B. LITERATURE REVIEW

B.1. PATHOGEN, ITS MODE OF ENTRY AND PATHOGENESIS.

*Helminthosporium oryzae* Breda de Haan Perfect stage, *Cochliobolus miyabeanus* (Ito and Kuribayashi) Drechsler ex Dastur, as a major pathogen of rice in India was appreciated when it caused severe epiphytotics in 1918-19 in Godavari delta, India (Sundararaman, 1922). In Bengal it was considered to be the major factor in reduction in yield in 1942, resulting in Bengal famine in 1943, (Report of the Famine Enquiry Commission (1943), cited by Padwick, 1950).

The pathogen attacks all parts of the rice plant, excepting roots, in all stages of development, symptoms accordingly may vary (Mundkur and Chattopadhyay, 1967; Ono, 1972), nevertheless the most characteristic symptom is the formation of discrete dark brown ellipsoidal to eye shaped spots on the upper surface of the leaves, glumes and other aerial parts of the affected plant.

Infection is most prominent on leaves, number being more and size of the necrotic spots larger in a susceptible variety than in a resistant variety. Susceptibility to brown spot disease has been observed to depend on the position of rice leaves within a variety. Ono (1953)
first reported that top most leaves are the most resistant against lesion expansion in brown spot disease of rice. Sato et al. (1958) also noted that epidermal tissues of a leaf sheath at higher position are resistant, but this was not the case in lower ones. These observations were confirmed by Oku and Nakanishi (1962). Similar observations on variation in susceptibility due to leaf position was noted by Das Gupta (1968) in brown spot and by Goto et al. (1960) in rice blast. Oku and Nakanishi (1962) showed that the antifungal activity of leaves were similar in leaves of all positions, so resistance or susceptibility could not be correlated to possible gradation in antifungal activity of leaves in different positions.

Nishikado and Miyake (1922) found that penetration of H. oryzae into rice leaves was mostly through cuticle and epidermis. Penetration through stomata without appreciable formation of appressoria was also noted. They found that within a few hours of germination, germ tubes were surrounded by thick mucilagenous sheaths which enabled them to adhere to a solid surface. The tips swelled to form lobed or branched appressoria in course of a few hours. Peg like infection hyphae were then extruded out from such appressoria. Tullis (1935) reported that apart from direct infection through the motor cells of the epidermis was also common.
These cells were invaded by the mycelium, later spreading to the intercellular spaces of the mesophyll. Further spread was impeded by vascular bundles in resistant varieties. Horino and Akai (1966) observed ninety eight per cent penetration through motor cells and two per cent through stomata. Nishikado (1943) observed that infection of grains could take place through the base of small hairs, the pathogen then entering into the surrounding epidermal cells. In resistant varieties, a yellowish brown substance was observed to be deposited in the intercellular spaces which checked spread of the fungus. These observations were supported by Goto (1952). Ono (1953) stated that the initial point of hyphal penetration appeared to be affected by culture conditions of the host plant. Oku (1962) noted that in resistant varieties at the time of appressorial formation, colour of adjacent cell contents became yellowish brown and browning was conspicuous. Horino and Akai (1968) made detailed observations on the process of penetration in susceptible and resistant varieties under the electron microscope. They found conidia of H. oryzae germinating equally well on leaves of both resistant and susceptible varieties, but germ tubes were shorter and fewer appressoria formed on leaves of resistant varieties. Goto (1958) observed closely the infection processes and formation of lesions in brown spot disease. He noted that at the time of appressorial formation, protoplasm streaming in host cells increased, and
the nuclei moved to a position near the appressorium. At the
time of separation of middle lamella, appearance of yellowish
granules was noted. Then following death of 2-3 cells, penetra-
tion took place. Observations of Goto were confirmed by Sato
(1964-65). Sato and Sakamoto (1960), in brown spot disease of
rice noted that the infection hypha having emerged from the
underpart of the appressorium found its way into the middle
lamellae (formation of infection cavity) and invaded the host
cell. The host nuclei tended to migrate towards the invading
hypha. The infected cells became discolored and protoplasm
showed granulation. In the early stage, infected cells showed
rapid necrotic reaction. Host cells around the necrotic cells
appeared to be still alive even after about 40 hours of infec-
tion. Sometime a slight discoloration of the infected cells
might be seen before complete invasion. Oku (1962) observed
that after 4 hours of inoculation of the 2nd leaf sheath
(from the top) germination of conidia was almost complete
and the germ tubes formed appressoria on the host cells. A
number of small granules was found to appear in the host cells
beneath the appressoria in brownian motion. The granulation
was more conspicuous in resistant varieties. These cells
showed abnormal plasmolysis on treatment with hypertonic
solution of potassium chloride, but did not show signs of
death. In all varieties, invaded cells lost the capacity of
plasmolysis, and the neighbouring cells were of slight brown
colour but still plasmolyzable in resistant varieties, while those in susceptible varieties were incapable of plasmolysis even before any colour change could be observed.

