THE UNIVERSITY OF MANITOBA

EFFECT OF BENZIMIDAZOLE, KINETIN AND THEIR RELATED COMPOUNDS ON CHLOROPHYLL METABOLISM AND RUST DEVELOPMENT IN DETACHED LEAVES OF KHAPLI WHEAT

A THESIS

SUBMITTED TO

THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF

THE REQUIREMENT FOR

THE DEGREE OF MASTER OF SCIENCE

by

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October, 1960

ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. E.R. Waygood and Dr. D. Wang for their continued guidance and encouragement throughout the course of this study and in the preparation of this manuscript.

Thanks are also due to Dr. P.K. Isaac and Dr. D. Nandi for their helpful suggestions for the improvement of the manuscript.

The present work was supported by a grant-in-aid (EMR-14) from The Canada Department of Agriculture.

ABSTRACT

Leaves of Khapli wheat, which are normally resistant to race 15B-1 of *Puccinia graminis tritici*, become susceptable and chlorotic when floated on water. This breakdown of resistance and development of chlorosis can be reversed by floating the detached leaves on a solution of benzimidazole or kinetin.

A study was made on the structural specificity of 18 benzimidazole related compounds, 9 kinetin related compounds and a few other compounds, in relation to their activity to stem rust development and chlorophyll metabolism in order to have some information which may contribute to our understanding of the nature of rust resistance as well as the physiology of detached leaves of Khapli wheat.

Results obtained show that any change of the molecular structure of benzimidazole and kinetin makes them lose their activity. Furthermore, compounds with methyl, nitro or other groups introduced into benzimidazole ring are either phytotoxic or antagonistically active to their parent compound. Kinetin related compounds are, however, more active as antagonists of benzimidazole than benzimidazole related compounds particularly in the maintenance of rust resistance.

An investigation into the effect of benzimidazole on the incorporation of carbon-14 labelled compounds into chlorophyll of detached leaves of Khapli wheat shows that benzimidazole enhances greatly the rate of incorporation of labelled glycine and succinate, but without any effect on the incorporation of labelled glutamate and urea.

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INTRODUCTION

The nature of resistance and susceptibility of wheat plant to stem rust is the result of the interaction of the physiology of the host and the parasite. Recent studies (11, 15, 16, 24, 32, 33, 35, 46, 47, 48, 49) have shown that certain chemical agencies may modify or change the rust reaction by interfering with the normal metabolism of the host and disturbing the balance of physiological interaction. Many of these studies were conducted by floating detached wheat leaves on water or aqueous medium (16, 32, 33, 35, 47, 48, 49). A disadvantage of this method is that detached wheat leaves when floated on water usually become chlorotic and fail to give their characteristic reaction to rust infection. However, a supply of benzimidazole in the medium can overcome this difficulty to a large extent (25, 32, 47, 50).

Despite the fact that benzimidazole has not yet been proved to be a naturally occurring factor, its importance in the maintenance of the normal physiology and of the rust resistance of the detached wheat leaves has been emphasized (32, 47, 50). It has been reported that the effect of benzimidazole on the rust reaction of detached Khapli wheat leaves can be overcome by 5, 6-dimethylbenzimidazole (47). Accordingly it appeared essential to study the structural specificity of benzimidazole in relation to chlorosis development and the rust reaction and also the effect of benzimidazole on the biosynthesis of chlorophyll in detached leaves of Khapli wheat. Such information may help to elucidate the site of action of benzimidazole in chlorophyll metabolism and perhaps provide some clues as to the nature of rust resistance.

The present study was conducted to investigate:

(1) the structural specificity of benzimidazole, kinetin and their related compounds in their activity in chlorophyll metabolism and stem rust (*Puccinia graminis tritici* Eriks. and Henn. race 15B - 1) reaction, and

(2) the effect of benzimidazole on the incorporation of carbon labelled compounds into chlorophyll in detached leaves of wheat (*Triticum dicoccum* Schuhler var. Khapli).

LITERATURE REVIEW

Physiological changes in plants following the invasion of obligate parasites have been widely studied. Most of these studies have dealt with changes in photosynthesis, respiration, and the metabolism of carbohydrates and nitrogen.

Sempio (37) reported a sharp increase in photosynthesis in wheat leaves infected with Erysiphe graminis DC. during the first two or three days following inoculation. During the expansion of the mycelium, there was a definite decrease in photosynthesis, which has been attributed to the destruction of chlorophyll in the mildewed leaves during the period of disease development (3).

An increase in the rate of respiration of rust-infected leaves has been observed by many investigators. Allen (3) considered the increased respiration to be a result of accumulation of carbohydrates. Pratt (27) reported that the respiratory intensity of wheat increased rapidly and reached a maximum nine days after infection of powdery mildew, with values almost triple the normal. Allen and Goddard (6) found that the respiratory intensity was at a maximum six days after inoculation, with values three to four times that of normal. They noted also a small increase in anaerobic respiration. Samborski and Shaw (34) reported that the increase in the respiration rate of rust-infected wheat was considerably reduced with increasing distance from the center of the infection site. Shaw and Samborski (40) also demonstrated that the increase in respiration was correlated with an increased participation of the pentose phosphate pathway in

the respiration of the host-pathogen complex.

The accumulation of carbohydrates and fed chemicals at infection sites is one of the striking consequences of infection with obligate parasites. The first demonstration of this was by Gottlieb and Garner (18) for wheat infected with stem rust and supplied with radioactive phosphorus. Results of similar studies have been reported for the infection of rust (1, 2, 22, 35, 39, 51), of powdery mildew (2, 26), of virus and other pathogens (38, 39, 51, 52). The accumulation of fed chemicals was regarded by Shaw and Samborski (39) as an 'active' process of the host since they could inhibit it by treating the plant with certain metabolic inhibitors, such as azide and 2,4-dinitrophenol. On the other hand, Staples and Ledbetter (44) showed that much more radioactivity from glycine tritiated at the 2-position was found in the young urediospores, their sporophores, and the distal portions of the fungal mycelia than in the host. Recent evidence by Wang (48) indicated that the apparent accumulation of radioactivity at the infection sites of rusted leaves shown on the radioautographs may not represent the actual distribution of the labelled compounds throughout the infected leaf. In the case of powdery mildew, the increase in phosphorus, glucose, sucrose, and starch was considered by Allen (4, 5) to be due to metabolic changes in the host.

