, the higher plants synthesize the vitamin, but microorganisms don't apparently form the vitamin or require it for growth. In spite of the relatively early laboratory synthesis of vitamin C, we still know very little about its specific functions in the body. No available knowledge about biochemical significance in plant tissues of the vitamin has been clarified. The studies carried out by the author aimed to elucidate the physiological function of the vitamin especially in plant tissues.

When the author initiated in 1936 his works on ascorbate,[38] only a few report had been presented with vitamin C in the daily foodstuffs such as tea, fruits and some vegetables, whereas little was known about it on plant green leaves. Also, there had been a difficulty in determination of the vitamin in red-colored fruits such as strawberry and fig by titration method with 2,4-dichlorophenol-indophenol: the extracted redish-pigments interfered usually with confirming the end point of the titration. Furtheremore, the indophenol method had a very weak point that the phenol aqueus solution was very unstable. The author found that the first defect could be easily removed by treating the samples with Japanese acid clay before providing for the vitamin determination, and that the phenol was extremely stabilized in a mixture of butanol and water. These facts stimulated the author to establish a spectrophotometric method for micro-determination of the vitamin in colored samples.[39]

Distribution of ascorbate in the vegetables was shown by using the spectrophotometric method. As shown in Table 7 and 8, it was found that the fresh green leaves in persimmon, wisteria, amaranth, tea and rice-plant contained about two to twenty-fold higher contents of vitamin C than in orange, and that the vitamin contents in the younger leaves markedly exceeded than in the mature leaves. The vitamin levels in flowers of pumpkin, lily, azalea, sweet flang, ilis, rape, sweet pea and pansy were high. These findings definitely indicate that ascorbate is localized in plant tissues with high metabolic activity, and its content was closely linked with the change of the tissue activity. The metabolic activity in green leaves might be terribly influenced by light. Table 9 represents effect of light on the vitamin content and catalase activity in green leaves of wheat and pumpkin. Shading the leaves only for 24 hours resulted in decrease in the vitamin content and catalase activity. The similar relationship among the levels in chlorophyll, catalase and the vitamin was detected in amaranth, which discolored its green leaves with its growth. As shown in Table 10, the leaves discolored into yellowish-red color involved only trace of chlorophyll, and lower amounts of the vitamin and catalase activity than the green leaves.

**Table 7. Ascorbic acid content (mg%) in various leaves**

| Sample | Total | Reduced form | Oxidized form |
|---|---|---|---|
| Persimmon | 846.0* | 612.4 | 233.6 |
| | 390.4** | 271.1 | 119.3 |
| Wisteria | 270.0* | 166.5 | 103.5 |
| | 142.3** | 69.2 | 73.1 |
| Amaranth | 214.9 | 64.3 | 150.6 |
| Fresh leaf of tea | 145.4 | 65.7 | 79.6 |
| Rice-plant | 107.1 | 28.2 | 78.9 |
| Orange juice | 41.2 | 32.0 | 9.2 |

*Young leaf **Mature leaf

**Table 8. Ascorbic acid content (mg%) in various flowers**

| Sample | Total | Reduced form | Oxidized form |
|---|---|---|---|
| Pumpkin | 46.1 | 10.8 | 35.3 |
| Lily(white) | 44.1 | 2.4 | 41.6 |
| Azalea | 36.2 | 11.5 | 24.7 |
| Sweet flag(Light purple) | 42.0 | 37.5 | 4.5 |
| Ilis | 85.4 | 68.3 | 17.2 |
| Rape | 86.5 | 39.8 | 46.4 |
| Sweet pea(Light purple) | 58.6 | 4.6 | 54.0 |
| Pansy (yellow | 133.9 | 114.8 | 19.1 |
| Pansy purple) | 106.8 | 46.5 | 60.3 |

