PHYSIOLOGY OF "BAKANAE" DISEASE

1 Effect of GA_3 on the Metabolic Changes in Germinating Rice Seeds[1]

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Introduction

"Bakanae" disease caused by *Gibberella fujikuroi* is a seed-born disease. When seeds of rice plants are infected by the fungus, the most characteristic symptom of the disease is the appearence of tall thin plants, markedly overgrowing their uninfected neighbors. The active metabolic products of the pathogen, gibberellins, were isolated and proved to play an important role in the pathogenicity of this organism. For the purpose of understanding the pathogenicity of this organism, the effect of the gibberellic acid (GA_3) on the germinating rice seeds was studied.

Perhaps the most striking aspect of the normal germination of cereal seed is the correlation between the simultaneous breakdown of reserved carbohydrate and protein in the endosperm and the growth of the embryo. During germination the activity of hydrolytic enzymes such as amylase and protease in the endosperm are largely increased. The liberation of sugars and amino acids is correlated with the changes in enzyme activity, and it is suggested that hydrolytic enzymes are responsible for mobilizing the reserves in the endosperm. Since breakdown of starch and protein in the endosperm is one of the most active metabolism in germinating seeds, it would be of interest to know the effect of GA_3 on those metabolic systems. The effect of GA_3 on intact germinating cereal seeds was studied by Hayashi (1940). He found that the amylase activity of germinating barley and wheat seeds was somewhat increased, on a weight basis, proportional to the quantity of gibberellin used. However, this did not apply to rice. Recently several reports appeared dealing with the effects of exogenous GA_3 on the metabolism of the endosperm

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of barley (Paleg 1960a, Paleg 1960b, Paleg 1961). He described the dependence of starch hydrolysis and protein release in excised endosperm on the presence of added GA_3. Nonethless, there was no study published on the study of the effect of GA_3 on the metabolic changes in germinating rice seeds. In our laboratory the effect of GA_3 on the biochemical changes in the germinating seed during the 5 day period of germination was studied. The investigation included the variation of sugar, amino acid and nitrogen fraction. Research was also made on the activity of the main hydrolytic enzyme of the seed. The results of these studies are reported in this paper.

Materials and Methods

Seeds of rice plant (Taichung 65) of 1966 harvest were used. Uniform sized seeds of rice were selected and dehusked by hand. They were rinsed with sterile distilled water and then sterilized by immersion in 0.08% (w/v) mercuric chloride with occasional mixing for 30 minutes. The grains were twice washed with sterile distilled water, then soaked in sterile distilled water for 3 hours. Normally, three grams of seeds were germinated on filter paper with 3 ml of test solution in sterile 250 ml Erlenmeyer flasks. The flasks were kept in a incubator at 28°C in dark. Aqueous solutions, containing 50 ppm GA_3 was used in this study and sterile water was used as a control. All culture vessels were sterilized by dry heat, GA_3 solution was sterilized by filtration through millipore bacterium-proof filter. All operations were conducted under aseptic conditions. At the end of the culture period any contaminated tlasks were rejected. The growth, fresh weight, dry weight, nitrogen fraction, sugar, amino acid and the activity of α-amylase, β-amylase and protease were measured daily for a period of 5 days.

The length of shoots was measured to the nearest $\pm$ 0.5 mm. The mean value of 10 kernels was recorded. Fresh weights were determined immediately after the samples were harvested. Dry weights were obtained after drying the sample at 65°C for 24 hours. The total nitrogen content of the dried material was determined by a standard Kjeldahl procedure (Steyermark *et al* 1951). For the determination of alcohol soluble nitrogen, reducing sugar and amino acid, a sample of the fresh material was dropped into boiling 70% ethanol. After cooling, it was homogenized in ethanol. The homogenate was clarified by filtration. The amino acid content of the ethanol extract was determined by the Ninhydrin method described by Yemn and Cocking (1955) using lysine as the standard. The reducing sugar content of the ethanol extract was determined by Schaffer-Somogyi method (Schaffer and Somogyi 1933, Somogyi 1952), using glucose as the standard. The soluble nitrogen content of the ethanol extract was determined as described for total nitrogen. The

total nitrogen minus the alcohol-soluble nitrogen was considered as insoluble nitrogen.

