STUDIES ON THE BIOLOGICAL ACTIVITY OF "KETOENOL-SUBSTANCES" (HAMAZAKI)

REPORT NO. 2 ON KETOENOL-SUBSTANCES IN PLANT KINGDOM AND THEIR GROWTH-PROMOTING ACTIVITY IN LACTOBACILLUS CASEI

By YUKIO HAMAZAKI, SHIZUO TSUJI, JIRO SATO, HITOSHI YAMAJI
AND NOBUO KADO

Department of Pathology, Okayama University

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While making studies on a certain "acid-proof substances" of animal tissues, one of the authors (HAMAZAKI) has witnessed the existence of Ketoenolsubstances (KES) in plant kingdom (1935) (1). But its amount was compared with those in animal kingdom, and it was so small that the physiological and pathological studies on it have been to this day, almost entirely limited to that of animals.

In the preceding paper (2), we have reported convincing series of experiments that KES (HAMAZAKI) must belong to one of the folic acids complex. What was urgently needed under the present circumstances of our investigations, was to make efforts to pursue the histological features and the biological significance of it in plant kingdom. That is why we have launched this achievement.

Plant-tissues were fixed by sublimate-mixture (HAMAZAKI) for two days. Then washed in tapped water, made in paraffin-section, stained by carbol-fuchsin-iodin-method (CFI-method) by HAMAZAKI. Sometimes fresh materials were made in thin sections by a sharp razor and they were treated as above mentioned.

Green onion was one of the best materials used for this research. It was separated in three different parts, the green part, the yellow part and the white root, which were made in paraffin-sections, respectively.

The green part shows KES in a moderate amount. In the tintless foundation of nucleus, i. e. karyoplasm, there can be seen several sharply margined, round granules, 1-3 μ in size. Apparently they leave the nucleus into the protoplasm, and become thinner and scattered around the nucleus among the chlorophyll bodies. The latter are homogeneously stained in violet color and in their edges several KEG about 1 μ in diameter may be found. These granules, very likely, correspond to the granas of the chloroplasts. The collecting cells around the vessels contain KEG only in their nuclei. They contain for many KEG than that of the above mentioned green cells, and in a convincing series, transformations from chromatinnodules to the KEG can be seen. Among the cells of the epidermis, KEG are located abundantly in the guard-cells of the stomata. The findings of these KEG are similar to the above mentioned, but they are smaller and more numerous, and stained darkly. Cuticle of the guard-cells appears in deep violet, containing minute KEG in small number. The other cells of epidermis generally have KEG only in traces.

In case of differentiating though baryt-water, all the KEG in protoplasm and nucleus

have lost their color-reaction by CFI-method, but in the contrary, the chloroplasts maintain their color-reaction.

In the white root, leukoplasts can be seen which are slightly stained by CFI-method. Neither in the cytoplasm nor in the nucleus KEG has been demonstrated. No stomata are found in the epidermis and the nuclei are large, but faint.

The yellow part of green onion shows roughly, transitional features between the green part and the white root; the plastids in this part are stained in light violet, and the KEG can rarely be found around them. The cell nucleus appear pale, but contains faint granules in small number. The stomata with guard cells is a solitary instance showing some violet KEG.

By Feulgen's method, the nucleus of the plant cells gives a positive reaction, while the chloroplasts and their granas are entirely negative. The KEG situated in-and-outside of the nucleus are also negative.

The cotyledons of Japanese radish possess also a number of KEG. It is notable that the juice of sieve tubes apparently contains KES which presents itself as small clots of "mercuraffin substance" (HAMAZAKI). In the companion cells of sieve tubes, a large number of fine KEG can be detected in various sizes. They may presumably, be scattered diffusely, be derived from the KEG implied in the tissue juice of the perivascular juice-crevice. By differentiation in baryt-water, they are all obscure. KEG have been far less demonstrated in any other young leaves of radisch than that in the cotyledons. Noticeable, is that, the above mentioned KEG in plants were not verified by fixation through $K_2Cr_2O_7$ -mixture, that is to say, plants are scarcely equipped with the KES (Cr).