Asada (1962) noted viability of the mycelium within the necrotic regions as well as in the regions bordering necrotic and chlorotic margins of the lesions. Oezaemia (1924a) observed that within 24 hours of inoculation initial symptom was observed while Asada (1962) found that about a week was noted to ensure maximum expansion of lesions under usual conditions.

The toxic effect of the culture filtrate of H. oryzae was first observed by Hemmi and Matsuura (1928) on Vicia faba. Goto (1953 and 1958) noted that it had a toxic effect on cells of rice plants. Asada (1956 II) however was of the opinion that pathogenesis was due to action of poetic enzymes, xylanase, and cellulase in the filtrate and the injurious action of the fungus on the rice plant was not due to toxin. Orsenigo (1956a,b and 1957) suggested presence of a toxic substance "cochliobolin" in a culture filtrate of Helminthosporium oryzae which reduced germinability and produced abnormalities in rice seedlings. Nakamura and Oku (1960) detected in the diseased leaves "ophiobolin" which was toxic to roots, coleoptiles, and leaves and caused wilting of plants at 2-5 ppm.
Mitsusawa (1950), Wakimota and Yoshu (1958) reported that phenolic substances increased around the brown necrotic lesions resulting from infection of rice by *Pyricularia oryzae* and *Helminthosporium oryzae*. The browning of the cell contents of rice leaf due to brown spot infection was ascribed to certain oxidation products of phenolic substances in leaves by the action of polyphenoloxidase in the causal fungus (Oku, 1958). Kaji and Tamari (1957b) observed the presence of chlorogenic acid in rice. Toyoda and Suzuki (1960) detected increased peroxidase activity and decreased catalytic activity during oxidation of phenolic compounds in rice leaf tissue and infected area. Oku (1961) observed that the fungicidal action of the polyphenols, the quinone, the brown substance, nature and mode of action of which were still unknown. Oku and Nakanishi (1962) found a phytoalexin-like substance in infected tissues.

B.2. EFFECT OF DISEASE ON CHLOROPHYLL.

Kuprevicilis (1947) in case of powdery mildew infection of oat leaves observed that chlorophyll and xanthophyll content was somewhat reduced while carotene content remained unaltered and even increased a little. Akai and Fukutomi (1958) reported that in rice plants infected with downy mildew content of protochlorophyll and chlorophyll a and b
of leaves was greater in the lower leaves than in the upper ones, while in the healthy plants the situation was reverse, the difference between the diseased and healthy ones being more clearly defined in young leaves. Leiley (1963) noted that leaves of head smut infected maize plant contained more chlorophyll than those of the healthy ones. Haspelova and Horvatovicova (1965) noticed a rapid decrease in chlorophyll content in varieties of barley plant susceptible to Erisyphe graminis, f.sp. hordei. Pavlov and Nechiporenko (1969) reported in potato infected with potato virus X, Y and K. that chlorophyll and water content of the leaves were reduced. In hoja blanca infected rice plant, upto seventy per cent reduction of chlorophyll content was observed in infected chlorotic leaves (Long and Black, 1970). Horino and Akai (1970) studied ultrastructural changes of chloroplasts in rice plant due to infection of brown spot disease. In infected leaves, chloroplasts swelled one and half to twice their size, becoming oblong spherical and the double membrane partly desintegrated. Adjacent to invading hyphae desintegration of the lamellar system of chloroplasts began in the grana stacks near the envelope gradually spreading to every stack, while finally degenerated, forming abundant small vesicles 0.1 - 0.5 μm in diameter. Similar observation were noted in barley plants infected with Barley mosaic virus.
B.3. EFFECT ON PROTEIN.

