## Result and Discussion

### Inhibition of *in vitro* Metabolism

The inhibition of *in vitro* metabolisms of various barbiturates by IP and DMI were determined at a given inhibitor concentration (1 μmole/g liver) to compare their inhibitory activities. The results are summarized in Table I. Both the inhibitors suppressed rather uniformally *in vitro* metabolisms of all barbiturates, indicating non-specific inhibition by IP and DMI. And it is likely that a major metabolite DMI is a more potent inhibitor on barbiturate metabolisms than the parent IP. This finding is not in agreement with the conclusion of a previous

Table I. Inhibitory Activities of Imipramine (IP) and Desmethylimipramine (DMI) on *in vitro* Barbiturate Metabolisms

| Barbiturates | Control metabolism[a] | Inhibition[b] (%) | |
|---|---|---|---|
| | | IP | DMI |
| Hexobarbital | 0.735 | 43.0 | 62.9 |
| N-Methylallobarbital | 0.130 | 41.8 | 55.8 |
| Amobarbital | 0.265 | 53.0 | 61.2 |
| Secobarbital | 0.314 | 68.2 | 70.9 |
| Thiopental | 0.098 | 56.8 | 61.5 |

*a*) The figures are mean values of 3—7 identical experiments with 2—6 pooled livers each, and expressed in μmole metabolized/hr/g liver.
*b*) The figures are mean values of 2—3 identical experiments with 2—3 pooled livers each. The amount of inhibitor added was 1 μmole per 1 g fresh liver.

work[4] which excluded DMI as a less remarkable inhibitor. The kinetic treatments have been frequently done to obtain a clue to characterize the microsomal drug enzymes in the inhibition[8,20-23] or induction studies.[24-25] In the former cases, when the inhibitor can be an alternative substrate and the metabolite(s) formed elicits considerable inhibitory activity, the observed kinetics is not attributed solely to the parent inhibitor, but to the mixed system which is complicated kinetically. In the present studies, therefore, merely as a measure of apparent affinity to the enzymatic system, the inhibitor constants of IP and DMI were determined with hexobarbital as a substrate. And it was found that the suggested type of inhibition was non-competitive for both IP and DMI, and inhibitor constants of IP and DMI were $1.7\times10^{-4}$M and $0.3\times10^{-4}$M, respectively, and they were smaller than the Michaelis constant of hexobarbital, $5.3\times10^{-4}$M which was estimated separately. These results suggest a possible contribution of DMI in the inhibition by IP. As an attempt to get further evidence for the DMI participation, some experiments were carried out in which IP was preincubated in the complete system before the addition of substrate. The results are shown in Table II. The inhibition observed was almost equivalent to that listed in Table I, although the control metabolisms were reduced with the lapse of time which may be due to the essential cofactor consumption. According to the quantitative estimation of IP and DMI metabolisms in the *in vitro* system similar to the present one by Bickel and Baggiolini,[9] IP was metabolized mainly to DMI (*ca.* 45% in 1 hr), on the other hand DMI was less susceptible to the susequent

20) L.A. Rogers and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **146**, 286 (1964).
21) R. Kato and M. Takayanagi, *Jap. J. Pharmacol.*, **16**, 127 (1965).
22) M. Ikeda, S. Tanaka and T. Katayama, *Mol. Pharmacol.*, **4**, 38 (1968).
23) A. Rubin, T.R. Tephly and G.J. Mannering, *Biochem. Pharmacol.*, **13**, 1053 (1964).
24) A. Rubin, T.R. Tephly and G.J. Mannering, *Biochem. Pharmacol.*, **13**, 1007 (1964).
25) K.J. Netter and G. Seidel, *J. Pharmacol. Exptl. Therap.*, **146**, 61 (1964).

metabolism(*ca.* 9% in 1 hr). Considering these findings, it seems possible to expect that metabolically formed DMI is accumulated and significantly participate in the inhibition of barbiturate metabolisms by IP in the *in vivo* situation.

Table II. Effect of IP Preincubation on *in vitro* Hexobarbital Metabolism

| Preincubation time (min) | Control metabolism ($\mu$M/hr/g) | Metabolism when IP[a] was added ($\mu$M/hr/g) | | Inhibition (%) |
|---|---|---|---|---|
| | | in preincubation | in properincubation | |
| 45 | 0.430 | 0.228 | — | 47.0 |
| 45 | 0.430 | — | 0.245 | 43.0 |
| 60 | 0.323 | 0.182 | — | 43.7 |
| 60 | 0.323 | — | 0.187 | 42.1 |

*a*) IP was added to the system in 1.0 $\mu$mole for g fresh liver. Both incubations were done under the conditions described in methods.

