Special Lecture

On the Histochemistry of Vitamin

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Despite of effects for localizing vitamins in the tissue, histochemical demonstration has only been in vit A, B-2, and C. Recently, techniques have been successively devised. Most of them have been performed in our laboratory. In this paper progress of histochemistry of vitamins has been dealt with.

Histochemistry of fat soluble vitamin.

1) Vitamin A and carotene

Histochemistry of vitamin A is firstly reported by Querner using fluorescence microscope and by Jojet-Lavergne and others with Carr-Price reaction. But there are still several disadvantages in these methods, especially in the later. In 1950, glyceroldichlorhydrine method has been developed by Takada and Katsui (1). They observed abundant vit A, distributing in the pyloral appendix of fish. Their findings have resulted in contribution to vitamin A industry. The superiority of this method has been confirmed by Araki and Tsuji and developed for the studies on the different spieces. Furthermore, Araki and others have improved fluorescence method and succeeded in observing both vitamin A and carotene in the animals (3, 4). They discovered intracellular transformation from carotene to vitamin A. In their observations they concluded that the transformation occurs in the columnar epithelium of small intestine.

After being absorbed, vitamin A is esterified in the columnar epithelium of the small intestine. On the absorption of vitamin A, they described that it is absorbed from columnar epithelium and esterified in the same cell. In addition, it is also described that esterification may occur in the histiocytes of submucosa. And then esterified vitamin A is transported into the general circulation through the lymph channel. Esterified vitamin A which in the liver the esterified vitamin A is taken into the Kupffer cells firstly, where the vitamin is transformed to A-alcohol by esterase and transported to the liver cells. Hence, the vitamin is found as alcohol form in liver cells (3). When the animals are fed with vitamin deficient diet, A-alcohol of liver cells is released, showing marked decrease of A content. At the same time, utilized form of vitamin A in Kupffer cells is transformed to A-alcohol and then transfered into liver cells in which the vitamin is successively released (5).

The results obtained by electronmicroscopic observation on the absorption of carotene are as follows (6). In lumen of small intestine the fat droplets
containing carotene are decreased in size with approaching the epithelium. The droplets measure about 75Å in diameter at the level of micro villi and penetrate through the plasma membrane.

They are taken by small vesicles in the cytoplasm. The fat droplets increase the size in the lumina of small vesicles and are gradually transferred into deeper portion of the cell. In Golgi field they become to be large granules measuring about 4µ in diameter. Accompany this progress, mitochondria of upper portion of the cells approach to the vesicles containing carotene and move into deeper portion with them. In this stage, fluorescent microscopical observation reveals that near the Golgi field fat droplets lose green fluorescence of carotene and become to have yellowish fluorescence as vitamin A (7). They pass through the slit at the terminal bar, which is located at 8µ deeper portion from brush border, being discharged into lymph space. From above-mentioned results, it is suggested that carotene is transformed to vitamin A in columnar cells of small intestine. The site portion where the transformation are performed is small vesicle of the cells. In this process, mitochondria, which localized in the apical area, have been considered to have an important function as donator of energy and enzymes.

It is said that vitamin A is essential as a precursor of rhodopsin in retina. Araki has studied the relationship between vitamin A and rhodopsin in the retina of rats, carps, bats and birds in light and dark accommodation (8). In carps and rats which can see both in the light and dark circumstance, the vitamin is demonstrated in the chorioidea and retina. The chorioidea is a significant depot of the vitamin and supplier to retina when the vitamin of retina is used. In the retina vitamin A distributes abundantly in the portion where many rods are detected, and scantly in the peripheral portion of papilla where cones are located. At light accommodation, the vitamin A is mostly found in the granular projection of pigmented cells, moderate reaction in the outer segment of rods, and few in outer segment of cones and granules of rods and cones. At dark accommodation, the reaction of vitamin A in the outer segment of rods is disappeared and a beautiful red rhodopsin is demonstrated in the same part. When the section is exposed to light the red rhodopsin in the retina changes the color to yellowish within 20 minutes, and then becomes to be colorless within 40 minutes. Vitamin A gradually appears when the rhodopsin changes to yellow, and then the reaction of vitamin A becomes to be prominently demonstrated when the color of rhodopsin is disappeared. In the retina of lovebirds and fowls, either vitamin A at light accommodation or rhodopsin at dark accommodation is not demonstrated. In these animals there were many gold-yellow fat droplets in the outer segment of the layer of see cells. These droplets are distributed upwards when the animals accommodate to dark circumstance, and that is converted at light accommodation. Hence, the different mechanisms of the sight is suggested in the birds.

