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STRUCTURE AND FUNCTIONS OF FUNGAL CELL SURFACES

Nozawa, Y.

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STRUCTURE AND FUNCTIONS OF FUNGAL CELL SURFACES

Nozawa, Yoshinari

(Department Biochemistry, School of Medicine, Gifu
University, Gifu, Japan)

I. Introduction

Information on the structure of the surface of fungal cell /147*
walls can be said to be very scarce compared to that for bacterial
cells. It is primarily for this reason that it is difficult to
isolate the structural substances of the surface layers of fungal
cells. That is to say, with respect to bacteria, the separation of
the plasma membrane and cell wall of bacteria has already been
accomplished for some time now, and based on the detailed analysis
of the chemical constituents, even the structure of the molecular
construction is being elucidated. Moreover, recently, even the
in vitro reconstitution of the structure of the superficial layers
has been attempted, and a large amount of important information has
been obtained as a result. On the other hand, differing from this
kind of situation with bacterial cells, in approaching fungi, the
research has been centering around the yeast-type fungi,
Saccharomyces and Candida, and this research also has focused on the
chemical analysis of the structure of the surface layers. It can be
thought that understanding of the construction of the structure at
the molecular level is a problem almost of the past. With respect
to pathological filamentous fungi, research has just entered only
the early stages, and now it is necessary to study this field
diligently. In this study, the discussion of the structure related
to the surface layers will be limited to pathological filamentous
fungi (Dermatophytes).

II. Chemistry of the Cell Wall and the Construction of the Structure

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*Numbers in the margin indicate pagination in the foreign text.

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1) Separation of the Cell Wall and Ultramicrostructure

The attempts at separating the cell walls from dermatophytes
began late compared to those for yeast type fungi. By 1968, Shah
and Knight [1] and Itoh et al. [2] had separated and purified the
cell walls from Trichophyton mentagrophytes, but both groups had the
same difficulty wth the cell wall fraction, that of the purity of
the fraction, and it was necessary to remove foreign substances
using other membrane systems. Nozawa et al. [3] achieved the
separation of an almost completely clean and purified fraction of
cell walls by means of using Epidermophyton floccosum, which is
thought by others to easily undergo massive distruction of the cell.
In other words, in that method, after creating a homogenous
suspension of fungi in a Waring blender, the suspension is put into a
Ribi cell fractionator (Sorvall Co., U.S.A.) and destruction of the
cells is performed under high pressure (30,000 psi) three times.
The resultant is rinsed with water, centrifuged in a sucrose
gradient, and treated with SDS (sodium dodecyl sulfate). In this
manner a purified fraction of cell walls was obtained. The results
of observing the cell wall fraction obtained in this way, by means
of scanning electron micropscope, are shown in Figure 1. It shows a
hollow cylindrical structure, and it is clear that there is very
little foreign substance originating from the inner membrane system,
including the plasma membrane. Even in the ultra-thin layer image
there are no other substances which are from non-membrane system
areas attached to the cell wall. From the standpoint of these
electron microscopic findings, it is certain that the separation of
the cell walls of E. floccosum as done using this method results in
a very high degree of purity.

Furthermore, the results of the observation of the structure of
the outer surface of this fraction of cell walls by means of
shadowing electron microscopy showed that the bundled microfibriles
were aligned mainly in the direction of the axis of the fungal
filaments, and with the microfibriles which crossed these, a net

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FIGURE 1. Scanning Electron
Microscopic Image
of Separated E. floc-
cosum Cell Walls
(X 10,000)