### Inhibition of *in vivo* Metabolism

In order to correlate the *in vivo* inhibition exerted by IP administration and the metabolic disposition of IP itself, the time courses of the inhibition of barbiturate metabolisms were studied. In these experiments, the metabolic activity of 9000×g supernatant fraction was estimated at various times after IP administration with hexobarbital and amobarbital as the substrates. The time courses of enzyme activities affected by IP are shown in Fig. 1. It was apparent from the results that IP was appreciably absorbed from the intraperitoneal route and inhibited the metabolisms of both barbiturates with the maximum effect at approximately 3hr after IP administration and the IP-produced inhibition lasted for 24 hr. This metabolic inhibition by IP was further confirmed by examining the kinetics of hypnotic action of hexobarbital. As shown in Fig. 2, the pharmacological action was influenced significantly

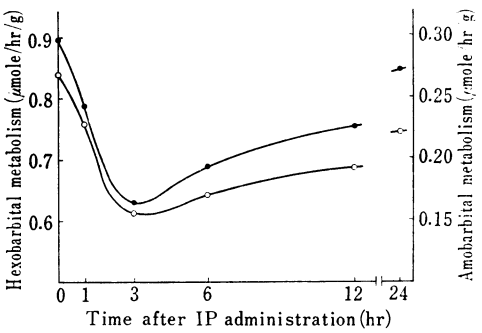


Fig. 1. Time Course of Inhibitory Activity of IP on Hexobarbital and Amobarbital Metabolisms

Each point represents the mean values of at least three rats, hexobarbital (○), amobarbital (●). Dose of IP was 30 mg/kg.

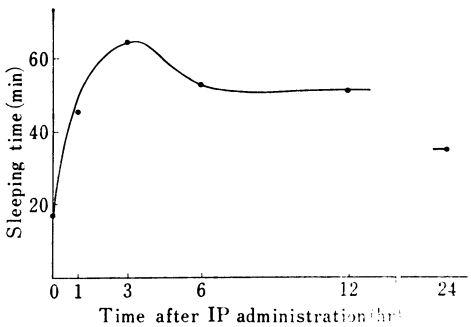


Fig. 2. Effect of IP on the Duration of Hypnotic Action of Hexobarbital

Each point represents the mean value of six rats, singnificantly different ($p<0.001$, $p<0.01$ for 24 hr) from the control sleeping time. Dose of hexobarbital was 100 mg sodium salt/kg.

by IP administration and coincided fairly well with the metabolic inhibition as shown in Fig. 1. This relationship seems to be indicative of the temporal and reversible nature of IP effect. IP has been known to have an intrinsic activity of increasing the sensitivity of the central

nervous system[3,26] in addition to its indirect effecct due to the metabolic inhibition, however considered from the recovery rate of the hypnotic potentiation and the metabolic inhibition observed, this direct effect may be relatively small. As predicted from the results of *in vitro* experiments, DMI formed *in vivo* may be an effective inhibitor on barbiturate metabolisms. According to Dingell, *et al.*,[10] the rats metabolize IP mainly to DMI more rapidly than they do DMI and the formed DMI accumulate considerably in the body. Therefore, if the pharmacokinetic parameters of DMI related to the processes of absorption and tissue distribution *etc.* are identical to those of IP, the inhibitory pattern after DMI administration will be similar to that observed after IP administration, although some deviations will be observed probably due to the different metabolic dispositions of IP and DMI. On this line of discussion, the time courses of the inhibition of barbiturate metabolisms elicited after DMI administration were examined. The results shown in Fig. 3 seem to be consistent with the above-mentioned trends. These findings suggest that the concentration of IP and its metabolite DMI present in liver cells around the microenvironment of the responsible microsomal enzymes is necessary for the effective inhibition. Further experiments were performed concerning with the effect of dose of IP on the enhancement of the inhibition. The results are shown in Fig. 4. For both the

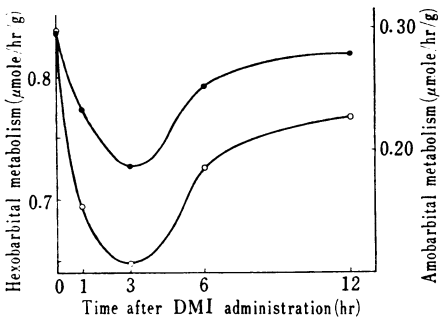


Fig. 3. Time Course of Inhibitory Activity of DMI on Hexobarbital and Amobarbital Metabolisms

Each point represents the mean value of at least three rats, hexobarbital (○), amobarbital (●). Dose of DMI was 28.7 mg/kg equimolar to 30 mg/kg of IP.