2) Calciferol

Histochemical technique of calciferol utilizing of yellowish primary fluorescence has been performed by Araki et al. (9). The fluorescence of calciferol
is much weaker than that of vitamin A and is less resistant to ultraviolet irradiation, therefore, its fluorescence has been overlooked for a long time. Figures 3 is a photomicrogram of fluorescence of calciferol crystal (D2). Histochemical distribution of calciferol in various animals observed by this technique is as follows: Majority of calciferol is not absorbed at the stomach but at the small intestine. The vitamin granules in resorption epithelium are small in size. Its granules in submucosa and central chylli are larger and have more prominent fluorescence. It is suggested that the vitamin may be combined with fatty acid and transforms to a ester form in submucosa. Absorption of calciferol is influenced by existence of bile juice. No absorption figures are observed in the case which common bile duct has been ligated. Most of absorbed calciferol from the intestine is carried to liver through portal stream. In the liver it is found in liver cells and Kupffer cells of peripheral portion of the acini, and gradually distributed in the centroacinal part. In the cells, calciferol is seen in cytoplasm but not in nucleus. By fat staining it is confirmed that the vitamin is coexisted with fatty substances. Other than liver calciferol is demonstrated in adipose tissue, adrenals, spleen and bone marrow. In adipose tissue the vitamin is found diffusely in cytoplasm. In adrenals it is located in the cortex, especially in zona fasciculata. In spleen it is demonstrated in reticuloendothelial cells. In bone the vitamin is not found in bony substance but only in reticulum cells of bone marrow. The vitamin is excreted chiefly from intestinal canal and liver cells. It is also excreted from the kidney if much dosis of vitamin is given to the animals.

3) Tocopherol

Although Emmerie-Engel, Nair-Mayer and Kofler reactions for tocopherol have been tested on tissue sections by Miyazaki, any of these techniques cannot be used for histochemical procedure. Primary fluorescence method is described as the most excellent method for histochemical observation of tocopherol (10). Fluorescence of tocopherol reveals violet in the intestine and yellowish green in the other organs. Maximal absorption of the former is noted at 445m\( \mu \) in wave length and the later at 540m\( \mu \). In Table 1. and 2 summarizes the physical and chemical characters of fluorescent granules of soluble vitamin. Histochemical results obtained by this method are as follows.

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<tr>
<th>Color</th>
<th>Vitamin A</th>
<th>Carotene</th>
<th>Calciferol</th>
<th>Tocopherol</th>
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<tr>
<td>A(_1) green</td>
<td>greenish</td>
<td>yellowish</td>
<td>violet</td>
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<td>A(_2) brown</td>
<td>greenish</td>
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<td>yellowish green</td>
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<tr>
<th>Resistance for ultraviolet light radiation</th>
<th>weak</th>
<th>strong</th>
<th>extremely weak</th>
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<td>A-alcohol 10 min.</td>
<td>weak</td>
<td>more 30 min.</td>
<td>about 5 min.</td>
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<td>A-ester 30 min.</td>
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Tocopherol which is absorbed from the small intestine is mostly transferred to liver. In liver it is firstly taken in liver cells. In the case of heavy administration, up-take of Kupffer cells is more prominent. In adrenal glands, fat tissue of various organs, distribution of the vitamin is also markedly observed. In testis, a small amount of tocopherol is found in cytoplasm of Sertoli cells. In ovaries, it distributes in the granular cells. In the vitamin deficient animals, the vitamin granules in liver cells are decreased in number at the beginning stage and then decreasing in adrenals is followed. But the vitamin content in the testis is preserved until later period.

**Histochemistry of water soluble vitamin**

1) Thiamine

The marked advance of histochemistry of thiamine has been made by thiochrome method. Araki and Chen (1948), and von Muralt (1943), have devised this method independently (11). Both potassium ferricyanide and cyanogen bromide method are available, but the former is simpler and more effective. In cells, thiamine distributes abundantly in cytoplasm and scanty in nucleus. In cytoplasm, thiamine distributes in mitochondria and diffusely in ground substance, but not in fat granules. In ground substance it is more marked in marginal zone of the cell body. In nucleus, it is detected in nuclear membrane and nucleolus but not in the nuclear plasm. Chen has studied the histochemical distribution of human organs in physiological condition. According to him thiamine distributes in parenchymatous cells of various organs, for instance liver cells in liver, nerve cells in brain, urinary epithelia in kidneys, myocardium in heart (12). Chen and Liao described more detailed distribution figures of nervous tissue (13). In the central nervous system content of the vitamin of nerve cells is different according to the localization of the cells. In cerebral cortex thiamine is mostly found in pyramidal and polygonal cell layer. In basal ganglia it is detected markedly in caudate nucleus, putamen and the nuclei of diencephalon. Fluorescence intensity of nerve cells is also changed according to its functional state. In cerebellum, thiamine is mostly distributed in Purkinje cells, and in spinal cord mostly in motor cells of anterior horn. In peripheral ganglia, both spinal and sympathetic ganglia, the vitamin is richly distributed in nerve cell and poorly in satellite cells. In nerve fibers, thiamine is more in axon and less in myelin sheath. About the Ranvier's nod, some nodes contain abundant distribution of thiamine and some show no distri-
bution. It is suggested that the vitamin enter into nerve fiber through the
nod in the same manner as inorganic matters, i.e. potassium, sodium, chloride
and calcium.