mesh like structure was exhibited (Figure 2a). On the other hand, /148
with respect to the inner surface, a net mesh like structure of
narrow microfibriles which was more pronounced than that seen in the
outer layer, and a condition in which amorphous substances filled
the spaces between the microfibriles, were observed (Figure 2b).
This morphological difference between the inner and outer surfaces
was clarified, and it suggests that there are differences in the
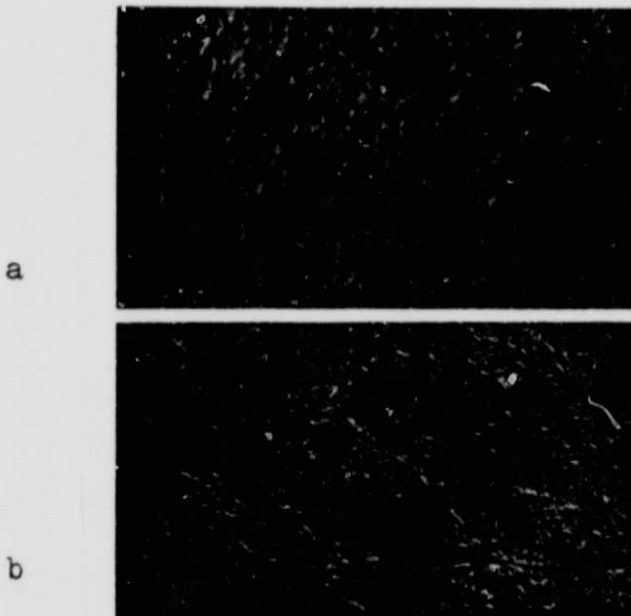chemical components which formed these structures.

FIGURE 2. Structure of the
Surfaces of Identi-
cal Cell Walls
(X 36,000); a: Outer
Surface; b: Inner
Surface

TABLE 1. Chemical Constituents of Individual Fractions of Cell Walls from Epidermophyton floccosum Extracted with Acids and Bases

Structural Components	Untreated Cell Wall	1N NaOH	1N NaOH + 1N NaCl	4N Acetic Acid	4N Acetic Acid + 1N NaOH	Exo-layer
Neutral Sugars	46.3	31.4	3.1	43.1	26.2	11
Glucose	35.2	26.5	2.9	37.2	22.3	trace
Mannose	5.3	3.0	0	5.5	2.0	10
Galactose	trace	0	0	0	0	trace
N-acetylglucosamine	30.9	66.7	95.3	47.2	68.4	17
Peptides	10.3	0.6	0.3	1.7	0.3	63
Lipids	4.6	0	0	0	0	0

2) Chemical Constituents of Isolated Cell Walls

The chemical constituents of the purified fraction of cell walls separated by means of the above-mentioned method, as shown in Tabel 1, included about 80% sugar components, this being the primary constituent as is the case with the cell walls from other types of cells. Other than this, there were a variety of peptides and lipids. Moreover, it was shown that the chemical constituents of the cell walls of T. mentagrophytes exhibited almost the same trend [4]. With respect to the sugar components, glucose and and glucosamine were the primary constituents, composing close to 70% of the sugars. On the other hand, with respect to the amino acid make-up, along with asparaginic acid, glutamic acid, proline, and glycine, there were the important ligands in which a sugar is added to alanine, namely serine and threonine. With respect to the lipid components, there were the phospholipids phosphatidalethanolamine and phosphatidalcholine and a number of neutral lipid ergosterols.

Next, having clarified the chemical constituents of the whole
cell wall, in order to clarify the correlations between the
structure of the constituents of the cell wall and the chemical
constituents, study of the dissolved fractions, obtained by treating
the purified cell walls with acid or bases, and the remaining
fractions was carried out [5]. And in particular in the latter
case, morphological observations were performed with the aid of
electron microscopy. Changes in the chemical constituents of the
cell walls which was obtained after the extraction with acids and
bases are shown in Table I. The peptides and lipids were disssolved
and removed by treating with the 1 N NaOH, and the proportion
showing the stable acetylglucosamine increased. When examined with
the aid of electron microscopy, mainly the changes in the inner   /149
surface of the wall were obvious. The contours of the microfibriles
became more distinct and the width of the fibers reached 15-20 nm.
Furthermore, when this fraction was treated with 1 N NaCl, a
fraction formed almost exclusively of acetylglucosamine was
obtained. Morphologically, it appeared to be linkages of thick rod-
like fibers. Because, it was identified as chitin by means of x-ray
diffraction, this acid and base resistant fraction was judged to be
chitin fibers. On the other hand, when the cell wall was initially
treated with 4 N acetic acid, the fiberous structure became blurred,
and a fraction was obtained in which microfibriles filled in within
the amorphous space-filling substance. Furthermore, when this
fraction was extracted with a base, a fraction with a small quantity
of glucose (the successor to glucan) in it was formed. Morpholo-
gically, the microfibriles reappeared distinctly. These fibers
differed from the ones initially formed with the treatment with the
base and were generally narrow.