For the determination of the activities of α-amylase, β-amylase and protease, the crud enzymes were prepared by the following procedure. The samples (3 grams of initial dry weight of seeds) were harvested after being incubated for a certain period and homogenized in a mortar by hand in 5 ml of cold 0.1 M citrate-phosphate buffer at pH 6.2. The homogenates were kept in a cold room (4°C) for 3 hours and centrifuged for 15 minutes at 10,000 xG. The supernatant was dialyzed four times against 3 liters of 0.01 *M* citrate-phosphate buffer (pH 6.2) in the cold room with constant stirring for 24 hours. It was then used for the assay of α-amylase, β-amylase and protease.

Total α- and β-amylase activity was measured by incubating 1 ml of enzyme preparation with 1 ml of 1% soluble starch in 0.016 M acetic buffer, pH 4.8 at 30°C for 3 minutes. The reaction was stopped by adding 2 ml of dinitrosalicylic acid reagent (Bernfeld 1951). The mixture was heated for 5 minutes in boiling water, cooled, diluted to 24 ml with water and the optical density was read at 540 mu against the blank obtained by treating the sample similarly except that dinitrosalicylic acid reagent was added before adding enzyme solution. The amount of reducing sugar formed was calculated from a standard curve prepared with known concentrations of maltose. α-amylase was obtained by heating crud enzymes with calcium acetate (end concentration 6×10^{-5}M) in a water bath at 70°C for 15 minutes to inactivate β-amylase (Berfeld 1951, Graesser 1946). Its activity was determined by incubating with potato starch. The activity of β-amylase was calculated by substracting the values for α-amylase from the total.

For quantitative test of α-amylase activity, a method described by Fuwa (1954) was used. Comparisons of the iodine color were made in a Erma photoelectric spectrophotometer model IV against control solutions.

For the assay of protease, the reaction mixture consisted of 1.0 ml enzyme solution and 1.0 ml of 1% "vitamine free" casein (N. B. Co.) solution in citrate-phosphate buffer pH 6.2 was incubated at 40°C for 2 hours. Then the reaction was terminated by adding 2.0 ml of 5% TCA. The extent of the proteolytic action was measured with the reagent of Lowry *et al* at 660 mu (Klein and Happaz 1965).

Results

The first response of the rice seeds to the application of GA_3 was the acceleration of the rate of germination. Generally the germination could be observed at about 20 hours in untreated seeds, compared to 16 hours with GA_3 treatment. After the germination, the growth of shoot was stimulated by

treatment of GA_3. As indicated in Fig. 1, the shoot of GA_3 treated seedlings was longer than untreated seedlings. Following the elongation of the shoot, the fresh weight of whole seedlings was increased. The fresh weight of treated seedlings was always heavier than that of untreated seedlings. The cause of increase in fresh weight was invastigated. As indicated in Fig. 2,

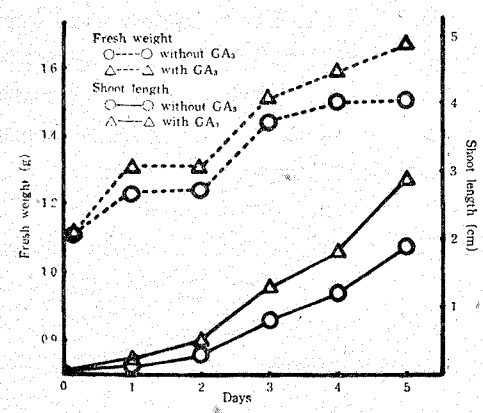

Fig. 1. The effect of GA_3 on the fresh weight and shoot length of germinating rice seeds.
Fresh weight started with one gram of dry seeds.

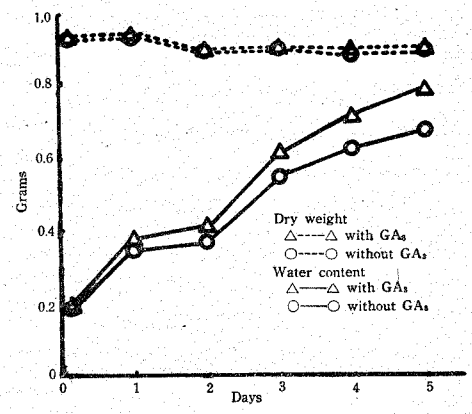

Fig. 2. The effect of GA_3 on dry weight and water content in germinating rice seeds.
Started with one gram of dry seeds.

the dry weights of both treated and untreated seedlings were almost the same during the 5 days period of germination. A possible small loss (4%) of dry weight was observed after 2 days. It might be due presumablely to a leaching of soluble components from the seedlings. The water content increased rapidly in germinating seeds. It was generally higher in treated seeds. It appears that the increase of fresh weight was mainly caused by the increased water content.