Kuga describes the histochemical figures of thiamine in the process of hepato-
toma formation produced in rats (14). The vitamin is markedly decreased in
early degenerative stage. In the period which exhibits hyperplastic change of
liver cells the vitamin contents are increased in hyperplastic cells. When carc-
inomatous development occurs, the vitamin is prominently decreased in the
lesion.

Ochiai observes the distribution of thiamine of human organs in various
pathological states (15). At dysfunctional state of liver or adrenals the vitamin
contents of organs such as liver, kidney, heart and brain is markedly decreased.
We also studies in same pathological conditions experimentally and describe
that phosphorylation of thiamine is inhibited in such conditions.

Nanzaki reports the vitamin distribution in the animals which are stressed
by such as scald, fracture etc. (16). According to his paper, in "alarm reaction"
the vitamin content in blood firstly decreases and then that of heart, and muscle
eetc. is markedly decreased, in "stage of resistance", the vitamin content recovers
to almost normal level and in the "stage of exhaustion" it is extremely decreased
again.

Taira describes the histochemical distribution of plants and animals and
from ontogenical and phylogenical point of view, and he discussed the specific
distribution pictures of thiamine in animals and plants (17).

2) Riboflavin

For histochemical technique of riboflavin, fluorescence method has been
introduced by Hirt and Wimmer in 1939. But this method is not satisfactory
because of poor differentiation from the other fluorescent substances in sections.
Araki et al has described the new differential method using hydrosulfate or
lumiflavin method (18). Chen and Yamauchi histochemically have devised
phosphatase method for conversion from flavinmononucleoidte and flavinadenine-
dinucleotide to free riboflavin (19).

Yoshikawa investigates the riboflavin distribution in rats which are
administered orally or subcutaneously and observes histochemical figures of
liver, kidney, heart and adrenals etc (20). He also describes little distribution
of riboflavin in human tumor tissues. Kushida certified this finding on the
experimentally produced rat hepatoma (21). He points out that administration
of riboflavin has an inhibitory effect for tumor development. Chen and Liao
report the phylogenical observation of riboflavin in experimental animals (22).
According to them, there are marked differences in the distribution of riboflavin
between the different species and also different individuals. The vitamin is
most abundantly detected in the organs with vigorous enzymatic activities such
as liver, kidney and heart etc. They emphasize that photochemical effect of
riboflavin cannot be overlooked, especially in chorioidea of aquatic animals.

Observing ontogenically, they describe that significantly less vitamin is
demonstrated in early fetal life and the vitamin increased gradually with the
lapse of fetal life, especially marked in the end of pregnancy.

Changes of the distribution pattern of the vitamin in pathological cases, especially in liver cirrhosis, are described; and disturbance of phosphorylation of the vitamin is also discussed (Chen and Liao).

Yamauchi observes the vitamin absorption of rats intestine supravitaly which is kept on riboflavin deficient for a week in order to decrease the vitamin level in the intestinal mucosa. He observes absorption of riboflavin from the jejunum, where riboflavin is partly phosphorylated into FMN in the area between Golgi apparatus and basal mitochondria in mucosal epithelium (23).

Chen and Yamauchi study the separate observation of three form of riboflavin (19). They describe that benzylalcohol is the most suitable for histochemical extraction of riboflavin. For conversion of the esterified riboflavin into the free form, the enzymatic method using phosphatase is the most adequate. According to them riboflavin distributes widely in living body, mostly as esterified form, especially FAD. However, the distribution varies according to the organs. Riboflavin is absorbed from the intestine, whereby it is phosphorylated into FMN, and is transferred to the liver and converted into FAD in the liver. In the carbon tetrachloride poisoned rats, the esterified form of the vitamin is decreased. In eyes, three forms of the vitamin are demonstrated in chorioidea and chiefly FAD and FMN form in retina. In kidneys, free form is abundant in glomeruli, esterified vitamin is abundantly demonstrated in urinary epithelia.