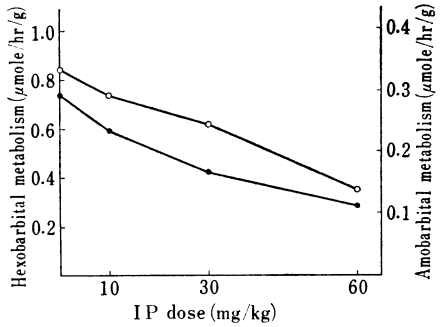


Fig. 4. Effect of IP Dose on Hexobarbital and Amobarbital Metabolisms

Each point represents the mean value of at least five rats, hexobarbital (○), amobarbital (●). Enzyme assays were done at 3 hr after IP administration.

substrates, the inhibitory effect of IP was found to be dose-dependent within the range examined, although the enhancement was not strictly linear. It can be assumed kinetically that the increase in dose will result in an increase in the effective concentration of the drug or its metabolite and the corresponding inhibition will be also increased if the physiological dispositions are of linear and passive nature. The present situation seems to be the case. The dose-dependent inhibition, however, has a possibility that it is associated with the alterations of the hepatic cell ultrastructure which are known to be causative of the change in drug metabolisms.[27] An attempt, therefore, was made to examine the effect of long-term pretreatment with IP on the enzyme activity as a possible criterion for the ultrastructural alterations. As shown in Fig. 5, any significant effect was not observed by the chronic treatment in both male and female rats. From the results obtained hitherto, it seems possible to assert that the concentration of IP and DMI present in the liver cells is an essentially

26) I. McCance, *Arch. Intern. Pharmacodyn.*, **148**, 270 (1964).
27) H. Remmer and H.J. Merker, "Proc. 2nd Intern. Pharmacol. Meet.," Vol. 4, Pergamon Press, Oxford, 1965, p. 299.

important factor in determining the extent of the inhibition and the concentration changes of these compounds with the lapse of time is related to the duration of the inhibitory effect after IP administration. Based on this reasoning, the liver levels of IP and DMI were determined after IP administration in male rats. The results represented in Fig. 6 offers a plausible explanation for underlying mechanism of the inhibition suggested above.

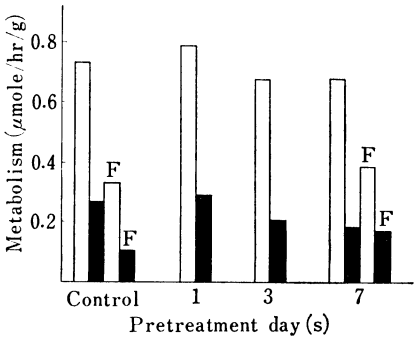


Fig. 5. Effect of Long-Term Pretreatment of IP on Hexobarbital and Amobarbital Metabolisms

Each bar represents the mean value of at least three rats. Enzyme assays were done at 24 hr after the last injection. Dose of IP was 30 mg/kg *i.p.* once daily.
□: hexobarbital
■: amobarbital
F: female rat

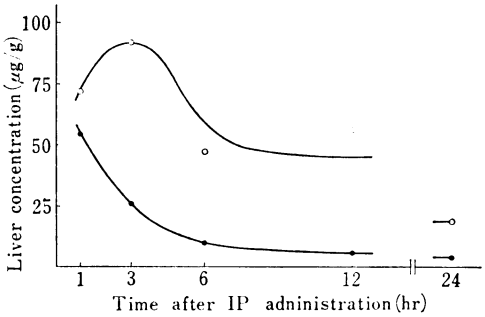


Fig. 6. Liver Concentrations of IP and DMI after a Single Dose of IP

Each point represents the mean value of two experiments, IP (●), DMI (○). Dose of IP was 60 mg/kg.

TABLE III. Species Difference of Inhibitory Activity of IP and DMI on *in vitro* Hexobarbital and Amobarbital Metabolism

| Animal species[a] | Hexobarbital | | Amobarbital | |
|---|---|---|---|---|
| | IP | DMI | IP | DMI |
| Rat | 13.5[b] | 33.6 | 26.9 | 56.6 |
| Mouse | 31.8 | 58.9 | 23.1 | 53.0 |
| Rabbit | 52.6 | 57.9 | 51.4 | 79.2 |
| Guinea pig | 13.2 | 15.3 | 14.2 | 12.2 |

*a*) Male adult animals were used and enzyme preparations were same as in rats described in Methods.
*b*) The figures are % inhibition and mean values of at least 3 experiments. The amount of inhibitor added was 0.2 μmole for g fresh liver.

The inhibitory activities of IP and DMI for *in vitro* barbiturate metabolisms were examined further in other animal species. As shown in Table III, in all species DMI was also active as an inhibitor. Because it is known that there is a marked species differences in the metabolic dispositions of IP,[10] these data can not be correlated with those in the *in vivo* studies which require further investigations.

**Physiological Availability of IP and DMI**

As the physicochemical properties affecting the physiological availability of a drug in the body, ionization constant and lipid solubility of drug molecule are often discussed.[28]

28) L.S. Schanker, *Ann. Rev. Pharmacol.*, **1**, 29 (1961).