Numerous studies of nitrogen metabolism in healthy and diseased plants have been conducted in an attempt to correlate the change of nitrogen metabolism with disease development. Samborski (31) found neither qualitative nor quantitative differences in free amino acids between leaves of susceptible and resistant barley varieties. However, he found that rust infection in wheat was accompanied by a decrease in bound amino acids. Wang (41)

found that rust infection induced systemic alterations in the nitrogen metabolism of wheat plants. A decrease in N^{15} concentration was found in the rust resistant (Khapli) variety, while the reverse was true for the susceptible (Little Club) variety. The semi-resistant (Golden) variety resembled resistant plants in their response to infection in that there was no change in the concentration of alcohol soluble nitrogen and little or no change in that of alcohol insoluble nitrogen, but resembled susceptible plants in that there was an increase in N^{15} concentration in both soluble and insoluble nitrogen, indicating an increase in the rate of both soluble nitrogen and protein metabolism.

The rust reaction of intact plants of wheat varieties is sufficiently stable under a wide range of environmental conditions (16). Nevertheless it is well known that the rust reaction may be influenced by a change of environmental factors, particularly temperature (17, 21). Most studies on the effects of temperature have been carried out with considerable variation of temperature, but in certain cases, a rise of only a few degrees will change the host reaction from resistant to susceptible (14). Rust reaction can also be modified by a supply of certain chemicals. Johnson (24) found that seedling leaves of Khapli wheat became susceptible to stem rust (race 17) a few days after being sprayed with DDT (dichlorodiphenyltrichloroethane). Similar results were obtained by Hotson (23) for race 56. DDT, according to Forsyth (13), alters the metabolism in such a way that free amino acids and sugars accumulate in the leaf. The supply of Zn^{++}, Co^{++}, and Mn^{++} in excess in culture solutions (15) and the treatment with maleic hydrazide (35) were reported to have the same effect in breaking down the resistance of wheat to rust. Samborski et al. (32) stressed the possible importance of

substrate concentration to the development of rust. Studies of this kind can be conveniently made with detached leaves. However, there are a number of disadvantages in the use of detached leaf cultures.

The early death of detached leaves is the greatest obstacle to their use for experimental purposes. When leaves are detached from a plant they usually die within 24 hours if deprived of moisture. Detached leaves supplied with water decline in vigor and develop chlorosis over several days and finally die. Beginning of chlorosis may be associated with exhaustion of available carbohydrates and with protein hydrolysis (53, 54), and death may be associated with an accumulation of ammonia (45, 54).

A second disadvantage of detached leaf cultures in the study of rust development is the fact that the rust reactions obtained are often not characteristic for the variety of plant in question (32). Browning (10) reported that detached leaves from oat varieties normally resistant to a given race of crown or stem rust frequently became susceptible when they were supplied with glucose or sucrose solutions.

In wheat, detached leaves retain their green colour for a few days only when floated on water and were usually chlorotic within a week (25). Leaves of Khapli wheat, normally resistant to race 15B-1 of stem rust, lose their resistance when they were detached and floated on water (32, 46).

The destruction of chlorophyll and the breakdown of the resistance of Khapli wheat to stem rust can be prevented by floating the detached leaves on benzimidazole solution. Person et al. (25), working with the rust-susceptible wheat variety Little Club, noticed that leaves floated on 100 p.p.m. benzimidazole retained their green colour and their capacity to support growth of the leaf and stem rust for a period up to at least one month. In their

investigations on leaves floated on 50 p.p.m. benzimidazole, a stable respiration rate as well as nitrogen metabolism were observed. The respiratory increase characteristic for detached leaves did not occur in benzimidazole treated leaves. There was also little change in the levels of soluble and insoluble nitrogen in treated leaves, while marked changes were observed in those floated on water. Amino acids, and particularly amides, were present in much smaller amounts in treated than in water control leaves. Recently Wang and Waygood (50) have studied the effect of benzimidazole on the chlorophyll metabolism in detached etiolated and green leaves of Khapli wheat. They found that benzimidazole stimulated the formation of chlorophyll and preventing its destruction in light and in darkness.

In a study of the changes of rust reaction in detached Khapli leaves Samborski et al. (32) found that detached leaves were susceptible on water, resistant on 40 p.p.m. benzimidazole, susceptible on 40 p.p.m. benzimidazole with 1 per cent glucose, and resistant on 60 p.p.m. benzimidazole with 1 per cent glucose. Little Club leaves were susceptible in all treatments. Wang (47) also reported that the effect of benzimidazole on rust reaction could be nullified by a supply of exogenous glucose or 5,6-dimethybenzimidazole. He also showed that the antagonistic effect of glucose and 5,6-dimethylbenzimidazole on benzimidazole could be eliminated by the addition of an appropriate concentration of cobalt or nickel ion solution.

In addition to benzimidazole, other chemicals have been reported to possess similar effects on chlorosis and rust development in detached wheat leaves. Nickel ion solution of 0.5 to 5.0 p.p.m.,as reported by Wang et al. (49), effectively inhibited the development of rust in detached Khapli leaves. Furthermore, nickel was very effective in the prevention of rust development

in the detached leaves of two susceptible varieties (Little Club and Marquis), although a slightly higher concentration of nickel was required in order to obtain the same degree of inhibition as that found in Khapli leaves. Wang and Waygood (50) have demonstrated that nickel ions were effective in preventing chlorophyll destruction, but decreased considerably the formative process of chlorophyll metabolism.

Richmond (29) reported that kinetin (6-furfuryl-amino-purine) reduced protein loss in detached leaves of Xanthium. Person et al. (25) floated detached wheat leaves on 5 p.p.m. kinetin and found that the effect of kinetin in preventing chlorophyll destruction is similar to that of benzimidazole. A recent report by Samborski and Forsyth (33) showed that in the presence of benzimidazole, a number of compounds inhibited the development of leaf and stem rusts in detached Little Club leaves. These compounds included thymine and its analogue oxythiamine, natural amino acids (histidine, isoleucine, methionine and serine), amino acid analogues (canavanine, ethionine, and p-fluorophenylalanine), and carbohydrates (lyxose, xylose, sorbose, adonitol, arabitol, dulcitol, erythritol, mannitol and sorbitol).

Studies on the biosynthesis of porphyrins in red blood cells have given evidence of the formation of porphyrin with glycine and succinate as the earlier precursors. Altman et al. (7, 8) fed rats with glycine labelled at the α-position with C^{14} and showed that this carbon was incorporated into heme. Radin et al. (28), using duck red cells in vitro, found that the α-carbon of glycine and carbon atoms of acetate were incorporated into heme. Shamin et al. (41, 42, 43) reported that glycine and succinate were the early precursors of porphyrin.

A hypothesis that the formation of chlorophyll follows the same path-

way as the synthesis of heme has been postulated by Granick (20). Salomon, et al. (30) found that α-carbons of acetic acid and glycine were used in the biosynthesis of chlorophyll in Chlorella cells. Later, Della Rosa et al. (12) showed that the carboxyl carbon of glycine was also incorporated into chlorophyll. Recently Brzecki and Rücherl (9) also reported that glycine was utilized by Chlorella vulgaris for the biosynthesis of chlorophyll. In the cells of a Chorella mutant, Granick (19) found an accumulation of protoporphyrin 9, which was isomerically identical with the protoporphyrin of blood heme. Granick (20) speculated that protoporphyrin 9 in Chlorella cells was synthesized from glycine and acetate and that this porphyrin was the common precursor of both the red blood pigment and the green plant pigment.