**Table 9. Effect of light on AsA content and catalase activity in green leaves**

| | Wheat | | | | Pumpkin | | |
|---|---|---|---|---|---|---|---|
| | Total AsA(mg%) | Catalase activities f. | | | Total AsA(mg%) | Catalase activities f. | |
| A | 96.6 | (62.1)* | 820 | A | 110.0 | (82.6)* | 433 |
| B | 75.6 | (50.3) | 514 | B | 106.8 | (51.5) | 257 |
| C | 170.9 | (103.0) | 1032 | C | 163.3 | (35.4) | 406 |
| D | 125.3 | (82.8) | 683 | D | 160.9 | (15.0) | 288 |

B,D: The leaves were covered with black paper for 24 hrs.
* Reduced from of ascorbic acid

**Table 10. Interrelation between chlorophyll, catalase and AsA of amaranth.**

| Chlorophyll (anhydrous) % | Catalase activities f. | Total AsA(mg%) |
|---|---|---|
| 1.46 | 91.4 | 172.9 (73.5+ 99.4)* |
| 1.10 | 100.3 | 182.8 (80.9+101.9) |
| trace | 33.6 | 130.5 (42.8+ 87.7) |
| 1.05 | 109.1 | 189.3 (86.3+103.0) |
| 0.95 | 127.4 | 165.5 (78.5+ 93.7) |
| 1.05 | 134.4 | 190.0 (91.6+ 98.4) |
| trace | 37.7 | 133.4 (76.6+ 56.8) |
| 1.46 | 160.4 | 192.4(100.0+ 92.4) |

* reduced from+oxidized form

Vitamin C contents in various tea products are compared in Table 11,[40] Gyokuro have a third to a quarter of the vitamin involved in Sencha and Bancha which are nearly equal to that in the fresh green leaves. Only trace of the vitamin is found in black tea. Manufacturing black tea product has been carried out though a step of fermentation of green tea leaves, during which the vitamin can be easily destroyed by action of such enzymes as ascorbate oxidase etc. On the contrary, the processes for green tea production includes in an early step steaming of the raw green leaves, whereby the enzymes destructive to act on the vitamin are rapidly inactivated. These are a fundamental reason to explain loss of ascorbate in black tea and presence of the equal amounts of the vitamin to that in the raw leaves. Tea green leaves supplied for making Gyokuro have been grown usually by shading the leaves from light, and so contains a slight amounts of vitamin C. At that time when the author started his workes on the vitamin, it had been believed that the vitamin might be very unstable against heating. But the author[41] found that ascorbate was indeed extremely unstable against oxidation, but relatively stable against heating under anaerobic condition, and that rapid heat treatment of green tea helps to maintain the vitamin rather under stabilized state by inactivation of oxidases.

**Table 11. The content and availability of AsA of various kinds of green tea**

| Sorts of tea | AsA contents mg.% | Conditions of infusion | | | | Availability of AsA(%) | |
|---|---|---|---|---|---|---|---|
| | | Sample g. | Amount of liquid ml | Temperature of liquid | Time min | First infusion | Second infusion |
| Gyokuro | 84.4 | 3 | 100 | 60°C | 5 | 49.2 | 30.6 |
| Matcha | 154.2 | 1 | 50 | | ** | 97.7 | — |
| Sencha | 334.4 | 3 | 100 | 100°C | 1 | 53.1 | 31.1 |
| Kawayanagi* | 281.4 | 3 | 100 | 100°C | 3 | 54.1 | 24.0 |
| Black tea | trace | — | — | — | — | — | — |

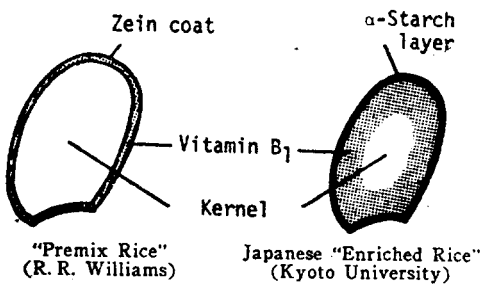
* Kawayanagi is a green tea of inferior quality. **Added hot water and stirred for 1 min.