The previously reported p$K_a$ values of IP and DMI are in the ranges of 7.94—9.5 and 9.22—10.2, respectively.[29-30] The molecular species of IP and DMI at physiological pH are predominantly in the charged formed, but the apparent partition coefficients of IP and DMI, determined with the pH 7.4 isotonic phosphate buffer–chloroform system at 37° were 1925 and 453, respectively. Considering the generalized theory of membrane permeation, these data suggest that IP and DMI are almost equivalently available in the absorption and tissue distribution processes.[31]

Further studies on the molecular-level correlation of drug enzyme inhibition and the inhibitor's dispositions with the other inhibitors should be of interest and required to chracterize the microsomal drug metabolizing enzyme systems.

29) R.A. Maxwell, P.D. Keenan, E. Chaplin, B. Roth and S.B. Eckhardt, *J. Pharmacol. Exptl. Therap.*, **166**, 320 (1969).
30) M.H. Bickel and H.J. Weder, *J. Pharm. Pharmacol.*, **21**, 160 (1969).
31) The absorption rate constants of IP and DMI from rat small intestine were determined and the value 1.43 $hr^{-1}$ was obtained for both the inhibitors.

[Chem. Pharm. Bull. 19(7)1402—1408(1971)]

UDC 581.192 : 547.458.02.05

# Studies on Fungicides. IX.[1] Chemical Structure of Chitin-like Substances of Cell Walls from *Cochliobolus miyabeanus*. (3)

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(Received December 11, 1970)

The chemical structure of the chitin-like substance, one of the main components of the cell wall of *Cochliobolus miyabeanus*, was studied. The results of X-ray powder pattern of this chitin-like substance suggested that the chitin molecules are connected with some radicals. The chitin fraction was submitted to enzymic digestion, acid hydrolysis, methylation and X-ray analysis. All the experimental results demonstrated that the hexosamine in the present fungal cell wall is composed of branching points conncected with α-N-acetylgalactosamine residue (connected through C-1) to the straight β-1,4-acetylglucosaminyl chain.

## Introduction

Although there are many reports on the structures of fungal cell walls, little is known of their detail. In the previous paper,[1,3] the authors reported the presence of the glucan (β-1,3-linked glucan having branched units connected through C-6 and C-1) and the chitin-like substance associated with a small amount of other sugars and peptides in the cell wall of *Cochilibolus miyabeanus*. The present paper deals with the fine chemical structure of the chitin-like substance described above.

### Material and Method

1) **Isolation of the Chitin-like Substance**——The fraction of cell walls free from cytoplasmic contamination was prepared in the manner described previously[3] and the isolation of the chitin-like substance from the cell wall was carried out as shown in Fig. 1. The chitin-like substance is expressed as Fraction A.

2) **Enzymolysis of Fraction A**——The enzymic hydrolysis of Fraction A was made with chitinase (Nutritional Biochemicals Co., Ohio) or lysozyme (Sigma Chemical Co., St. Louis). Fraction A was incubated with the enzyme under the following conditions.

a) Substrate, 25 mg; chitinase, 1000 μg; McIlvaine buffer, 0.01 M, pH 6.55, 3.5 ml; toluene, one drop; at 37° for 72 hr.

b) Substrate, 25 mg; lysozyme, 500 μg; phosphate buffer, 0.01 M, pH 6.25, 5.0 ml; toluene, one drop; at 28° for 75 hr.

3) **Acetolysis of Fraction A**——Acetolysis of Fraction A was performed according to the method of Matsuda, *et al.* described previously,[1] except that Fraction A dissolved in the acid mixture was kept at 37° for 130 hr rather than at 25° for 72 hr.

4) **Methylation of Fraction B**——Fraction B was methylated according to the method of Hakomori.[4]

5) **Methanolysis and Acid Hydrolysis of the Methylated Fraction B**——Methanolysis and acid hydrolysis of the methylated Fraction B were performed according to the methods described previously,[1] except that the methylated Fraction B was dissolved in 10% methanolic HCl and heated at 125° for 5 hr in a sealed tube.

6) **X-ray Analysis of the Compounds**——Powdered Fraction A was analysed in a diffraction meter (Rigaku Denki Co.) with nickel-filtered copper target at 40 kv, 1.5418 Å. A crustacean chitin (Seikagaku Kogyo Co., Tokyo) was used as a standard.

7) **Identification of the Compounds**——i) Paper Chromatography (PPC): PPC analysis for hexosamine was run on Toyo Roshi No. 50 paper with (a) butanol–acetic acid–water (4:1:5) or (b) phenol saturated with water containing 1% $NH_3$, and the analysis for methylated amino sugar was run with butanol–ethanol–water (5:1:4), and sprayed with Elson–Morgan's reagent or aniline phydrogen phthalate.