MATERIALS AND METHODS

The procedures used to study:

(1) effect of benzimidazole on the biosynthesis of chlorophyll, and

(2) effect of benzimidazole, kinetin and their related compounds on stem rust and chlorosis development in detached leaves of Khapli wheat are described seperately. The plant material used in these studies was the primary leaves of Khapli wheat.

I. EFFECT OF BENZIMIDAZOLE ON THE BIOSYNTHESIS OF CHLOROPHYLL

This study involved the incorporation of radioactive compounds into the leaf tissue and the subsequent separation of the chlorophyll (chloroform fraction). Radioactive compounds used were glycine-2-C^{14} (1.56 mg./10 ml. stock solution), succinic-2-3-C^{14} acid (0.86 mg./10 ml. stock solution), glutamic acid -C^{14} (1.64 mg./10 ml. stock solution) and urea-C^{14} (0.95 mg./10 ml. stock solution). The feeding solution consisted of 0.2 ml. of carbon-^{14} labelled compound having a radioactivity of 2 μc and made up to a final volume of 0.6 ml. with either water or solution of benzimidazole (200 p.p.m.).

Primary leaves of greenhouse grown Khapli wheat seedlings were excised nine days after seeding. The detached leaves were floated on water or benzimidazole solution in Petri dishes, which were then placed in a growth chamber at 21°C under eight-hour daily illumination for two or four days. The light source was a bank of 18 fluorescent lamps (made by General Electric Co.) of which nine were warm white (F72T12/WWX) and nine daylight (F72T12/D), giving a light intensity of 650 ft-candles at a distance of 23 in. from the

lamps. At the end of culture period twelve leaves were selected from each culture solution. One-centimeter section was cut off the base of each selected leaf and discarded. The leaves were allowed to stand in a vial containing the feeding solution for 5 hours, in the growth chamber. Whenever necessary, an equal number of water drops was added to each vial to prevent the leaves from wilting. At the end of the feeding period the sample from each treatment was placed in weighing bottle and the fresh weight of the leaves was determined. Leaves were cut into small sections (about 1 cm. in length) and immediately killed by immersion for 5 minutes in 40 ml. boiling 95% ethyl alcohol in a beaker. The leaf sections were then transfered with forceps into a boiling flask and refluxed for 30 minutes twice with 20 ml. each of 80% ethyl alcohol and once with 20 ml. of 40% ethyl alcohol. Each of the three alcoholic extracts was removed from the flask with a dropper and then combined with the original 95% alcohol extract. The combined extract was evaporated at room temperature under a stream of air. The residue was dissolved in 10 ml. of water. This solution was then partitioned with 10 ml. of chloroform. The chloroform layer which contained essentially chlorophyll and carotenes was carefully transferred with a pipette into a beaker. The aqueous solution was washed three more times with chloroform (5 ml. each time). These chloroform washings were combined with the original chloroform extract. The combined chloroform fraction was then washed three times with water (5 ml. each time) and each washing was transferred to original beaker containing aqueous extract. The aqueous extract was used in other studies which will not be described here. The chloroform fraction, having a total volume of 25 ml., was evaporated to dryness at room temperature under a stream of air. The residue was taken up

with chloroform and made up to 1 ml. in a volumetric flask. 10 µl. aliquot of the pigment extract was transferred with a micropipette onto a circular coverglass (18 m.m. diam.). Duplicates were made for each treatment. After drying the radioactivity of each sample was determined with a thin mica end-window Geiger-Müller counter connected to Philips scaler, model PW 4035. The background was determined in a similar manner except that a clean glass planchet was used. The net count of each determination was obtained by subtracting the back ground from the sample count. Total radioactivity in c.p.m. for each treatment was calculated on fresh weight basis.

II. EFFECT OF BENZIMIDAZOLE, KINETIN AND THEIR RELATED COMPOUNDS IN DETACHED KHAPLI LEAVES.

Benzimidazole; kinetin; eighteen benzimidazole related compounds; nine kinetin related compounds and three unrelated compounds were used in this study. The names and structures of these compounds are presented in the Appendix.

The culture of Puccinia graminis tritici (race 15B-1) was maintained on Little Club wheat seedlings. Urediospores used in this study were collected and stored in a stoppered vial in a cold room at about 1 - 4°C.

Nine days after seeding in greenhouse, the primary leaves of Khapli wheat seedlings were inoculated by rubbing with fingers a suspension of urediospores on both upper and lower leaf surfaces. The inoculated leaves were incubated in a moist chamber for 24 hours. Five inoculated leaves were excised and floated on 20 ml. of aqueous medium in a petri dish. The aqueous medium consisted of 20 ml. of compound to be tested or when two compounds were used, 10 ml. of each compound of the mixture. Duplicates

were made for each treatment. The detached leaf cultures were placed in a growth chamber at 21°C under an eight-hour daily illumination. Observations on the development of rust and chlorosis of leaves were made periodically up to the ninth or tenth day. The degree of chlorosis or rust development was expressed numerically. The extent of chlorosis and rust development was arbitrarily set as 5 for water and 0 for benzimidazole (50 p.p.m.) treated leaves.

EXPERIMENTAL RESULTS

I. EFFECT OF BENZIMIDAZOLE ON THE BIOSYNTHESIS OF CHLOROPHYLL

The effect of benzimidazole on the incorporation of carbon-14 labelled compounds into the chlorophyll (chloroform fraction) of detached Khapli leaves is shown in Table I. Results obtained from leaves fed with glycine-2-C^{14} showed that, after a culture period of two days, the amount of radioactivity incorporated into the chlorophyll fraction of benzimidazole treated leaves was approximately double that of the water control, e.g. the average of five experiments was 13,600 c.p.m. for water control and 30,300 c.p.m. for the treated, giving a ratio of benzimidazole treated to water control 2.20. In experiment 3, the radioactivity in both control and treated leaves was relatively low in comparison with that in other experiments. This low rate of incorporation of glycine-2-C^{14} may be due to unexpected changes of environmental factors, or errors in manipulation which might have occurred during the course of the experiment. The data from this experiment were therefore not included for the calculation of the average. Despite the relatively lower values, the ratio remained more or less the same, at least a positive effect of benzimidazole on the incorporation of glycine-2-C^{14} into the chlorophyll of detached leaves is apparent.

Results obtained from two experiments with succinic-2,3-C^{14} acid showed an even higher ratio of benzimidazole treated to water control. The ratio is 2.56 for one experiment and 5.04 for the other. On the other hand, benzimidazole showed no effect on the incorporation of labelled glutamic acid and urea.