### 7. Enrichment of Rice with Vitamin $B_1$ and Lysine

Since vitamin $B_1$ plays an essential role in the metabolism of carbohydrate, it is very suggestive that 86% of natural vitamin $B_1$ supplied through diets in Japan is produced by tissues rich in starch such as rice and wheat grains and potato tubers. And it seems to be quite rational that rice-eating races make a particular effort to enrich with vitamin $B_1$. Although half-polished rice contains enough vitamins, its digestibility, taste and resistance against rice weevill are inferior to polished rice.

In South Eash Asia it has long been a common practice to parboll rice, resulting unintentionally in enriching the polished rice. The authors invented an acid-parboiling method to transfer vitamin $B_1$ of bran and embryo into endosperm. As an application, a method, to add the vitamin into polished grains by penetration has been originated in the authors' laboratory,[42~44] or the vitamin may be smeared on the surface by an alternative method started by Williams[45] in the United States(see Scheme 9). Each of these two methods have strong and weak points. The most serious defect of the former is cracking of the grain, which damages the appearance; causes the grain to break and increases the loss of the added vitamin in washing and cooking. When, however, organic solvents like acetone or ethanol, were employed as soaking medium, no crack was formed. Mixing water in the solvent up to 30 to 40 volume per cent did not affect this favorable property. Since water-insoluble vitamin $B_1$ derivatives are soluble in these solvents, the loss of the vitamin in washing and cooking could be completely eliminated.

As this improved method is introduced, the $B_1$-enriched rice is being widely adopted in Japan and contributes to the promotion of the health of the entire nation.




**Scheme 9. Structure of vitamin $B_1$-enriched rice grains**

Even now, rice supplies Japanese 25% of their total protein uptake. The authors has demonstrated by using electron-microscopy that most of protein in rice endosperm is located in "protein bodies", the subcellular storage particles (see Fig. 4).[46,47,48] It is theoretically and experimentally well known that the rice protein is the best in terms of nutritive quality and the protein quality can be further improved by supplementation with lysine. No rice kernel enriched with lysine by means of a premic formula has been commercialized as yet, however. This appears to be due to the technical difficulty of applying relatively large amounts of this ingredient to the grain.

The soaking method developed for the production of vitamin-enriched rice can be effectively employed for this purpose.[49] By employing a 1% acetic acid solution saturated with lysine as soaking medium, one can produce kernels enriched with lysine up to 66 mg per g of kernel. The steaming process is effective in preventing loss of lysine in washing. Threonine, a second limiting amino acid in rice for human nutrition, can be introduced together with lysine by the soaking method. The product obtained after processing the kernel first in lysine solution and then in a solution of water-insoluble vitamin $B_1$ derivatives is found significantly resistant to washing. The technique for enrichment by this double-soaking method is quite feasible for fortifying staple food with both vitamin $B_1$ and lysine. Its appearance is close to that of ordinary white rice; neither cracking nor discoloration is detectable.

Enriched rice with vitamin $B_1$ and lysine will contribute to the improvement of protein malnutrition found in rice-eating nations of Asia, and to the promotion of their health.

*Addendum: An Application of Vitamin $B_1$ for the Crop Yield of Rice.*

In Japan we have long been studying biochemistry of vitamins in plants, as mentioned in the previous Chapters. For one of examples, physiological significance of vitamin $B_1$ had been elucidated by following the change in its content and behavior in different