1) Part VIII: H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **19**, 448 (1971).
2) Location: *Motoyama-cho, Higashinada-ku, Kobe.*
3) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **19**, 252 (1971).
4) S. Hakomori, *J. Biochem.* (Tokyo), **55**, 205 (1964).

ii) Gas-Liquid Chromatography (GLC): GLC analysis was performed according to the method described previously.[3]

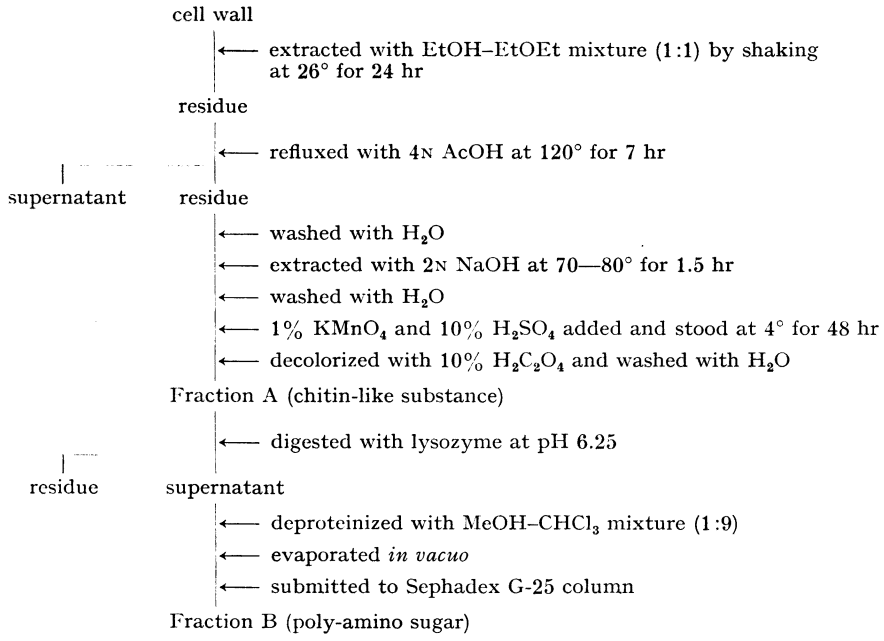


Fig. 1. Fractionation of Chitin-like Substance from the Cell Wall of *Cochliobolus miyabeanus*

## Result and Discussion

The main components of the cell wall of *Ascomycetes spp.* were recently characterised as β-glucan and chitin-like substance,[5] and the chemical structure of the β-glucan layer in the cell wall of *Cochliobolus miyabeanus* was clarified by the present authors.[1] Therefore, the fine structure of the chitin-like substance in this fungus was studied in detail.

As described previously,[3] a large amount of glucosamine and a trace of galactosamine were found in the hydrolysate of Fraction A by PPC or GLC analysis, and the enzymolysis of this fraction also N-acetylglucosamine identified by PPC or GLC analysis (Table I), and methods of Svennerholm[6] or Reissig[7] (Fig. 2).

The infrared (IR) spectrum of Fraction A was almost identical with that of crustacean chitin and exhibited an absorption peak at around 884—900 $cm^{-1}$ which represents the β-configuration of a polysaccharide[8] (Fig. 3). Consequently, this fraction was regarded as a chitin-like substance which consisted mainly of N-acetylglucosamine molecule, while this Fraction A gave an X-ray powder pattern almost identical with that of crustacean chitin except for the presence of one clear band indicated by an arrow in Fig. 4a. This pattern suggests that the chemical structure of Fraction A is different from that of crustacean chitin and that it is composed of chitin molecules connected with some radicals.

In order to clarify this assumption, Fraction A and crustacean chitin were refluxed with 2N HCl for 2 hr and the supernatants were checked by PPC or GLC analysis. A fair amount

5) S. Bartnicki-Garcia, *Ann. Rev. Microbiol.*, **22**, 87, (1968).
6) L. Svennerholm, *Acta Soc. Med. Upsaliensis*, **61**, 287 (1956).
7) J.L. Reissig, J.L. Strominger and L.F. Leloir, *J. Biol. Chem.*, **217**, 959 (1955).
8) S. A. Barker, A. B. Foster, M. Stacey and J. M. Webber, *J. Chem. Soc.*, **1958**, 2218.

Table I. Paper and Gas–Liquid Chromatographic Analysis of Amino Sugars produced from Fraction A by Enzymolysis

| Sample | PPC (*Rf*) | | GLC ($R_t$) |
|---|---|---|---|
| | Solvent a | Solvent b | OV-17 (180°) |
| Hydrolysate of Fraction A by chitinase | 0.31 | 0.72 | 15.1 |
| Hydrolysate of Fraction A by lysozyme | 0.30 | 0.73 | 15.0 |
| Authentic N-acetylglucosamine | 0.31 | 0.72 | 14.9 |

solvent a: BuOH–AcOH–$H_2O$=4–1–5
solvent b: phenol (+1% $NH_3$)
filter paper: Toyo Roshi No. 50
detector: Elson–Morgan's reagent