TABLE I. Effect of benzimidazole on the incorporation of carbon-^{14} labelled compounds into chlorophyll (chloroform fraction) in detached leaves of Khapli wheat.

Experiment No.	Compound fed	Radioactivity (c.p.m.)		Benzimidazole/Water
		Water	Benzimidazole	
1	Glycine-2-C^{14}	11,200	26,000	2.32
2	Glycine-2-C^{14}	14,500	29,600	2.04
3	Glycine-2-C^{14}	5,000	8,000	1.60
4	Glycine-2-C^{14}	11,800	23,300	1.97
5	Glycine-2-C^{14}	16,500	43,300	2.62
6	Glycine-2-C^{14}	14,600	29,300	2.01
7	Succinic-2,3-C^{14} acid	8,200	21,000	2.56
8	Succinic-2,3-C^{14} acid	7,400	37,300	5.04
9	Glutamic acid-C^{14}	2,200	2,500	1.14
10	Glutamic acid-C^{14}	1,000	1,300	1.30
11	Urea-C^{14}	3,500	2,500	0.71
12	Urea-C^{14}	2,300	3,200	1.40

When a comparison was made on the incorporation of carbon-14 labelled glycine between the immediately detached leaves and those floated on water or on benzimidazole solution, it was found that benzimidazole maintained the normal rate of incorporation even after a four day period of detachment (Table II). In contrast, the amount of radioactivity from glycine in the two-day water control was only half the value of immediately detached leaves. After four

days on water, the rate of incorporation of glycine in the detached leaves decreased further to a value of approximately one quarter of that of the immediately detached leaves, whereas those treated with benzimidazole showed only a slight decrease.

TABLE II. Effect of benzimidazole on the incorporation of glycine-2-C^{14} into chlorophyll (chloroform fraction) in detached leaves of Khapli wheat.

Pretreatment (day)	Radioactivity (c.p.m.)		Benzimidazole/ Water control
	Water Control	Benzimidazole	
0	30,000	30,300*	1.01
0	34,600	30,000*	0.88
2	14,600	29,300	2.01
4	8,400	21,400	2.54

* An average of five experiments with detached wheat leaves which were pretreated with benzimidazole for two days.

II. EFFECT OF BENZIMIDAZOLE, KINETIN, AND RELATED COMPOUNDS ON THE DEVELOPMENT OF RUST AND CHLOROSIS IN DETACHED LEAVES OF KHAPLI WHEAT.

(1) Effect of Benzimidazole and Related Compounds.

Benzimidazole and eighteen related compounds were tested for the effect of their structural specificity on the development of stem rust (race 15B-1) and chlorosis in detached leaves of Khapli wheat. Results are pre-

sented in Table III. Detached leaves were susceptible to stem rust on 5, 10, 20, or 30 p.p.m. of benzimidazole solution, but resistant on 50 p.p.m. or on higher concentrations. Samborski et al. (32) reported that Khapli wheat leaves maintained their resistance to stem rust on 40 p.p.m. of benzimidazole. In the present study, experiments with this concentration of benzimidazole were conducted repeatedly in order to explore its effectiveness in preventing rust development in detached leaves. However, results consistently showed the development of rust, though to a lesser extent than in leaves on lower concentrations. This discrepancy might be due to the fact that experimental conditions and the physiological age of the leaves adapted by the two groups were not identical. The effect of various concentrations of benzimidazole on chlorosis development in detached leaves was similar to that on rust development except that the critical concentration for inhibiting chlorosis was 40 p.p.m.

Among the benzimidazole related compounds, only six were effective in preventing rust development. They are 2-amino-benzimidazole, 6-nitro-benzimidazole, 2-benzoxazole-thiol, benzoxazolone, 5-chloro-2-methyl-benzothiazole and quinoxaline. The effective concentration for each compound varied considerably. For example, a concentration of 10 p.p.m. of 2-benzoxazolethiol or benzoxazolone has the same degree of effectiveness as benzimidazole on the prevention of rust development, whereas it required a concentration of 20 p.p.m. of 5-chloro-2-methyl-benzothiazole, 30 p.p.m. of 6-nitro-benzimidazole, 80 p.p.m. of 2-amino-benzimidazole, or 100 p.p.m. of quinoxaline. A number of compounds tested were found to be phytotoxic at various concentrations. They are 2-benzoxazolethiol, benzoxazolone, 5-chloro-2-methyl-benzothiazole, 6-nitro-benzimidazole, 2-amino-

TABLE III. Effect of benzimidazole and related compounds on the development of stem rust (race 15B-1) and chlorosis in detached leaves of Khapli wheat. 1/

Compound	Development of rust and chlorosis															
	Concentration of test compound (p.p.m.)															
	10		20		30		40		50		60		80		100	
	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C
Benzimidazole	5	5	4	2	2	1	1-0	0	0	0	0	0	0	0	0	0
2-amino-benzimidazole	5	4	5	4	3	4	-	-	2	5	1	5	0	5	*	5
5-amino-benzimidazole	-	-	5	4	-	-	-	-	4	4	-	-	4	4	4	4
5-amino-2-benzimidazolethiol	4	4	-	-	-	-	-	-	4	4	-	-	4	4	4	4
Benzimidazolethiol	-	-	-	-	-	-	5	5	-	-	-	-	-	-	-	-
6-nitro-benzimidazole	3	4	2	4	0	5	-	-	0	5	-	-	Toxic		Toxic	
5,6-dimethyl-benzimidazole	3	4	3	4	2	5	-	-	-	-	-	-	-	-	-	-
Benzoxazole	-	-	5	5	5	5	5	5	5	5	-	-	-	-	-	-
Benzoxazolethiol	0	4	0	5	Toxic		Toxic		Toxic		Toxic		Toxic		Toxic	
Benzoxazolone	0	3	-	-	0	3	-	-	0	3	0	3	Toxic		Toxic	
2-methyl-benzoxazole	Toxic		Toxic		Toxic		-	-	Toxic		Toxic		Toxic		Toxic	
2-o-hydroxy-phenylbenzoxazole	Toxic		Toxic		Toxic		-	-	Toxic		-	-	-	-	-	-
Benzothiazole	-	-	5	5	5	5	5	4	-	-	-	-	-	-	-	-
2-chloro-benzothiazole	-	-	4	4	-	-	4	4	2	5	Toxic		Toxic		Toxic	
5-chloro-2-methyl-benzotriazole	-	-	5	5	5	5	5	5	5	5	-	-	-	-	-	-

contd.