By the way, this type of fibrous structure appeared to be
wholly without any import. It has been ascertained even
morphologically that there are substances presenting a membranous
structure on the outermost layer of the cell wall, and the fraction
with this structure generated a lot of interest. Nozawa et al.

FIGURE 3. Electron Microscopic
Image of "Exo-layer"
(X 60,000)

compared this structure to the structure of other cell walls. It
showed a peculiar resistant to Helix pomatia enzyme, and even after
treating with this enzyme the membranous structure remained intact.
This fraction was obtained by centrifugation and was named the "exo-
layer" [6]. The electron microscopic presentation of this
structure, as shown in Figure 3., shows a membranous structure, but
the structure is not like that commonly found in biological
membranes, the typical two-layer structure of a simple membrane.
Even from the standpoint of the chemical analysis of the fraction,
because no lipids were detected, this was not judged to be a so-
called membrane structure. The characteristics of this structure
were that of a type of sugar protein with the sugar portion composed
only of mannose. According to disc electrophoresis, the molecular
content of the primary peptide fractions (I-V) consisted of various
molecules with molecular weights ranging from 42,000 to 154,000.
Among these, fraction I (molecular weight, 154,000) was judged to be
a sugar protein containing mannose which stained positively as a
sugar. Also, with respect to the amino acid content, as a item of
deep interest, it was found that the primary constituents were the
amino acids which are seen often in bacterial cell walls, namely,
asparaginic acid, glutamic acid, glycine, and alanine. However,
while the biochemical significance of this unique outer layer
structure is currently completely unknown, it is possible that it

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may have some important function involving immunological activity. At any rate, both of these may be answered by the results of current research.

3) Structure of the Cell Walls of Dermatophytes

Based on the above-described changes in the structure of the cell walls treated with acids and bases and in the chemical constituents, the basic components of the structure of the basic cell wall was inferred. Accordingly, it is indicated that the cell wall of Epidermophyton floccosum is formed by the primary component, 2 layers, an inner and an outer, each approximately 0.1-0.2 microns thick, and of an outermost layer, a thin "exo-layer." Moreover, the outermost layer exhibits a coarse net mesh like structure composed mainly of sugar proteins; the outer layer present under this is formed of finely integrated microfibriles. These microfibriles (200 Angstroms) are suggested to be composed of a core region of chitin fibers surrounded by a layer of glucan, and all of this is covered with a base resistant, acid-dissolvable polysaccharide. The inner layer of the cell wall is thought to be formed from almost identical microfibriles and amorphous substances which are base dissolvable polysaccharides which fill in the spaces between the microfibriles.

However, this model of the components of this structure is no more than a tentative proposal, and a more detailed molecular structure should be shown by the current elucidation on the form of bonding of the polysaccharides. Recently, the results of the systematic chemical analysis of T. mentagrophytes cell walls by Poulain et al.[7] led them to report that the cell wall structure determined from the results matched the model proposed by Nozawa et al rather well. This suggests that there are similarities in the structure of the cell walls of both types of fungi. On the other hand, Nozawa et al have already discussed the similarities and differences with the model of the cell wall of E. floccosum from the standpoint of the analysis of the chemical components and the

morphological observation of the cell wall of T. metagrophytes, and the resemblances have been pointed out [8,9].

Next the most recent general remarks by Kitashima et al.[10] on details of such things as the cell walls of other types of fungi is presented.