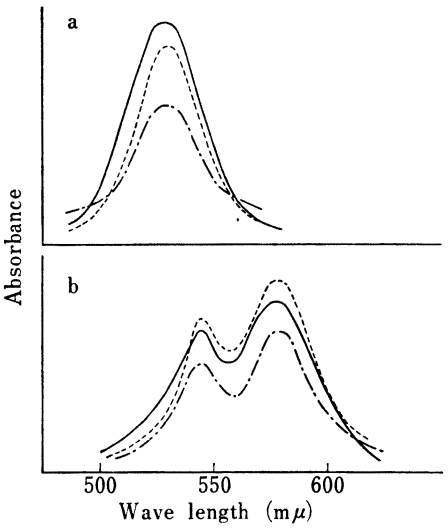


Fig. 2. Absorbance profiles of Amino sugar from Fraction A

**a**: by the method of Svennerholm
**b**: by the method of Reissig
------: crustacean chitin
—·—: Fraction A
——: authentic N-acetylglucosamine

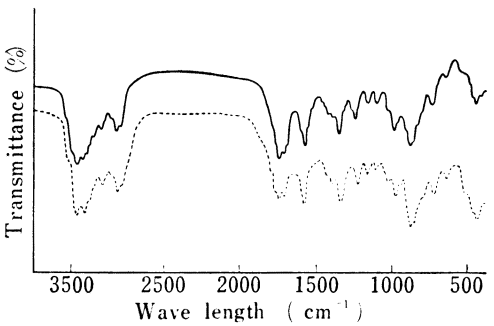


Fig. 3. Infrared Spectrum of Fraction A and Crustacean Chitin

——: crustacean chitin
------: Fraction A

Table II. Paper and Gas–Liquid Chromatographic Analyses of Amino Sugars from Acid Hydrolysis of Fraction A

| Sample | PPC (*Rf*) | | | | GLC ($R_t$) | |
|---|---|---|---|---|---|---|
| | Solvent a | | Solvent b | | OV-17 (180°) | |
| Hydrolysate of Fraction A by 2N HCl | 0.73 | 0.69 | 1.61 | 1.67 | 16 | 14.3 |
| Hydrolysate of Crustacean by 6N HCl | 0.73 | | 1.60 | | 16 | |
| Authentic glucosamine-HCl | 0.73 | | 1.61 | | 16 | |
| Authentic galactosamine-HCl | | 0.69 | | 1.68 | | 14.3 |

solvent a: BuOH–AcOH–$H_2O$=4–1–5
solvent b: phenol (+1% $NH_3$)
filter paper: Toyo Roshi No. 50
detector: Elson–Morgan's reagent

of galactosamine and a trace of glucosamine were found in the supernatant of Fraction A, while that of crustacean chitin gave only a trace of glucosamine (Table II), and the peculiar band in the X-ray powder pattern of Fraction A was extinguished by this acid treatment (Fig. 4b).

Further, N-acetylglucosamine and N-acetylgalactosamine were released from Fraction A by chitinase digestion (37°, 150 hr). The chitinase used in this experiment gave 5 bands by acryl amide gel electrophoretic analysis (Fig. 5), and this result indicated that this chitinase was composed of at least 5 kinds of protein and some of them might have α- or β-N-acetylgalactosaminidase activity. In order to determine the specificity of this enzyme, the chitinase preparation was incubated with phenyl-N-acetyl-α-galactosaminide or phenyl-N-acetyl-β-galactosaminide under the following conditions: 0.01 M phosphate buffer (pH 6.25) 1 ml, substrate 15 mg, 1000 γ chitinase 2 ml, and toluene (one drop) at 37° for 150 hr. The released galactosamine was detected by GLC analysis. As shown in Fig. 5, the liberation of galactosamine was detected only in the case of

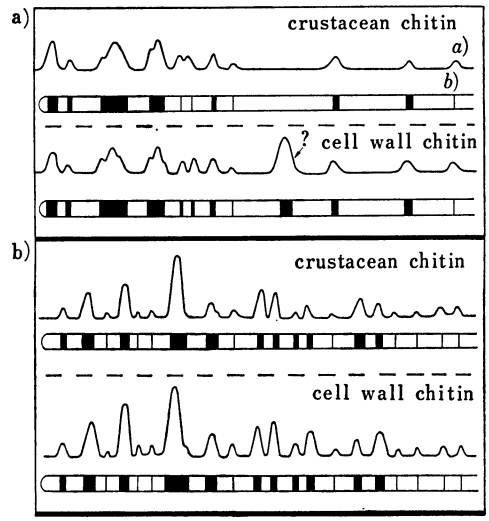


Fig. 4. X-Ray Powder Patterns of the Acid-treated Fraction A and Crustacean Chitin

*a*) X-ray diagrams from photograph by densitometer
*b*) patterns of analytical photograph of X-ray powder analysis


Analytical pattern

| Substrate | Enzyme activity[a)] |
|---|---|
| Phenyl-N-acetyl-α-galactosaminide | + |
| Phenyl-N-acetyl-β-galactosaminide | − |

*a*) Enzyme activity was determined by GLC analysis of released N-acetylgalactosamine.