Spinach is a solitary instance of plant that is histologically equipped with KES by fixation of Cr-mixture and they demonstrate biologically the growth-promoting factor of lactobacillus casei. By histological examination through CFI-method, the guard cells of the epithelium which are laden with many KEG by Hg-mixture fixation, do not especially demonstrate many KEG (Cr), but they are distributed through out the epithelium diffusely. These KEG are spherical $2-3\mu$ in size. In the parenchyma cells of the spinach, numerous KEG being attached to chloroplasts, are dituated diffusely almost in the same manner as those of the green onion. The KEG (Cr) are exclusively spherical and $0.5-1.0\mu$ in size. It is notable that the nuclei of parenchyma cells used to contain some spherical KEG about 1μ in size, some of which are going to pass over the nucleal membrane to cytoplasm. In the in-and-outside of the sieve tubes some KES can be seen in coarse irregular forms.

It is very interesting that the KEG in clovers exhibits a seasonal polymorphism. The clover leaves collected in June were equipped with a large amount of KEG which were distributed in the guard cells of the stomata, in the edges of chloroplasts of parenchyma cells, in the companion cells of sieve tubes, et. al. And the granules present in those places are sharply defined and takes up a dark nuance by CFI-method. At that time, the amount of the KES extracted from the clover leaves has become as much as 0.2 g per 400 g of fresh leaves and its growth promoting activity in lactobacillus casei was notable

The clover leaves collected in August diminished in number of KEG which seemed to somewhat faint as compared with that of June-clovers, and the biological activity was less notable. The KES extracted from these clover leaves amounted to 0.093g per 400g of leaves in fresh state. In September, there can be seen a small number of faint KEG in the guard cells of stomata, but no KEG could be detected in any other places. To our consternation the amount of the KES extracted from those fresh leaves were as small as 0.017 g per 400 g of leaves, and the biological response could hardly be recognized (see the accompanying table 1). The above mentioned clover leaves collected during the three season were, by outward appearance, similarly well nourished and distinctly green.

According to Wright & Welch (1943) (3) the effects of liver cell suspension were proved in the clover extracts. The extract from June-clovers has successfully revealed production of growth-promoting response in *Lactobacillus casei* (see the table), but the those from August and September clover were quite slight.

By administering Hg-mixture (4), the KES being precipitated from clover extract exhibits a spherical body of about 8μ in crystal form, rarely ranging to 40μ in size. The larger one is usually a combined body of two or three small crystals. The spherical crystal appears in gray-brownish silver gloss and it consists of an infinite number of feeble needles in radial arrangement. When the precipitation was made in smear preparation and was stained by carbol-fuchsin-iodin-method, the radial structure disappeared and instead of this structure, concentric tiny layers in violet color appeared. In the center of crystal is always to be seen a little globus which is colored in deep clear violet.

The problem whether nucleic acids in plant and animal-kingdom are the same or not long been discussed. Carbol-fuchsin-iodin method (HAMAZAKI), which is a chemical reaction producing a new sort of violet dye, gives a positive reaction on desoxyribonucleic acid, while a negative one on ribo-nucleic acid. Nucleoprotein also can not generally react on this method. As a rule, plant tissue has no KES (Cr), but KES (Hg). The former represents nucleotide conjugates and the latter purinbase complex. Therefore, it is presumed, except in spinach, that KES (Cr) which is mainly of desoxyribo nucleotides, does not exist in plant tissue.

KES in plant tissue exist in company with chloroplasts and they are most numerous in the beginning of summer. It is nothing to be wondered about to see the highest biological activity (L. c. f.) of KES, extracted from the plant growing at that season. KEG, at least a part of them, originate in nuclei of cells which contain chloroplasts.

Lactobacillus casei factor of clover KES (crude).
(Titration acidity in 5 cc Snell-Peterson's medium)

gathering time gain of KES (Factor γ) Droplets	June	August	October
	0.20/400 g	0.093/400 g	0.0166/400 g
0	0.45		
1/4 (37)	0.43		

1/3 (4 r)	0.48	0.44	0.45
1/2 (6 r)	0.47	0.47	
1 (12 r)	0.53	0.47	0.45
4 (48 r)	0.54	0.53	0.47
8 (96 r)	0.54	0.48	0.48

Effects of libercell suspension on L. c. f. of clover KES (crude).

(Titration acidity in 5 cc Snell-Petterson's medium)

Droplets (Factor r)	Clover KES	Salinesol lcc Libercellsusp 3 drop	Clover KES lcc Libeycellsusp 3 drop	Multiplication Coefficient
0	0.45			
1/3 (4 r)	0.48	0.60	0.78	1.90
1/2 (6 r)	0.47	0.62	0.82	2.04
1 (12 r)	0.53	0.80	1.30	2.09
4 (48 r)	0.54	1.75	2.09	1.43

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