In infected plants, marked changes in the major metabolic processes were noted to take place, which induced protein metabolism. Sempio (1950) put forward the idea that a shift in the relative rates of different aspects of metabolism might be triggered by the pathogen, resulting in development of an altered chemical environment.

Shishiyama et al. (1965) proposed that the process of metabolic changes in the fungus and rice plant during infection was as follows: "Spore germination using reserve substrates through dicarboxylic acid cycle \(\rightarrow\) change of respiration system in the germinating spore from DCA to TCA cycle \(\rightarrow\) formation of appressorium \(\rightarrow\) invasion into host cell \(\rightarrow\) disturbance of host metabolism by the toxic substance like ophiobolin and acceleration of the respiration pathway in the host plant \(\rightarrow\) occurrence of abnormal metabolism of carbon and nitrogen in the host tissue \(\rightarrow\) temporary accumulation of amino acid and organic acid brought by the disturbance \(\rightarrow\) use of amino acid and organic acid by the fungus, deamination, decarboxylation and transamination of the amino acids in the host cells or the synthesis of abnormal protein such as r-globulin like substance \(\rightarrow\) decrease of glutamate and aspartate \(\rightarrow\) inhibition of energy metabolism of the fungus due to synthesis.
of abnormal protein or formation of necrotic barrier → interruption of fungal extension i.e., stoppage of disease spot development".

High doses of nitrogen in the form of inorganic salts or urea were found to increase the susceptibility of plants to fungal diseases (Alten and Orth, 1941; Kiraly, 1954 and Otani, 1959). Nitrogen metabolism played a part in the host pathogen interaction between plant and pathogen. Correlation between total nitrogen content or amino acid contents of the plant and susceptibility was demonstrated for genetically susceptible plants under different conditions or of different ages (Samborski and Forsyth, 1958; Weinhold, 1964), as well as when comparisons were made among the varieties differing in degree of susceptibility (Rubin and Artsikhovskaja, 1963). Horsfall et al. (1932) pointed out that as a result of infection with pathogenic fungi the total nitrogen content in plant tissues was reduced.

In 1948, Holden and Tracey observed that well fertilized tobacco plants infected with TMV contained more total nitrogen than the uninfected ones. Grümmer (1955) noted decrease in total nitrogen content in poppy leaves, infected with *Helminthosporium papaverae*. Takahashi (1957), Baba (1958), Baba and Tajima (1960) noted that rate of accumulated
soluble nitrogen to protein nitrogen increased appreciably in blast infected rice leaves. In rust infected wheat, as the disease progressed, amino acid content of the tissue usually started to decrease (Rohringer, 1957; Shaw and Colotelo, 1961). Considerable increase in total nitrogen, protein nitrogen, and particularly non-protein nitrogen was found in Phaseolus vulgaris inoculated with TMV, by application of nutrients (Hofferek, 1967).

In bean plants susceptible to halo blight, there was an increase in ornithine, histidine, methionine, asparagine, glutamine, B-alanine and lysine in inoculated leaves, but little difference was noted between healthy and infected plants of resistant variety in this respect (Patel and Walker, 1963). Multiplier disease affected strawberry plants showed much less accumulation of protein and amino acid than in healthy plants (Sehgal and Boone, 1964). Significant changes in amino acid content in leaves of barley plants infected with Erysiphe graminis f.sp. hordei was noticed particularly with reference to resistance of host (Fric, 1964). No difference in buffer soluble protein was observed between healthy and E. graminis infected barley plants for 2-9 days, but both qualitative and quantitative differences in protein patterns were revealed by staining polyacrilamide gel columns with amide schwarz dye (Johnson, Brannamara and Zscheile, 1966). In
susceptible wheat infected with *Puccinia graminis tritici* as early as 2 days after inoculation, there was a decrease in histone and marked increase in RNA and acidic protein (Bhattacharya, Naylor and Shaw, 1965). There was a striking increase in total nitrogen per gm of fresh weight in infections of stem rust on little club wheat and an increase in the ratios of soluble to insoluble nitrogen. Only slight and transitory increases in nitrogen occurred at infection on Khapli wheat and infected leaves quickly reached a stage at which soluble and insoluble nitrogen and the ratios of soluble to insoluble nitrogen and free to protein bound amino acids declined drastically. Particularly striking decreases occurred in glutamic acid (Shaw and Colotelo, 1968). Rai and Sinha (1968) observed increase in total nitrogen content and a considerable change in free amino acid composition in *Sclerospora graminicola* infected bajra plant. Tryptophan was present only in diseased tissues.