Fig. 5. Disk Electrophoretic Analysis and the Specificity of Chitinase

TABLE III. Paper and Gas–Liquid Chromatographic Analyses of Acetolyzate Solution of Fraction A

| Compound | PPC (*Rf*) | | GLC ($R_t$) OV-17 (160°) | |
|---|---|---|---|---|
| Crustacean chitin | 0.18 | | 15.5 | |
| Fraction A | 0.18 | 0.13 | 15.5 | 12.0 |
| Authentic N-acetylglucosamine | 0.18 | | 15.5 | |
| Authentic N-acetylgalactosamine | | 0.13 | | 12.0 |

solvent: BuOH–AcOH–$H_2O$=4-1-6
filter paper: Toyo Roshi No. 50
detector: Elson–Morgan reagent or aniline hydrogen phthalate

phenyl-N-acetyl-α-galactosaminide. This result indicated that this chitinase contains α-N-acetylgalactosaminidase.

Although the oligosaccharide composed of glucosamine and galactosamine was detected in the acetolyzate solution of Fraction A by PPC or GLC analysis, the monomers of both amino sugars were found (Table III), and it was likely that Fraction A was composed of chitin molecules (β-1,4-linked polymer of N-acetylglucosamine) connected with N-acetylgalactosamine.

The contents of amino sugars in acid hydrolysate or acetolyzate solution of Fraction A were determined by the Blix method[9] for glucosamine and the Ludowing-Benmaman method[10] for galactosamine, and the molar ratio of these amino sugars is given in Table IV.

TABLE IV. Molar Ratio of Glucosamine and Galactosamine in Fraction A

| Sample | Molar ratio | |
|---|---|---|
| | Glucosamine | Galactosamine |
| Hydrolysate of Fraction A by 6N HCl | 11.8 | 1.0 |
| Hydrolysate of Fraction B by 6N HCl | 11.3 | 1.0 |
| Acetolyzate solution of Fraction A | 11.4 | 1.0 |

By incubating Fraction A with lysozyme at 27° for 72 hr, the soluble poly-N-acetylglucosamine connected with N-acetylgalactosamine (Fraction B) was released into the supernatant of the reaction system. In order to determine the molecular weight of Fraction B and poly-hexosamine released from Fraction B by elimination of N-acetylgalactosamine, an aliquot of Fraction B and the hydrolysate of Fraction B by refluxing with 2N HCl for 2 hr at 100° were chromatographed on a column (2×60 cm) of Sephadex G-25 (coarse type). Tannic acid (mol. wt. 1701.25), Eosin Y (mol. wt. 691.91) and the compound formed by the combination of tannic acid and Eosin Y (mol. wt. 2393.16) were used as the standard. As shown in Fig. 6, the molecular weight of Fraction B was about 2200—2350 (degree of polymerization of hexosamine: 10—11) and this value was scarcely changed by elimination of N-acetylgalactosamine.

If Fraction B is composed of 10—11 N-acetylglucosamine and one N-acetylgalactosamine molecule linked in a straight chain (Fig. 7a), the molecular weight should be reduced by elimination of N-acetylgalactosamine. In the present experiment, the molecular weight of poly-hexosamine was not reduced by elimination of N-acetylgalactosamine (Fig. 6), and this result suggests that N-acetylgalactosaminyl radical was connected with poly-N-acetylgucosamine chain by branches linked as shown in Fig. 7b.

There have been few papers on the methylation of chitin of chitin-like substance because no suitable solvents for methylation was present, but Fraction B or lysozyme-treated crustacean chitin can be methylated by the method of Hakomori. After repeated methylation and checking with IR spectrum, a completely methylated compound was subjected to methanolysis in a sealed tube with 10% methanolic HCl at 125° for 5 hr and demethylated by acid hydrolysis. An aliqout of the product was submitted to PPC analysis. As shown in Table V, the result of PPC (Elson-Morgan reagent and aniline hydrogen phthalate used as a detector) demonstrated the presence of 3,4,6-tri-O-methyl-D-galactosamine and 3,4,6-tri-O-methyl-D-glucosamine, and unidentifide compounds remained at the original point by this solvent system, while the chromatogram of the methylated crustacean chitin treated with lysozyme showed the presence of 3,4,6-tri-O-methyl-D-glucosamine and unidentified com-

9) G. Blix, *Acta Chem. Scand.*, **2**, 467 (1948).
10) J. Ludowig and J.D. Benmaman, *Anal. Biochem.*, **19**, 80 (1967).