The effect of exogenous GA_3 on the reducing sugar content in germinating rice seeds was shown in Fig. 3. At the start there was no significant difference in reducing sugar content in both GA_3 treated and untreated seeds. However, the content of reducing sugar in germinating seeds was gradually increased during the 5 days period, and the sugar content in GA_3 treated seeds was much higher. It appeared that the GA_3 treatment accelerated the breakdown of starch and enhanced the release of reducing sugar. Since α- and β-amylase were main enzymes involved in the system, the effect of GA_3 on the activities of both enzymes in germinating seeds was to be examined naturally. In Fig. 4, it gave the results of using Fuwa's method (1954), specific for α-amylase activity. No α-amylase activity was detected prior to germination. After 24

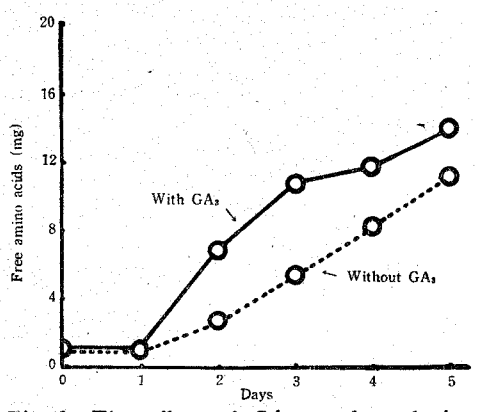

Fig. 3. The effect of GA_3 on the reducing sugar content in germinating rice seeds. Reducing sugar was expressed by mg equivalent of glucose per gram dry weight of seeds.

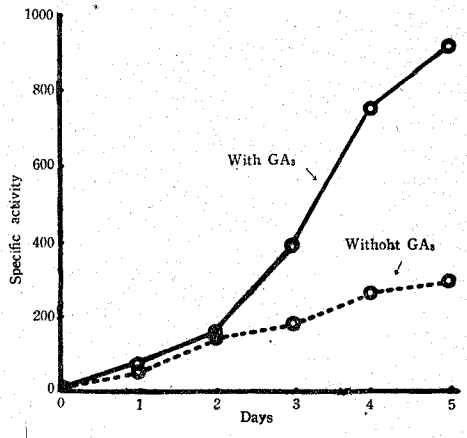

Fig. 4. The effect of GA_3 on the level of α-amylase activity in germinating rice seeds.
Specific activity=10% decrease of color intensity/3 min./g of dry weight.

hours only little α-amylase activity was detected. The activity increased sharply after 48 hours. After which the enzyme activity in GA_3 treated seeds had much higher than that of the untreated seeds. At the end of the fifth day, GA_3 treated seeds showed about 3-fold increase in α-amylase activity. β-amylase was also examined in GA_3 treated and untreated seed. As indicated in Fig. 5, β-amylase activity showed a sharp increase during the first 3 days, after which there was a decrease in activity. The pattern of rise and fall of β-amylase activity in treated germinating seed was similar to that in untreated seeds. However, the β-amylase activity in treated seeds was lower than that in control seeds at the fifth day. In Fig. 5, it showed that the total amylase activity in treated seed was higher than that in untreated seeds after 3 days. The increase of total amylase activity was generally in accordance with the increase of sugar content as being showed in Fig. 3. It appeared that GA_3 had no effect on the level of β-amylase activity. The increase of sugar content in GA_3 treated seeds was the result of the increase in the level of α-amylase.

There was no change observed in the total nitrogen content in both treated and untreated seeds during the 5 day period. When the conversion of insoluble nitrogen to soluble nitrogen during germination was examined, as being indicated in Fig. 6, there was a decrease in insoluble constituents which was accompanied by an increase in soluble material. The rate of conversion of nitrogenous compounds in GA_3 treated germinating seeds was faster than that in untreated seeds. It seems that GA_3 stimulated the change of insoluble nitrogenous constituents to soluble nitrogenous compounds.