Rice plants fed with asparagic or glutamic acid developed larger but not a greater number of spots after inoculation with *Helminthosporium oryzae* (Akai, Shishiyama and Egawa, 1958).

In variety susceptible to *Helminthosporium oryzae* protein increased 24 hours after inoculation. The increased protein fraction from inoculated leaves had no inhibitory
effect on spore germination and germ tube growth. Protein fraction from diseased leaves contained more aspartic acid and less glutamic acid than from healthy leaves (Shishiyama, Oguchi and Akai, 1968). Both aspartic and glutamic acids decreased in rice leaves after inoculation with Helminthosporium oryzae and lesion enlargement was greater in plants. Change in amino acid concentration was delayed for two days in resistant plants. Aspartate rich protein was found in diseased plant and peroxidase activity increased twice. The acidic amino acids which decreased in diseased leaves might have been utilized for incorporation into newly synthesized protein in the diseased metabolic system, and in the early stage of infection β-D-glucosidase might be involved in splitting of the phenolics from their glucosides (Shishiyama, Egawa, Mayama, and Akai, 1969). Increase in amino acid content might be due to decomposition of host protein or decreased protein synthesis. Diseased tissues in general often showed higher proteolytic activity than healthy tissue (Kiraly and Farkas, 1959; Rubin and Artsikhovskaja, 1963). On the other hand, protein bound amino acids were sometimes found to increase simultaneously with free amino acids (Brushovetz, 1954; Shaw and Colotelo, 1961), in which case amino acids might have been translocated from other parts of the host to the infected tissue (Rubin and Artsikhovskaja, 1963; Shaw and Colotelo, 1961), or amino acid synthesis must be increased as well as protein synthesis.
(Shaw and Colotelo, 1961). Synthesis of amino acids by the growing fungus would also cause an increase in the amino acid content of the invaded cells. A large increase in glutamine content was found in rust infected wheat leaves at the time when sporulation began and the rust uredospores were shown to contain much glutamine (Rohringer, 1957).

Decrease in amino acid content in infected tissue was sometimes attributed to utilization by the pathogen, but the disappearing amino acids were not always the ones used most easily by the fungus in vitro. The activity of enzymes involved with amino acid metabolism often increased in diseased tissue. Part of these enzymes were found to be of fungal origin (Farkas and Kiraly, 1961), but the similarity in the changes in amino acid content and enzyme activity induced by infection and by mechanical injury (Minamikawa and Uritani, 1964; Rohringer, Stahmann and Walker, 1958) indicated that the changes observed did not necessarily constitute a specific response to presence of the pathogen.

B.4. EFFECT ON CARBOHYDRATES.

A careful analysis of the available literature on the relation of sugar content on resistance of plant tissues to level of infection led Roemer, Fuchs et al. (1938) to conclude
that there was no relationship between sugar content and resistance of plant tissues to infection. Later works however showed existence of a relationship.

Baba, Ishamu, Takahashi, Iwata, Iwaho and Tajima (1956) observed high negative correlation between susceptibility and starch content or content of acid hydrolysable polysaccharides and starch, in rice plant infected with blast disease. Positive correlation was found with soluble nitrogen/starch or soluble nitrogen/protein nitrogen and starch but no significant correlation with soluble nitrogen/protein nitrogen and K₂O/nitrogen was noted. Respiratory rate also showed no significant correlation with the occurrence of disease, but respiratory rate/starch content showed high correlation. So the susceptibility of rice variety seemed to be closely connected with the content of the respiratory substrate in the plant.

Goto et al. (1958) and Goto and Ohata (1960-61) commented that resistance of rice leaves to blast disease was accountable due to accumulation of total carbohydrates including sugars in the leaves.

Resistant varieties of plants (wheat, rice and banana) to different leaf spot diseases were found to contain more sugar than those in susceptible ones (Lyles, 1959
Lilly and Barnett (1958) observed increased resistance of plant to infection in general with increased sugar concentration; while Lilles et al. (1959) reported that rust resistant wheat contained higher sugar levels than the susceptible varieties. Norman, Torneau and Hart (1961) noted greatly decreased sugar content mostly the sucrose fraction in rust infected susceptible wheat leaves.