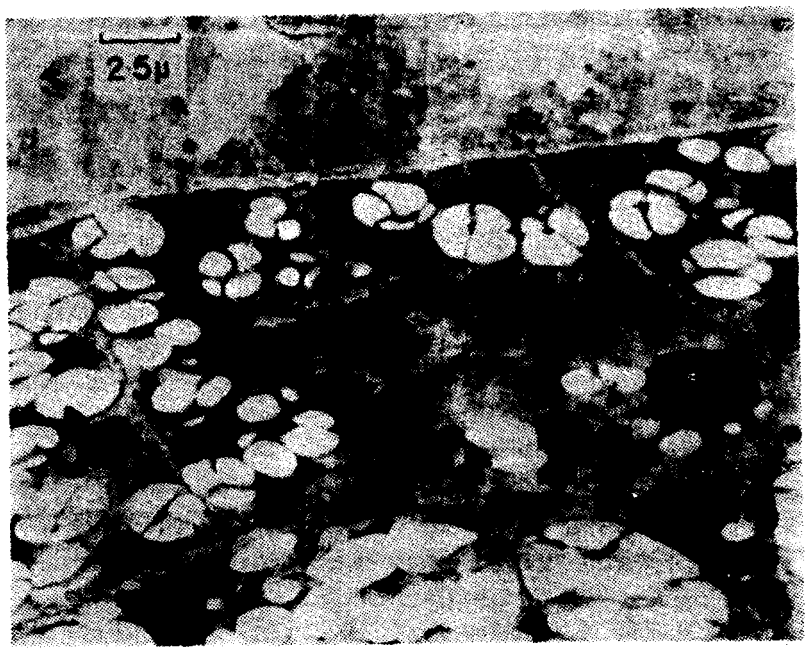
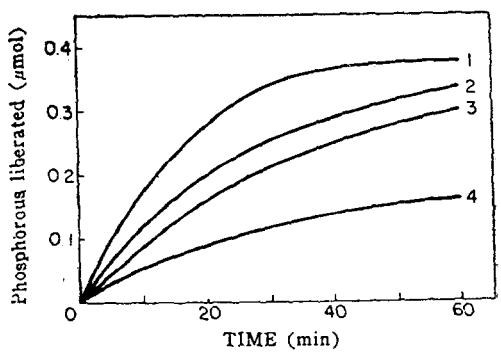



**Fig. 4. Electron-Micrograph of the periphery of young rice endosperm fixed with 1% osmium tetraoxide. The thin sections were stained for 20 min. with uranyl acetate saturated in 50% ethyl alcohol solution. Electron-dense and translucent bodies represent protein and starch granules respectively.**

part of plant over the entire period of growth in connection with its biosynthesis. As an application we have established that vitamin $B_1$ sprayed on leaves is effective in increasing the crop yield of rice, wheat and barley, as well as in strengthening pest resistance and in preventing harmful effects of agricultural chemicals to the plants.[50]

### 8. Preservation of Inosinic Acid in the Raw Food Products by Nucleotides.

Now, inosine-5′-phosphate (IMP) is used as a new chemical seasoner added to foodstuffs, as well as sodium glutamate. However, IMP added has been known to be gradually hydrolyzed by phosphatase present in raw diets, and this causes to decrease the seasoning effect of the nucleotide. As described in the previous Chapter, the authors found that hydrolysis of FMN by acid phosphatase could be blocked by ATP and other nucleotides. This finding can be employed for preventing the IMP decomposition.[27] Although ATP is the most effective to inhibit acid phosphatase acting on IMP as shown in Fig. 5, ATP may be unpractical to be used as a diet additive, since this compound is very expensive. ADP, AMP and




0.35μmol of IMP. 0.5μmol of $MgSO_4$, 0.2ml of 5′-ribonucleotidase, 95μmol of Tris buffer(pH 8.5) and various amounts of ATP in 2.2ml; 1) no ATP, 2) ATP 0.005μmol, 3) 0.01μmol, 4) 0.05μmol

**Fig. 5. Inhibition of 5-ribonucleotidase by ATP**

UMP can similarly inhibit phosphatase, and may be rather favorable as the additives used in respect of their lower cost compared with that of ATP. Exactly, addition of these nucleotides can maintain the seasoning effect of IMP in the raw food products.