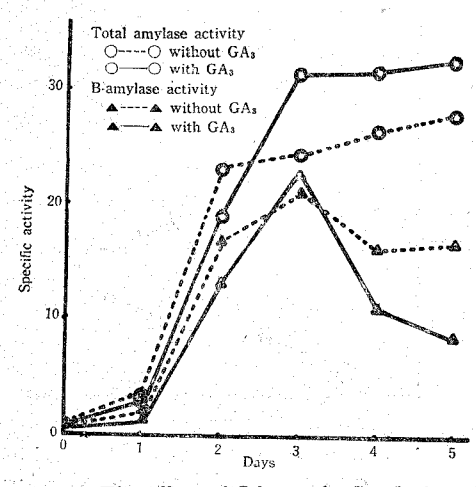

Fig. 5. The effect of GA_3 on the level of total amylase and β-amylase activities in germinating rice seeds.
Specific activity=mg maltose formed/3 min./g of dry weight.

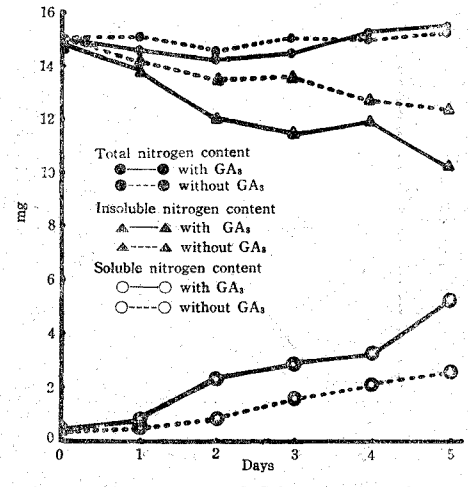

Fig. 6. The effect of GA_3 on the change of nitrogen fraction in germinating rice seeds.
Started with one gram of dry seeds.

The total amino acid content present in the seed increased progressively with germination. The amino acid content in GA_3 treated seeds was higher than that in untreated seeds (Fig. 7). The main enzyme, protease, responsible for the breakdown of reserve protein and release of free amino acids, was examined.

As indicated in Fig. 8, the enzyme activity in GA_3 treated seeds was higher than that in untreated seeds and release of amino acids was correlated with the activity of protease.

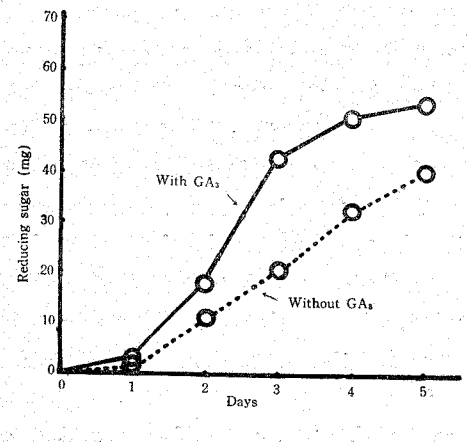

Fig. 7. The effect of GA_3 on free amino acids in germinating rice seeds.
Free amino acids were expressed by mg equivalents of lysine per gram dry weight of seed.

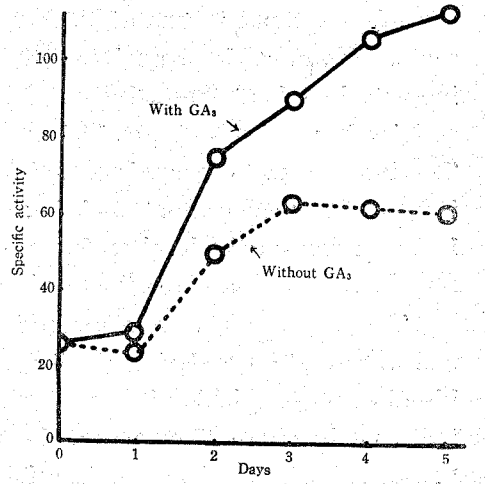

Fig. 8. The effect of GA_3 on the level of protease activity in germinating rice seeds.
Specific activity=μg tyrosine released/2hr/g dry weight.