In Pinto bean leaves, infection of Uromyces phaseoli induced a concomitant increase in starch accumulation with the increase in chlorophyll content at the rust infection site (Wang, 1961). But decrease in carbohydrate content due to different virus infections were noted in barley leaves (Orlob and Army, 1961; Ramkrishnan et al., 1968; Sutic et al., 1959).

Akai et al. (1958-66) found accumulation of starch surrounding the diseased spots. Accumulation was due to a decreased activity of &beta;-amylase activity and increased activity of invertase near the spot. Substances which inhibit starch decomposition were found in the culture filtrate which inhibited also &beta;-amylase activity. These substances were considered to have diffused out of the lesions and
inhibited β-amylase activity in the tissues surrounding the lesions. An increase in catalase activity was also noticed by them within twenty four hours of inoculation.

Asada (1957) also noticed reduction of soluble carbohydrates and increase of insoluble carbohydrates following infection.

Sato (1962) studied distribution of starch near the shoot and root apices of the rice plant. He found that environment has a greater effect on the starch content of the leaf sheaths, and internodes than of the tissues near the tips of root and shoot.

Total soluble sugar decreased in the leaves of Traill Barley as infection by Helminthosporium sativum progressed, total soluble sugar decreased. Sucrose was lower in diseased than in healthy tissue, while reducing sugar increased with infection, followed by a decline. The duration of sugar increase was inversely proportional to disease intensity (Imman, 1965).

Mirocha and Zaki (1966) observed fluctuation in the amount of starch in host plants, bean and oat infected by rust and mildew fungi, respectively. Within twenty four hours of inoculation with Uromyces phaseoli var. typica
race 32, starch content of bean decreased ninety per cent. Just before sporulation it increased sharply and then decreased again.

Padhi and Sinha (1972) noted that in mosaic infected papya leaves, lamina contained lesser amount of chlorophyll and water and more sugar content than in the healthy leaves.

B.5. EFFECT ON PHOSPHORUS, NUCLEIC ACID (DNA AND RNA).

Changes in different fractions of phosphorus content due to infection were noted by different workers. Yoshii (1958) found that rice plants infected with dwarf virus contained much less inorganic but substantially more organic and nucleic acid phosphorus than healthy ones, total phosphorus content being slightly higher in the former. The virus did not appear to influence the quantity or quality of the nucleoprotein and nucleic acid in the host.

No effect of phosphorus on the susceptibility of brown spot either alone or in combination with nitrogen was noted by Chakraborty (1956). No other records of similar observation could be traced in the literature. Asada (1962, 1967) studied the causes of greater susceptibility of "Akiochi" rice plants to infection of *H. oryzae*. It was observed that
In normal leaves oxidative phosphorylation took place efficiently, whereas in leaves of "akiochi" plants, substrates for respiration were utilized without production of energy rich phosphate compounds. Susceptibility was ascribed to lower metabolic activity which was probably due to uncoupling of oxidative phosphorylation caused by respiration. Tokunaga et al. (1965) however observed susceptibility of rice to blast increased with the increase in available phosphorus and it was greater in young mature tissue than in the old.

TMV infected tobacco leaf showed higher content of dry matter, soluble and total nitrogen, nucleic acid and protein. At the time of maximum protein and nucleic acid synthesis the insoluble phosphates temporarily increased. Total phosphate showed no significant increase but soluble phosphate was considerably lower than in the healthy control (Elbertzhazen, 1958). Mukherjee and Shaw (1962) observed that total phosphorus, inorganic and organic phosphorus and all other phosphate fractions per unit of fresh weight, increased in stem rust infected little club wheat and highest concentration occurred at the loci of rust pustule, probably due to the transportation of phosphorus from uninfected parts of the leaf to the pustule, since the concentration of phosphorus was lower in uninfected portions of infected leaves.
Brown rust infected rye leaves showed considerable increase in the acid soluble, total, inorganic and organic phosphorus content. Concentration of high energy compounds forming the acid soluble P-fraction and the amount of nucleic acid also increased in the period before sporulation (Kupri-vich and Ustinova, 1966).