TABLE III. continued

Compound	Development of rust and chlorosis Concentration of test compound (p.p.m.)															
	10		20		30		40		50		60		80		100	
	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C
Benzotriazole	-	-	5	5	5	5	5	5	5	5	-	-	-	-	-	-
Indole	-	-	5	3	5	4	5	4	Toxic		-	-	-	-	Toxic	
4,5-dicarboxylic acid imidazole	-	-	5	4	-	-	-	-	5	4	-	-	-	-	-	-
Quinoxaline	-	-	4	4	-	-	-	-	2	4	-	-	1	5	0	5

1/. R. stands for rust development and C for chlorosis development.

The arabic numerals are used to express the extensiveness of rust and chlorosis development; 5 is equivalent to that of water control and 0 to that of benzimidazole (50 p.p.m.) treated leaves.

* Necrosis occasionally appeared at rust infection sites.

benzimidazole, quinoxaline, 2-methyl-benzoxazole and 2-o-hydroxy-phenyl-benzoxazole. 2-methyl-benzoxazole and 2-o-hydroxy-phenyl-benzoxazole were toxic to leaves even at a concentration as low as 10 p.p.m. (which was the lowest concentration tested). Indole and 2-chloro-benzothiazole had no inhibitory effect on rust development within the range of their nonphytotoxic concentrations. For the remaining eight compounds, 5-amino-benzimidazole, 5-amino-2-benzimidazolethiol, benzimidazolethiol, 5,6-dimethyl-benzimidazole, benzoxazole, benzothiazole, benzotriazole and 4,5-dicarboxylic acid imidazole, it was found that they were neither effective in preventing rust development nor toxic to detached leaves. All the compounds tested showed no apparent effect on the chlorophyll metabolism as compared with water control.

(2). Effect of Kinetin and Related Compounds.

Kinetin and nine related compounds were tested for their activity on rust and chlorosis development in detached leaves of Khapli wheat. The results are shown in Table IV. At lower concentrations (0.2 to 0.5 p.p.m.) kinetin slightly inhibited chlorosis, but did not prevent rust development. When the concentration was, however, increased from 0.5 to 2.0 p.p.m. it prevented both rust and chlorosis development. These inhibitory effects were not evident when leaves were treated with certain kinetin related compounds, with the exception of 2-furoic acid and 2-furamide. 2-furamide showed a pronounced effect in preserving green pigments. 2-furoic acid was phytotoxic at all concentrations tested, but the development of rust was more or less unaffected. All the other compounds reduced slightly the chlorosis of detached leaves.

TABLE IV. Effect of kinetin and related compounds on the development of stem rust (race 15B-1) and chlorosis in detached leaves of Khapli wheat. 1/.

	Development of rust and chlorosis							
	Concentration of test compound (p.p.m.)							
	20		50		80		100	
Compound	R	C	R	C	R	C	R	C
Kinetin **	0-1	0-1	0	0	0	0	0	0
Adenine	4	4	3	4	-	-	2	4
Hypoxanthine	4	4	4	3	4	3	4	3
6-mercaptopurine	3	4	3	4	2	4	2	4
Xanthine	5	3	5	4	5	4	4	4
8-Azaxanthine	5	3	5	3	5	4	4	4
8-chloroxanthine	5	3	5	3	4	4	4	4
8-Azaguanine	5	3	5	4	5	4	4	4
2-Furoic acid	2	5*	3	5*	-	-	3	5*
2-Furamide	5	1	5	1	-	-	5	1

1/ See note under Table III.

* phytotoxicity appeared.

** the concentrations of kinetin in increasing order are 1.0, 2.0, 3.0, and 5.0 p.p.m., respectively. Other concentrations of kinetin have also been tested. The results of rust and chlorosis development in detached leaves on 0.2, 0.5, and 0.8 p.p.m. of kinetin were 5 and 1-5, 5 and 1-4, and 3 and 1-3, respectively.

Since kinetin consists of two components, a purine base and a furan derivative, experiments were then carried out to test the activity of combinations of certain purine and furan derivatives in an attempt to replace kinetin.

It was found that 2-furamide when used together with hypoxanthine or adenine gave a reading of 5 for rust development and a reading of 1 for chlorosis (Table V). Evidently, 2-furamide is responsible for the action of these mixtures in reducing chlorosis as 2-furamide alone gave the same reading (Table IV). 2-furoic acid and hypoxanthine in combinations were ineffective in reducing rust and chlorosis.

TABLE V. Effect of combination of purine and furan derivatives on the development of stem rust and chlorosis in detached leaves of Khapli wheat. 1/.

Furan derivative	Purine compound	Concentration of purine compound. p.p.m.	Development of rust and chlorosis: Concentration of test compound (p.p.m.) 20		50		100	
			R	C	R	C	R	C
2-Furamide	Hypoxanthine	20	5	1	5	1	5	1
2-Furamide	Hypoxanthine	50	5	1	5	1	5	1
2-Furamide	Adenine	20	5	1	-	-	5	1
2-Furamide	Adenine	50	5	1	-	-	5	1
2-Furoic acid	Hypoxanthine	20	5	5	5	5	5	5
2-Furoic acid	Hypoxanthine	50	5	5	5	5	5	5

1/ See note under Table III.

(3) Effect of Compounds Related to Benzimidazole or Kinetin on the Activity of Benzimidazole.

It is evident from the results of proceeding experiments that only

benzimidazole or kinetin, when used alone, could effectively maintain the rust resistance as well as the normal chlorophyll metabolism of the detached leaves. These results suggest that there is a specific molecular structural requirement. However, the relation of rust resistance to chlorophyll metabolism in the leaves is unknown. In order to elucidate this intricate and complex problem studies were made on the action of benzimidazole and kinetin related compounds on benzimidazole in respect to rust resistance and chlorophyll metabolism of detached leaves and the results are shown in Tables VI and VII.

A total of 20 compounds, 12 related to benzimidazole and 8 to kinetin, in combination with benzimidazole (50 p.p.m.) were used for this purpose.

Among the benzimidazole related compounds tested (Table VI), 2-chlorobenzothiazole was most active in reversing the action of benzimidazole on rust development. It was also highly antagonistic to benzimidazole in preventing chlorosis.

Some of these compounds with certain range of concentrations did not affect the activity of benzimidazole in detached leaves. The activity of benzimidazole in preventing rust development was not at all affected by quinoxaline, with all concentrations tested. But, the activity of benzimidazole in inhibiting chlorosis decreased with the increasing concentration of quinoxaline. For leaves floated on 10 to 60 p.p.m. of 6-nitro-benzimidazole with benzimidazole, neither rust nor chlorosis developed at lower concentrations. Phytotoxicity was, however, observed with high concentrations (80 and 100 p.p.m.) of 6-nitro-benzimidazole. At 10 and 20 p.p.m., benzoxazole and benzoxazolthiol failed to overcome the effect of benzimidazole. When

TABLE VI. Effect of benzimidazole related compounds on the action of benzimidazole on stem rust and chlorosis development in detached leaves of Khapli wheat.1/.