## III.   The Plasma Membrane as the Site of the Effects of Polyene Antibiotics

A method to separate the plasma membrane from dermatophytes with a high rate of recovery and a high degree of purity has not yet been established. Consequently, in order to understand the cytoplasmic membrane which incorporates many functions even in the layers on the surface of fungal cells, a very significant approach is to obtain information by introducing polyene antibiotics which have a particular localization of effect on this membrane. The effects of the polyene antibiotics on this membrane are described below.
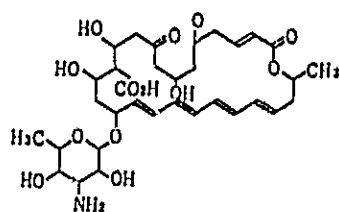
1)   Chemical Structure of Polyene Antibiotics

Since the discovery of the antifungal substances (the current Naistatin) produced by Streptomyces noursei which was separated from the soil of cattle ranches in Virginia by Hazen et al. in 1951, many polyene antibiotics have been reported. Actually, there are not a large number of varieties which can be put to practical service, and those are limited to only a few. Here, if one is to show a representative chemical structure, it would be like Figure 4. As the names indicate, there are many trans-type conjugated double bonds in the macrolide rings. Among the larger ringed molecules are nai-statin and amphotericin B, and among smaller ones is filipin. Also, they are classified according to the number of double bonds. In-cluded in the tetraenes are naistatin, pimaricin, and etholscomicin, in the pentaenes is filipin, and in the heptaenes is amphoteriin B.

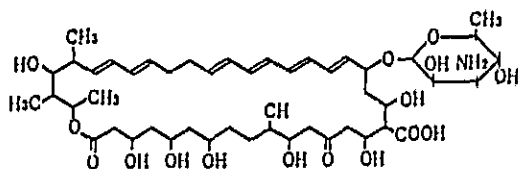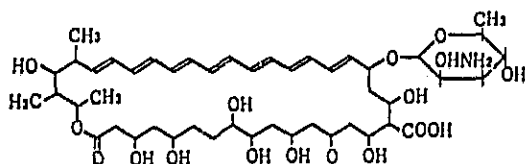図4　代表的なポリエン抗生物質の化学構造

Filipin

Pimaricin

FIGURE 4. Chemical
Structure of Representative
Polyene Antibiotics
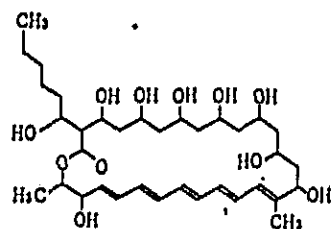
Naistatin

Amphotericin B

In all of these substances, as clarified by the structure, there are hydroxide groups on one side of the macrolide ring, and on the other side there are a series of double bonds; it is a so-called substance that alternates between both parents.

2) Morphological Changes in the Cytoplasmic Membranes of Fungi Due to Polyene Antibiotics