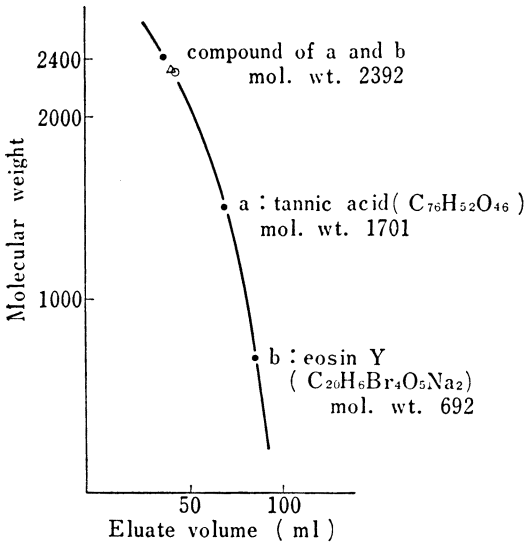


Fig. 6. Change in Molecular Weight of Fraction B by Elimination of N-Acetylgalactosamine

column condition: Sephadex G-25 (coarse type) 2×60 cm, eluted with $H_2O$

△: Fraction B

▲: Fraction B minus N-acetylgalactosamine

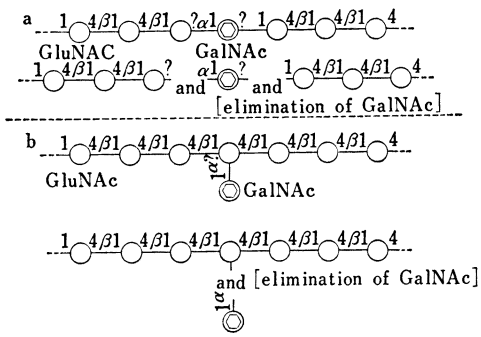


Fig. 7. Conversion of the Chemical Structure of Fraction B by Elimination of N-Acetylgalactosamine

TABLE V. Paper Chromatographic Analysis of the Methylated Amino Sugars derived from Fraction B and Enzyme treated Crustacean Chitin

| Methylated compound | *Rf* | | |
|---|---|---|---|
| Fraction B | 0.02 | 0.27 | 0.25 |
| Crustacean chitin | 0.02 | 0.28 | |
| Authentic N-acetylglucosamine | | 0.28 | |
| Authentic N-acetylgalactosamine | | | 0.25 |

solvent: BUOH-EtOH-$H_2O$=5-1-4
filter paper: Toyo Roshi No. 50
detector: Elson-Morgan reagent or aniline hydrogen phthalate

pounds remained at the original point spot. This result demonstrated the presence of some branching points connected by α-N-acetylgalactosaminyl linkage (connected through C-1).

These experimental results demonstrate that hexosamine in the present fungal cell wall are composed of β-1,4-linked 2-acetamide-2-deoxy-D-glucose having branched units of 2-acetamide-2-deoxy-D-galactose connected through C-1 by α-glycosidic linkage as shown in Fig. 7b.

Since early in the 19th century, the alkali-resistant substance of mushroom was named "fungine" by Braconnot,[11] Odier[12] also discovered an alkali-resistant compound named "chitin." Wisselingh,[13] Wettstein,[14] Nabel,[15] and a number of workers, described in the

11) H. Braconnot, *Ann. Chem.*, **79**, 265 (1811).
12) A. Odier, *Mem. Soc. Hist. Nat. Paris*, **1**, 29 (1823).
13) C. Wisseligh, *Jahrb. Wiss. Botan.*, **31**, 619 (1898).
14) F. Wettstein, *Akad. Wiss. Wien. Math. Naturw. Kl.*, **Abt. 1**, 130 (1921).
15) K. Nabel, *Arch. Microbiol.*, **10**, 515 (1939).

review by Aronson[16] and by Bartnicki-Garcia,[5] have taken up study of the chemical constitution of chitin in fungal cell walls of *Zygomycetes*, *Phycomycetes*, *Basidiomycetes*, and *Ascomycetes* species. Recently, the presence of chitin in the cell wall of *N. crassa* by X-ray method was reported by Manocha,[17] that of *Aspergillus spp.* by Johnston[18] and Ruiz-Herrera,[19] and of *Aspergillus oryzae* by Horikoshi.[20] Harold[21] isolated a poly-galactosamine which is capable of binding with polyphosphate, and Johnston, *et al.*[22] found a galactosamine in the cell walls of *Aspergillus niger*, *Neurospora sitophila*, and *Botrytis cinerea*. Applegarth, *et al.*[23] also reported the presence of galactosamine polymer in the cell wall of *Helminthosporium sativum*. However, all these investigations demonstrated that chitin in the fungal cell wall is composed of a straight-chain $\beta$-1,4-N-acetylglucosamine polymer. There seems to be no mention of the chitin-like substances as described in this paper in the fungal cell wall. Korn[24] reported the presence of an amino sugar associated with mannan, glucan, or protein in the cell wall of baker's yeast. Further research is planned to find the possible role of N-acetylgalactosamine branch in the chitin molecule.

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