In highly susceptible variety of potato to potato leaf roll virus, a decrease in total phosphorus due to infection was noted. During autumn, some of the moderately susceptible varieties showed a slight decrease in total phosphorus. Acid soluble organic phosphates in the stems of affected plant were higher than in the healthy ones (Ulrychova, Marie and Limberk, 1968).

Using a slight modification of methyl-green-Pyonin Y stain (Kurnick, 1955) and fulgen stain, Craigie (1959) observed that in infected cells the host nucleoli, the rust nucleoli and the infective hyphae from which the haustoria arose, gave a strong RNA reaction and the fulgen stain gave positive DNA reactions for the nuclear chromation of both the host and the pathogen. Weak fulgen staining of the cytoplasm of haustoria and their supporting hyphae might indicate presence of a fulgen positive fungal metabolite rather than DNA, since cytoplasm of the germinating uredospores and of
other fungal structures frequently gave a similar reaction (Savile, 1939). The DNA response of the chromatin of the rust nuclei was very weak, both to fuulgen and to methyl green, probably because the nuclei in the structures examined occurred largely in the 'expanded' state, with the chromatin dispersed over a relatively large area (Savile, 1939; Crigie, 1959). As evidenced by nuclear enlargement and the appearance of the perinucleolar sphere, the infected host cell also gave evidence of increased metabolic activity. Basing only on visual observation, Person (1960) observed histochemical localization of DNA and RNA in rust infected wheat leaves.

Kuplia and Stern (1961) studied DNA content of internodes of broad bean (Vicia fava) in connection with tumor induction by Agrobacterium tumifaciens. There was no doubt that as a consequence of tumor induction there was heightened DNA synthesis, but such heightened synthesis was not the initial act in tumor induction.

RNA of both healthy and mildew infected soyabean leaves remained essentially constant in tenth and eleventh days of old plant (Millikan, Wyllie and Pickett, 1965). On the twelfth day diseased leaf showed an eighteen per cent decrease in RNA, whereas healthy leaves remained nearly
unchanged in RNA content. Comparable reduction in RNA content for the diseased leaves was observed on thirteenth and fourteenth days. At the thirteen days' age RNA of healthy plants remained unchanged but an eight per cent decrease occurred in the fourteenth day, indicating that half of the observed decrease in RNA could be due to ageing as noticed in developing apple leaves (Kessler, et al. 1962).

Immediately after the appearance of symptoms of powdery mildew, an increase occurred in the RNA content of barley leaves (Malca; Zscheile, and Gulli, 1964).

Quick and Shaw (1964) in wheat leaves of susceptible Little club variety, infected with *P. graminis* observed an increase in the amount of RNA/dry weight, or more conspicuously per fresh weight which was paralleled roughly by the increase in respiration following infection. No increase was apparent in rust infected resistant Khapli plants. In Little club, only on fresh weight basis fifteen per cent increase in DNA occurred. Decrease in histones and marked increase in RNA and acidic proteins as early as two days after inoculation in the nuclei of the mesophyll cells of a susceptible wheat infected with *P. graminis tritici* was noted by Bhattacharya, Naylor and Shaw (1965). Johnson et al. (1967) found total nucleotide content of NaCl extractable RNA on
an average was forty three per cent higher in infected than in healthy leaves after six days of infection of host by pathogen.

In TMV infected tobacco leaves the amount of RNA increased in 2-9 hours after inoculation (Rottger, 1965). Virus infection also altered the RNA content of apple leaf during the early season growth. These effects not only varied according to the strain of the virus, but also according to the sensitivity of the variety (Millikan and Koirtyokhan, 1966). Barley straw mosaic virus infected barley leaf showed increased ribonuclease activity in first and second leaves and evidently ribonuclease was more active in degradation than in synthesis of RNA (Pring, 1969).

Germination of fungal spore was accompanied by RNA synthesis and subsequent changes in the ribosomal activity. Host-parasite interaction occurred between host gene and pathogenic or infective RNA. Locally infected tissue both in fungus and virus disease had a common shift in the metabolic pattern including respiratory increase, accumulation of phenolic compounds and peroxidase activity and ethylene production. The rate of carbon-di-oxide fixation increased in both infections at an early stage and there was a change in the same direction of the nucleic acid metabolism, including synthesis and turnover of RNA, r RNA and t RNA (Hirai, Tokuzo, 1970).