3) Vitamin B₂
Vitamin B₂ has a bluish white fluorescence which has a character of labile to acid and stable to alkali. It loses fluorescence at about pH 3.0 and maintains over the pH 7.0. At the pH 7.0 or more higher level of pH pyridoxine (PIN) and pyridoxamine (PAM) have same colored fluorescence, on the other hand fluorescence of pyridoxal (PAL) is discolored to brilliant yellow. By addition of NaNO₂, the violet blue fluorescence of PAL is changed to yellow but the fluorescence of the other forms of the vitamin are not influenced. These characters are demonstrated both in test tube and on tissue sections. Using these characters the distribution of the vitamin is studied in rats which are orally administered with the vitamin (24). The vitamin is most predominantly taken into liver, kidneys and heart. Up-take of stomach, intestine, pancreas, spleen and brain is also prominent. In Langerhans’ islet of pancreas the vitamin is detected abundantly in β-cells and scanty in α-cells. In the case of alloxan-diabetic rabbits, the β-cells show marked decreasing of the vitamin contents. It is suggested that there is an intimate relationship between insulin production and the vitamin.

4) Folic acid
Folic acid reveals feeble bluish white primary fluorescence which is intensified by addition of potassium permanganate and changed to yellowish green in color decreasing its intensity by HCl. Utilizing these characters a fluorescent method is devised for histochemical study (25). Folic acid is absorbed not only from the small intestine but also from stomach and large intestine. In the stomach the absorbed folic acid is taken into glandular cells. On the other
hand, in deficient animals of folic acid experimentally produced the degenerative changes and hemorrhagic erosion are reported by Chen and Okamoto.

Therefore, it is suggested that folic acid has a special significance in the stomach. In intestine, the absorbed folic acid from columnar epithelia is demonstrated abundantly in histiocytes and leucocytes of submucosa. In the liver it is chiefly taken into liver cells and partially into Kupffer cells. It distributes chiefly in cytoplasm of the liver cells. In the nucleus it is difficult to be detected. The folic acid is abundantly excreted in bile ducts from liver cells. In kidney the acid is found most markedly in epithelium of urinary tubules, especially proximal convulated part. In hematopoetic organs it is detected in the walls of capillary of bone marrow, spleen and lymph nodes, and is not in the parenchymatous cells. It is suggested that folic acid is stored in liver, and transferred to hematopoetic organs by blood stream when it is required.

5) Vitamin B₁₂

No satisfied reaction have been reported histochemically. Araki et al. observe autoradiographically the incorporation of the vitamin in liver and kidney of rats which are loaded with Co⁵⁹-B₁₂ (26). In the liver, the vitamin is taken into liver cells and Kupffer cells. In liver cells the vitamin is detected abundantly in cytoplasm and in small amount in nucleus. In Kupffer cells, it is detected only in cytoplasm. Excretion from liver cells into bile ducts is demonstrated. In kidneys, though some of the vitamin is found in glomerular capillary and lumina of urinary tubules, majority of the vitamin is noted in lining epithelium of urinary tubules.

6) Orotic acid

Orotic acid has a whitish blue primary fluorescence which is intensified by adding H₂O₂. Utilizing this property a histochemical method has been devised (27). Using the method, a distribution of orotic acid in the rats loaded with orotic acid is reported. The orotic acid is absorbed from intestine and distributes in liver, kidneys, nervous system, testis and hematopoetic organs. In nervous system it is abundantly detected in Nissl substances of nerve cells. In bone marrow it is found in capillary, reticulum cells and hematopoetic area. In spleen the acid is demonstrated in lymphoid follicles. In testes orotic acid is remarkably found in seminiferous tubules. It appears mostly in Sertoli cells and seminiferous cells. In the later cells it is the richest in spermatogonia, followed by spermatocytes and spermatoids, but scarcely in sperm itself.

In the carbon tetrachloride poisoned animals the acid is markedly noted in regenerative portion (28). From the findings, mentioned above, the acid is distributed in the tissues which reveal high activity of nuclear protein synthesis.

7) Ascorbic acid

Though histochemical detection of ascorbic acid (silver method) is available since Bourne, there are many substances which reduce silver in living bodies. Indophenol method is suggested by Kudo (29). But no practical results are obtained in this field.

8) Flavonoids

Flavonoid shows yellowish fluorescence which is remarkably intensified on
addition of alkali. After Wang's observation on histochemical distribution in rats which are administered with rutin or hesperidin (30). Both rutin and hesperidin are absorbed from columnar epithelium of the small intestine and detected in liver, kidney, heart, spleen and lungs. For specific distribution figures of flavonoid in animals it is pointed out that flavonoids are detected predominantly in the capillary wall of various organs. If flavonoids are significant as capillary stabilizer, it is suggested that flavonoids are directly distributed in the vessels and inhibited the activity of hyaluronidase.

The mentioned above is the outline of present state in histochemical field of vitamin in our laboratory. Though there are many vitamins which cannot be detectable histochemically and several technical difficulties are yet remained in this field, the more brilliant development in this field is expected in near feature.

References