### 9. Prevention of the Development of Haze in Beer by Vitamin C

In 1937, the author found that vitamin C added to bottled beer was very effective in preventing the development of haze in beer. The beer haze appears when a minute amount of oxygen dissolved in beer deteriorates protein and causes it to precipitate during storage in summer. The added ascorbate prevents it by removing the oxygen through the strong reducing action of its endiol group. Furtheremore, the ascorbate was found to be stabilized by carbonic acid in beer and the coloured bottle was expedient to protect the vitamin from the destructive action of sunlight. However, at this time, 15 to 20 mg of the vitamin to be added to a bottle of beer costed more than five times of the price of the beer. Naturally this situation made my idea look quite unpractical about thirty years ago. But now the cost of the vitamin does not add anything more than 0.05% to the cost of beer. And two-sided beneficial effects of the vitamin, one in nutritional and the other in quality improvement, makes it an indispensable additive to beer now.[51]

### 10. The Development Researches Founded on Basic Research on Catalase: a New Storage of Cereal Grains and a New Utilization of Microbial Protein as Foodstuff

Animals are endowed with the inherent devices to survive unfavorable or even lethal conditions in the surroundings. One of these is hibernation, by which animals can be protected against a futile wastage of energy leading to their death: in the unfavorable conditions animals become into an inactive state of physiological and metabolic functions, and again they can be reactivated by improvement of the surrounding conditions or by their inherent capacity. This is a common phenomenon observed with poikilotherms. Differed from homoiotherms can not regulate their body temperature, and the temperature is essentially indentical with that of their surroundings. Thus, the internal metabolism in poikilotherms are very easily influenced by the change of the enviromental temperature. Hibernation is thought as an unavoidable adaptation to the surrounding changes for the survival of poikiotherms. The similar phenomena have been observed with some mammals.

The authors have been extremely interested in essential differentiate in catalytic properties of the enzymes existing between in homoiotherms and in poikilotherms. To this aim, the authors tried to isolate catalases in crystalline states from ox and toad livers.[52] In the course of studies on the catalytic properties of these enzymes, the authors found that the maximal activities of these enzymes are closely related with their body temperature: 40° for ox catalase, and 15° for toad catalase. These findings suggested that ox enzyme differs markedly from toad enzyme in respect to the fundamental protein conformation, and that an aspect of differentiae in adaptation to the enviromental changes between homoiotherms and poikilotherms are reflected in the level of the enzyme molecule. Also, the authors observed the similar phenomenon with the catalases isolated from various summer and winter vegetables.

From the works on catalase, the authors have get two precious fruits leading to food and agricultural development researches. Highly-training techniques acquired are successfully employed for the isolation and the utilization of microbial cell protein (MIPRON) for human food.[53] Conception of hibernation is led to the under-water and under-ground storage of cereal grains.[54] These problems will be presented in detail by our other articles.

## Summary

Reviewed biosynthetic pathways in green leaves of thiamine, riboflavin and folate as disclosed by the authors, and the regulatory systems operating on their biosynthesis and biodegradation as revealed since a potent inhibitory activity of ATP was found for the phosphatase reactions in 1965. Physiological roles of ascorbic acid in plant were evidenced by its higher content in green and flower leaves; the content in persimmon leaves for example was found ten or more

times of that in citrus fruits. A close relationship existing between basic and development researches was illustrated by examples chosen from the author's researches. Examples cited were as follows; basic researches on vitamins were extended to the creation of thiamine enriched rice, to the growth promotion of rice plant by foliar application of thiamine, and to the use of ascorbic acid to prevent the appearance of beer cloudness; histological and biochemical studies on protein bodies in rice endosperm turned out amino acid enriched rice; techniques acquired in the studies on catalase were successfully employed for the utilization of single cell protein (MIPRON) for human food; conception of hybernation was led up to under-water or under-ground storage of cereal grains.

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