Benzimidazole related compound	Development of rust and chlorosis															
	Concentration of test compound															
	10		20		30		40		50		60		80		100	
	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C
2-Amino-benzimidazole	1	0	1	0	1	0	1*	0	2*	1	3*	1	3*	1	4*	1
5-Amino-benzimidazole	-	-	3	2	-	-	-	-	2	1	-	-	1	1	1	1
5-amino-2-benzimidazolethiol	1	1	-	-	2	1	-	-	2	1	1	1	1	1	1	1
6-nitro-benzimidazole	0	0	0	0	0	0	0	0	0	2	0	2	Toxic		Toxic	
5,6-dimethyl-benzimidazole	2	0	2	2	3	3	-	-	-	-	-	-	-	-	-	-
Benzoxazole	0	1	0	1	1	1	1	2	1	3	2	3	2	3	2	2
2-benzoxazolethiol	0	4	0	5	Toxic		Toxic		Toxic		Toxic		Toxic		Toxic	
2-chloro-benzothiazole	5	1	5	2	-	-	5	2	5	2	5	4	Toxic		Toxic	
Benzotriazole	1	1	2	1	2	1	2	2	2	2	3	2	4	3	5	4
Indole	-	-	2	1	-	-	2	2	3	3	-	-	-	-	-	-
4,5-decarboxylic acid imidazole	-	-	2	2	-	-	-	-	3	3	-	-	-	-	3	3
Quinoxaline	-	-	0	1	-	-	-	-	0	3	-	-	0	4	0	4

1/ See note under Table III. Concentration of benzimidazole used in these experiments was 50 p.p.m. except that 40 p.p.m. was used in experiments with indole.

* Necrosis appeared at rust infection sites.

higher concentrations of benzoxazole were used, rust developed considerably in the detached leaves. Phytotoxicity was observed in all concentrations higher than 30 p.p.m. of 2-benzoxazolethiol. In the presence of benzimidazole 2-amino-benzimidazole caused necrotic areas at the rust infection sites. The most interesting fact is the differential sensitivity of the leaf tissue towards these compounds. Relatively low antagonistic effect was observed with the remaining benzimidazole related compounds like 5-amino-2-benzimidazole-thiol, 5,6-dimethyl-benzimidazole, benzotriazole, indole, 5-amino-benzimidazole and 4,5-dicarboxylic acid imidazole.

All the kinetin related compounds investigated were antagonistically active to benzimidazole in preventing rust development in detached leaves (Table VII). Adenine, 8-chloroxanthine and 8-azaguanine overcame entirely

TABLE VII. Effect of kinetin related compounds on the action of benzimidazole on rust and chlorosis development in detached leaves of Khapli wheat. 1/.

Kinetin related compound	Development of rust and chlorosis Concentration of test compound							
	20		50		80		100	
	R	C	R	C	R	C	R	C
Adenine	5	2	5	2	5	2	4	2
Hypoxanthine	4	2	4	2	4	2	4	2
6-mercaptopurine	2	2	2	2	2	2	2	2
xanthine	3	1	4	2	4	2	4	2
8-azaxanthine	4	2	4	2	4	2	4	2
8-chloroxanthine	5	2	5	2	5	2	4	2
8-azaguanine	5	2	5	2	5	2	4	2
2-furoic acid	2	0	-	-	-	-	3	1

1/. See note under Table III. The concentration used in these experiments was 50 p.p.m.

the affect of benzimidazole in preventing rust development. 6-mercapto-purine and 2-furoic acid were less active in this respect. On the other hand, all kinetin related compounds showed only slight activity in respect to chlorophyll metabolism of the detached leaves.

(4) Effect of Benzimidazole, 2-chloro-benzothiazole, Adenine or 2-furoic acid on the Activity of Kinetin.

It has been shown in the preceeding experiments that neither benzimidazole at a concentration lower than 40 p.p.m. nor kinetin at a concentration lower than 1.0 p.p.m., when used alone, was effective in preventing rust and chlorosis development in detached leaves. However, when 0.5 p.p.m. kinetin was combined with benzimidazole of varying concentrations satisfactory results were obtained (Table VIII). Rust and chlorosis were entirely controlled by the complementary activity of these two compounds even when the concentration of benzimidazole used was as low as 5 p.p.m.

The activity of adenine, 2-chlorobenzothiazole or 2-furoic acid on kinetin was investigated. Kinetin at 1.0 p.p.m. gave readings of 0-1 and 0-1 for rust and chlorosis development (Table IV). Same readings of 4,4 was obtained for adenine without benzimidazole (Table IV) and 5,2 for adenine with benzimidazole (Table VII). Table VIII shows that when 1.0 p.p.m. kinetin was combined with 1 to 20 p.p.m. adenine, rust and chlorosis were effectively controlled. Similar results were obtained for 2-chloro-benzothiazole. 2-furoic acid was active in decreasing the effect of kinetin in preventing rust development, but it did not affect the latter's activity of chlorosis inhibition. Since

kinetin and benzimidazole showed similarity in their activities in detached wheat leaves, similar results might be expected when kinetin is substituted for benzimidazole in the above experiments. However, on the basis of the above evidence this assumption may not be correct.

TABLE VIII. Effect of kinetin in combination with benzimidazole, 2-chloro-benzothiazole, adenine and 2-furoic acid on rust and chlorosis development in detached leaves of Khapli wheat. 1/.

		Development of rust and chlorosis															
		Concentration of test compound															
	Kinetin	0.5		1.0		5.0		10		20		40		50		100	
Compound	(p.p.m.)	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C
Benzimidazole	0.5	-	-	-	-	0	0	0	0	0	0	0	0	0	0	-	-
2-chloro-benzothiazole	0.5	-	-	-	-	-	-	3	0	5	0	5	0	5	0	-	-
2-chloro-benzothiazole	1.0	-	-	-	-	0	0	0	0	1	0	3	0	4	0	-	-
Adenine	0.5	1	0	2	1	-	-	3	1	3	1	4	1	-	-	-	-
Adenine	1.0	-	-	0	0	-	-	0	0	0	0	1	0	-	-	-	-
2-furoic acid	0.5	-	-	-	-	-	-	-	-	2	0	-	-	3	0	5	0
2-furoic acid	1.0	-	-	-	-	-	-	-	-	4	0	-	-	4	0	4	0

1/. See note under Table III.

(5) Effect of Choline, Phenyl-urea or Diphenyl-urea, with and without Benzimidazole.

The results of this study are presented in Table IX. Choline and phenyl-urea at lower concentrations (10 p.p.m. to 40 p.p.m.) were not effective in reducing rust development. The latter, however, considerably

reduced chlorosis. Choline at higher concentrations (50 p.p.m. to 100 p.p.m.) and diphenyl-urea at all concentrations (10 p.p.m. to 100 p.p.m.) investigated were phytotoxic and prevented rust development in detached leaves of Khapli Wheat. When used with benzimidazole Choline , at lower concentrations, prevented chlorosis. On the other hand phytotoxicity of diphenyl-urea was not affected at all. Phenyl-urea could not overcome the action of benzimidazole in preventing rust and chlorosis development, a fact which might suggest that the former is not an antagonist of the latter.