Discussion

In the course of normal germination the starch and proteins of storage cells are hydrolyzed. It was recognized that aleurone layer is a gland which secretes the hydrolytic enzymes responsible for liquifying the reserve starch. Yomo in Japan (Yomo 1960) and Paleg in Australia (Paleg 1960a, 1960b) began to realize that gibberellin was the chemical signal. This hormone, secreted by the embryo, activates the cells of the aleurone layer into secreting hydrolytic enzymes. The amount of hormone in the germinating seeds is important in the regulation of the secretion of hydrolytic enzymes. Excess of gibberellic acid in germinating seeds modifies the metabolism of starch and protein hydrolys s. When rice seeds are infected by *Gibberella fujikuroi*, which produced an excess of GA_3, the modification of metabolism of starch and protein hydrolysis can be expected. The first apparent response of the rice seeds to the reaction of GA_3 is the acceleration of the rate of germination, followed by an increase of the length of the shoots and the fresh weight. As indicated in Fig. 1 and Fig. 2, the increase of shoot length and the fresh weight is the result of the uptake of water and GA_3 has no obvious effect upon the total dry weight. Apparently the response of rice seedlings to the GA_3 is on the rate of water uptake, but it may also involve other reactions as well. The reaction of the germinating seeds to the applied GA_3 appeares to involve all the major components i. e. starch and protein. The effect of GA_3 on the germinating rice seeds was quite similar to the reactions obtained on the endosperm of barley (Paleg 1960a, 1960b) and corn (Ingle and Hageman 1965). Treatment of germinating seeds with GA_3 induces the release of amino acid and reducing sugar. Their increases are correlated with the increase of amylase and protease activities.

The general pattern for making reserve starch available to the embryo in cereal seeds seems to be due to the hydrolysis of starch to glucose in the endosperm by α-amylase, β-amylase and maltase. In our experiment GA_3 treatment increased the level of α-amylase markedly but not β-amylase. And α-amylase activity was mainly responsible for the increase of sugar. Detailed studies were made by many works on the GA_3 stimulation of α-amylase activity in barley endosperm in an attempt to elucidate the mechanisms of this action. Paleg (1960b) suggested that the hormone activities or the releases of protease would in turn activate or release the α-amylase, Macleod and Millar (1962) proposed that GA_3 brought about the release of hydrolytic enzymes, including α-amylase from lysosome like particles. Recently, however Briggs (1963) reported that there was an enhanced *de novo* synthesis of proteins in the aleurone cells of barley endosperm in response to GA_3, and Varner (1964)

demonstrated the stimulation of *de novo* synthesis of α-amylase in the same tissue. In regard to the β-amylase activity in rice seedlings, there was an increase during germination, however, GA_3 has no effect on the increase of β-amylase activity. Rowsell and Good (1962) demonstrated that β-amylase was present in the developing wheat seed. This activity fell to a low level at maturity. The activity increased several fold during germination. This was however not due to new synthesis, but to the activation of latent β-amylase which could be activated in vitro by reducing agents.

During germination, seed proteins are hydrolyzed into peptides and amino acids and they are translocated to the growing parts of the embryo. The protease activity is stimulated by GA_3. Protease is active in the resting rice seeds. Its activity in germinating rice seed is increased and stimulated by the addition of GA_3. The effect of the GA_3 treatment on protease activity appears to be complex. However, the regulation of protease activity of germinating seeds is largely an unanswered question. Ingle and Hageman (1965) suggested that the production of amino acids by protease activity is only partially under hormonal control.

Summary

The changes of various chemical components, nitrogen fraction, sugar, and the activities of hydrolytic enzymes, α-amylase, β-amylase and protease in germinating rice seeds which were treated with and without GA_3 were studied over a 5-day germination period. Many changes in these chemical components and hydrolytic enzyme activities were observed, indicating that these changes were affected by the treatment of GA_3. Treatment of germinating seeds with GA_3 induced the release of amino acid and reducing sugar. The increase of the released sugar and amino acid were correlated with the increase in amylase and protease activities.

GA_3 had no effect on the level of β-amylase activity while the level of α-amylase was markedly stimulated by GA_3 in germinating rice seeds. The increase of sugar content in GA_3 treated seed seemed to be the result of the increase of α-amylase activity. The level of protease activity in germinating seeds was also stimulated by GA_3.