TABLE IX. Effect of choline, diphenyl-urea and phenyl-urea, with and without benzimidazole on rust and chlorosis development in detached leaves of Khapli wheat. 1/.

	Development of rust and chlorosis																	
	Concentration of test compound																	
	5		10		20		30		40		50		60		80		100	
Compound	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C	R	C
Choline	5	3	5	3	5	3	-	-	-	-	0	5*	0	5*	0	5*	0	5*
Choline-benzimidazole (50 p.p.m.)	4	0	4	0	5	0	-	-	-	-	-	-	-	-	-	-	-	-
Diphenyl-urea	-	-	0	5*	0	5*	-	-	-	-	0	5*	0	5*	0	5*	0	5*
Diphenyl-urea-benzimidazole (50 p.p.m.)	-	-	-	-	-	-	-	-	-	-	2	5*	2	5*	2	5*	2	5*
Phenyl-urea	-	-	-	-	5	1	5	1	-	-	-	-	-	-	-	-	-	-
Phenyl-urea-benzimidazole (40 p.p.m.)	-	-	-	-	0	0	0	0	1	0	-	-	-	-	-	-	-	-

1/. See note under Table III.

* Phytotoxicity appeared.

DISCUSSION

Allen (3) reported that in mildewed leaves of wheat, chlorotic spots appear beneath each colony and enlarge until they fuse. Before the completion of the fusion, at about the time of the maximum respiration of the leaves, chlorophyll is re-formed under the center of each mildew colony and spreads outward to form green island of about the size of a mildew colony. Unpublished observations made on bean leaves in this laboratory showed that the chlorophyll in the circular zones surrounding the rust colonies is not destroyed while the leaves become chlorotic. An accumulation of starch is also observed in these areas. Evidence has been shown that benzimidazole is effective in maintaining normal chlorophyll metabolism and rust reaction in detached wheat leaves (25, 32, 47, 50). It is therefore reasonable to assume that chlorophyll metabolism plays an important role in the host-parasite relation in rust-infected leaves of wheat.

A total of 32 compounds were investigated in the present study for their activities in detached Khapli leaves. It is evident from the results that only benzimidazole or kinetin could effectively maintain the rust resistance as well as the normal chlorophyll metabolism of the detached leaves. The results also reveal a complementary effect of these two compounds in their activities in detached leaves. These suggest that there is a specific molecular structural requirement for the maintenance of normal physiology in detached leaves.

Besides benzimidazole and kinetin only two compounds, 2-furamide and phenyl-urea reduced chlorosis considerably. Six benzimidazole related compounds (Table III) within certain range of concentrations prevented rust development

entirely. The fact that these six compounds prevented effectively rust development but failed to inhibit chlorosis implies that both benzene ring and imidazole ring of benzimidazole are essential for the maintenance of the normal physiology and the rust resistance of detached Khapli leaves, and that an introduction of a methyl, amino, or other groups into the molecule of benzimidazole will alter its activities in detached leaves.

The effect of benzimidazole and kinetin related compounds, choline, phenyl-urea and diphenyl-urea on the activities of benzimidazole in detached leaves were also studied. At certain concentrations four benzimidazole related compounds, 6-nitro-benzimidazole, benzoxazole, 2-benzoxazolethiol and quinoxaline, had no adverse effect on the activity of benzimidazole in preventing rust development. (Table VI). However, only 6-nitro-benzimidazole at 40 p.p.m. or lower concentrations did not alter the effect of benzimidazole in chlorosis inhibition. 2-benzoxazolethiol at 10 and 20 p.p.m. was highly antagonistic to the activity of benzimidazole in inhibiting chlorosis. 10 p.p.m. quinoxaline with benzimidazole considerably reduced chlorosis. With increased concentration of quinoxaline, however, severe chlorosis development was observed. On the basis of these results, it might be assumed that the antagonistic effect of an antagonist on the activities of benzimidazole in preventing rust development is different from the antagonistic effect on the latter's activity in inhibiting chlorosis. Further evidence supporting this assumption can be found in the experiments with 2-furoic acid with benzimidazole (Table VII) and choline with benzimidazole (Table IX).

In general, kinetin related compounds are more active as antagonists of benzimidazole (Table VII), particularly in reversing the latter's activity in

the maintenance of rust resistance, than benzimidazole related compounds, except 2-chloro-benzothiazole (Table VI).

Since kinetin and benzimidazole showed similarity in their activities in detached wheat leaves, one would expect similar results for experiments with compounds combined with either benzimidazole or kinetin. However, results obtained in the present study failed to support this assumption.

Although structural specificity is required for the maintenance of normal chlorophyll metabolism and rust resistance, it is apparent from the discussions above that the structural specificity required for inhibiting chlorosis and that required for preventing rust development are different from each other. Further studies on this problem would be very helpful in the understanding of the physiology of host-parasite relation.

A hypothesis has been proposed by Granick (20) that a common pathway exists in the biosynthesis of heme and chlorophyll. Della Rosa *et al.*(12) have demonstrated the utilization of glycine by *Chlorella* in the biosynthesis of chlorophyll. However, no evidence of the incorporation of succinate into chlorophyll has yet been noted.

In the present study it is noted that benzimidazole has a positive effect on the incorporation of glycine and succinate into the chlorophyll of detached Khapli leaves. This evidence furnishes further proof of the stimulating effect of benzimidazole on chlorophyll formation proposed by Wang and Waygood (50), and is in agreement with the findings of Della Rosa *et al.*(12) and Granick's Hypothesis (20).

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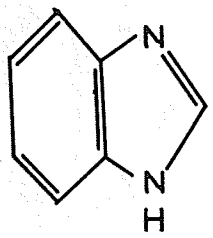
A P P E N D I X



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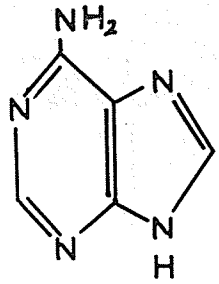

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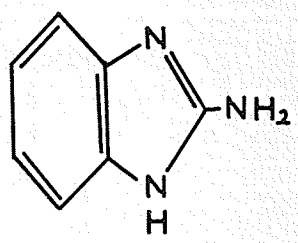

2-Amino-
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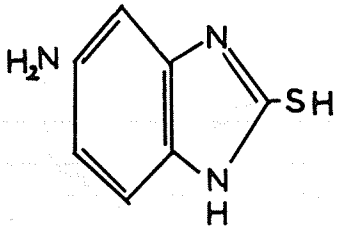

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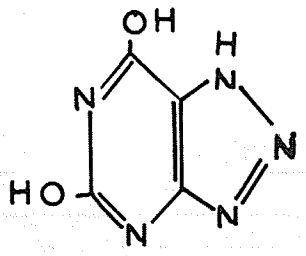

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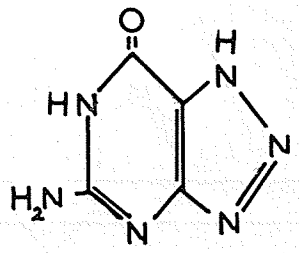
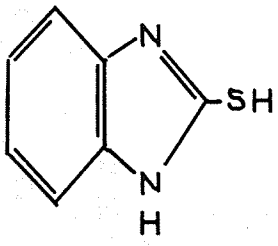


8-Azaguanine



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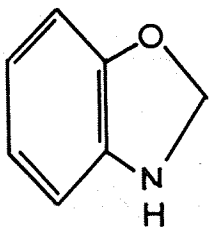
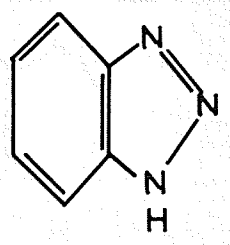


Benzoxazole



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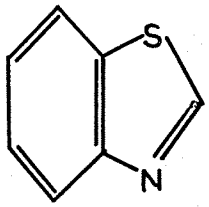

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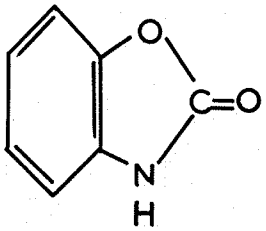

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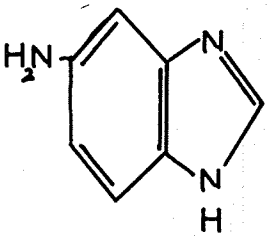

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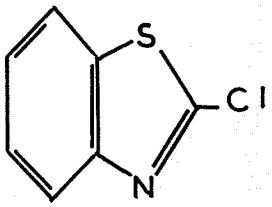

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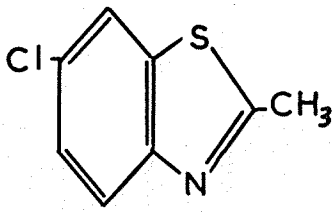

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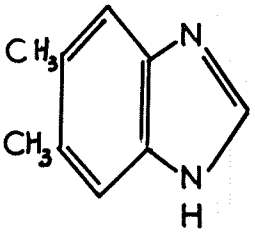
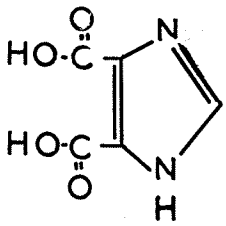
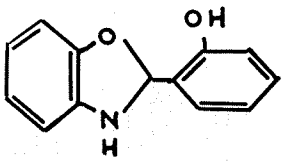


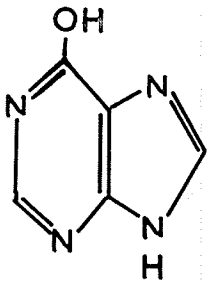
5,6-Dimethyl-benzimidazole



4,5-Dicarboxylic acid imidazole



2-O-Hydroxy-phenyl-benzoxazole



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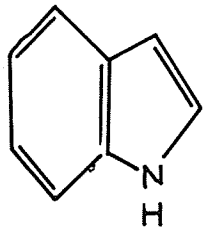

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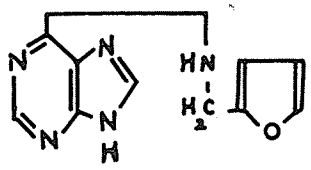

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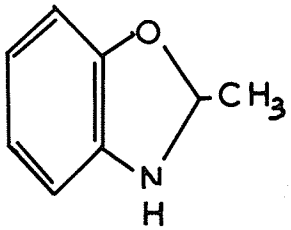

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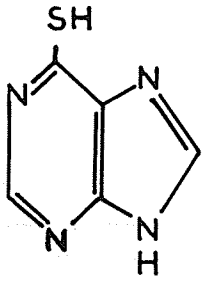
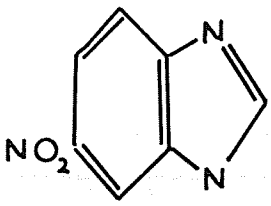
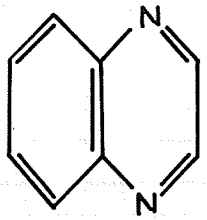


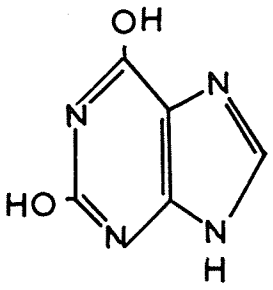
6-Mercapto-purine



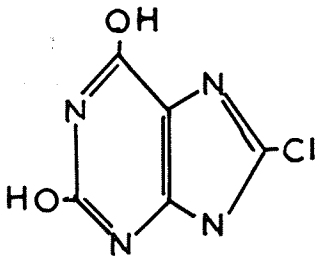
6-Nitro-benzimidazole



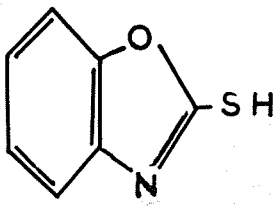
Quinoxaline



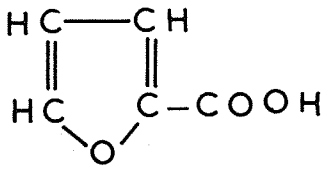
Xanthine



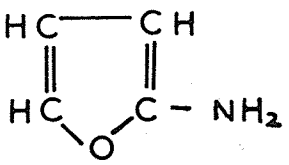
8-Chloro-xanthine



Benzoxazolethiol



2 Furoic acid



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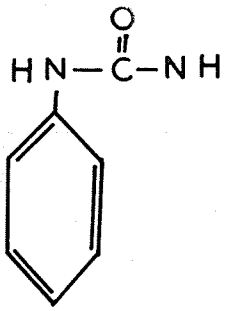

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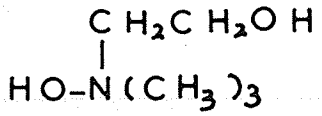

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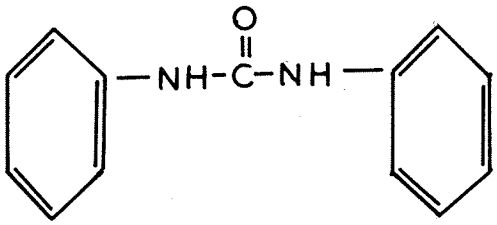

Di-phenyl-urea