水稻徒長苗病之生理研究

1. Gibberellic acid 對水稻種子發芽之新陳代謝之影響

郭宗德 楊素娥

水稻徒長苗病原菌能產生 Gibberellic acid, 此種代謝物與水稻發芽過程中之新陳代謝有密切之關係。水稻發芽最主要之代謝仍將貯藏在胚乳之澱粉及蛋白質分解成可溶性之還原糖及氨基酸，此種可溶性糖及氨基酸將自胚乳輸送到胚之部分，以供胚之生長。本實驗直接將 Gibberellic acid 處理發芽之水稻，分析水稻開始發芽五天內上述新陳代謝之變化，經 Gibberellic acid 處理之發芽水稻，其內含之游離還原糖及氨基酸均比無處理者爲高，其增高之樣式與糖類分解酵素及蛋白質分解酵素之活性相同。分析與分解澱粉有關之酵素發現 Gibberellic acid 能促進 α-amylose 之形成，而對 β-amylase 則無影響，在經 Gibberellic acid 處理之發芽種子，其內含之還原糖之增加，α-amylase 之增加可能爲一重要原因。分析蛋白質分解酵素發現 Gibberellic acid 亦有促進 Prolease 形成之作用。

Literature Cited

BERNFELD, P. Enzymes of starch degradation and synthesis. Advances in Enzymol. **2**: 380-424, 1951.

BRIGGS, D. E. Biochemistry of barley germination, action of gibberellic acid on barley endosperm. J. Inst. Brewing **69**: 13-19, 1963.

FUWA, H. A new method for microdetermination of amylase activity by the use of amylose as the substrate. J. Biochem. (Japan) **41**: 583-603, 1954.

GRAESSER, F. R. and P. J. Dax. Simple method for the determination of alpha- and beta-amylase in malt. Wallerstein Lab. Comms. **26**: 43-48, 1946.

HAYASHI, T. Biochemical studies on "Bakanae" fungus of rice. VI Effect of gibberellin on the activity of amylase in germinated cereal grain. Bull. Agr. Chem. Soc. Japan **16**: 531-538, 1940.

INGLE, J. and R. H. HAGEMAN. Metabolic changes associated with the germination of corn. III Effects of gibberellic acid on endosperm metabolism. Plant Physiol. **40**: 672-675, 1965.

KLEIN, M. and I. HAPPAZ. Proteolytic activity in Maize leaves. Phytochemistry **4**: 327-332, 1965.

MACLEOD, A. M. and A. S. MILLAR. Effects of gibberellic acid on barley Endosperm. J. Inst. Brewing **68**: 322-332, 1962.

PALEG, L. G. Physiological effects of gibberellic acid. I On carbohydrate metabolism and amylase activity of barley endosperm. Plant Physiol. **35**: 293-299, 1960a.

PALEG, L. G. Physiological effects of gibberellic acid. II On starch hydrolyzing enzymes of barley endosperm. Plant Physiol. **35**: 902-906, 1960b.

PALEG, L. G. III Observations on its mode of action on barley endosperm. Plant Physiol. **36**: 825-837, 1961.

ROWSWELL, E. V. and L. J. GOOD. The constituent of wheat binding latent β-amylase. Biochem. J. **84**: 73P. 1962.

SHAFFER, P. and M. SOMOGYI. Copper-iodometric reagents for sugar determination. J. Biol. Chem. **100**: 695-713, 1933.

SOVOGYI, M. Notes on sugar determination. J. Biol. Chem. **195**: 19-23, 1952.

STEYERMARK, A. L., H. K. AIBER, V. A. ALUISE, E. W. D. HUFFMAN, J. A. KUCK, J. J. MORAN and C. O. WILLITS. Recommended specifications for microchemical apparatus. micro-kjeldhal nitrogen. Anal. Chem. **23**: 523-528, 1951.

VARNER, J. E. Gibberellic Acid Controlled synthesis of α-amylase in barley endosperm. Plant Physiol. **39**: 413-415, 1964.

YEMN, E. W. and E. C. COCKING. The determination of amino acids with ninhydrin. Analyst **80**: 209-213, 1955.

YOMO, H. Studies on the amylase activating substance. IV. On the amylase activating action of gibberellin. Hakko Kyokaishi **18**: 600-602. 1960.