The creation of a "pit" structure when polyene substances are applied to a membrane containing sterols as membrane components has been clarfied already by Kinsky et al. (1967) [11]. However, the question of whether or not the resulting "pit" structure observed by means of negative staining electron microscopy is the "gap" commonly found in membranes has been argued since that time. Since the introduction of freeze fracture electron microscopy, it has become possible to observe the structure of the channel region within the double-layers of living membranes. Thus, Nozawa et al applied various polyene antibiotics to Epidermophyton floccosum and observed the morphological changes. As a result, it was shown that changes in the distribution of the membrane protein particles (80-105 Angstroms) were induced, and this trend was particularly evident on the inner fracture face (PF) [12,13]. It is thought that the movement of membrane proteins and the change in the distribution pattern was brought about by the rearrangement of the membrane lipids. In other words, by means of the interaction of the polyene antibiotics and the ergosterols within the membrane, rigid and fluid areas were created in the lipids, and the membrane protein particles moved like icebergs from solid areas to fluid areas. Consequently, it may be permissible to think of this also as a type of phase separation. As shown in Figure 5, the morphological changes in the cytoplasmic membrane can vary according to the type of polyene anti-biotic. These have been divided into 3 major types [14]. Type I is the "pit" form type like in the case of filipin; a consistent struc-ture with 250-300 Angstrom diameter elevated areas can be seen on the PF surface, and 70 Angstrom diameter depressions can be observed in the center of the elevated areas. Because of this information, it has been suggested that the large gaps previously thought to be common in membranes do not exist. Actually, the correlation of the depression in the central area of the pits with the unusual
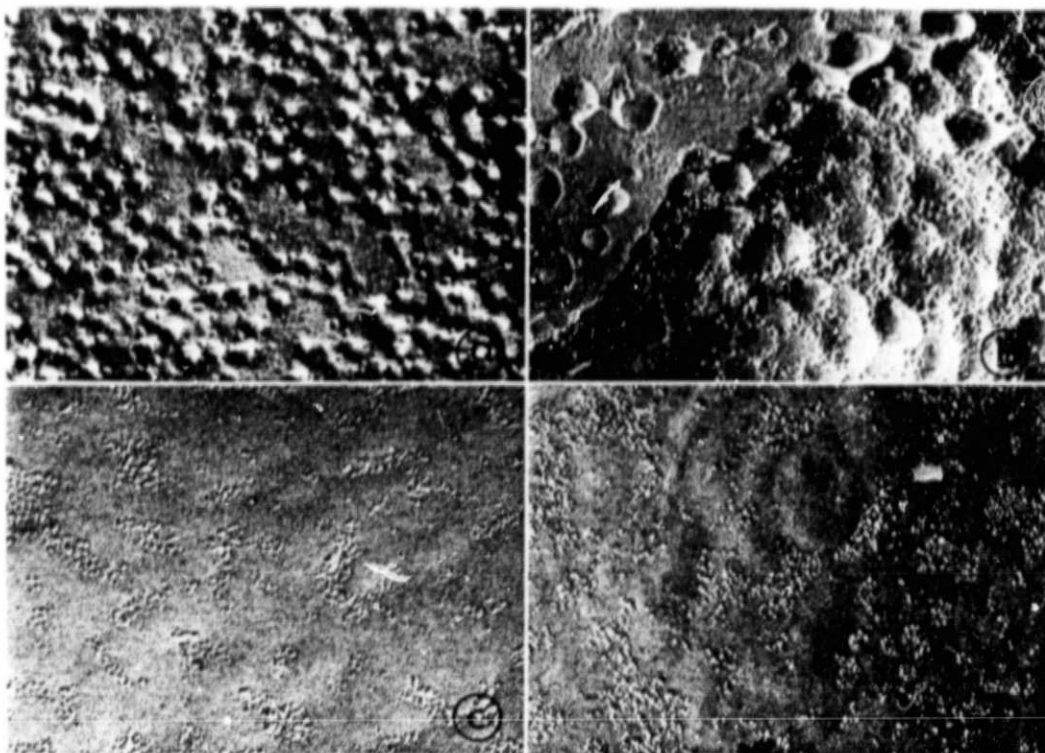
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FIGURE 5. Morphological Changes in the Plasma Membrane of E.
floccosum Due to the Polyene Antibiotics Shown in Figure 4.
(X 80,000)   a: Filipan   b: Pimaricin   c: Amphotericin B
d: Naistatin

permeability of the inside and outside of the membrane can be ade-
quately explained. Type II is the net mesh type of structure as in
the case with pimaricin. On the PF surface there are many protein
particles forming a net mesh shape on the periphery of a circular
elevated structure that is deficient of membrane protein particles
and has a diameter of 1,000 Angstroms. On the EF surface one can
observe depressed areas which correspond to these protruding parts.
And finally, Type III, is an irregular type, and is seen in the case
of antibiotics as amphotericin B and naistatin; there is no pattern
to the arrangement of the membrane proteins and one can only see
agglutination of various large membrane proteins. Thus, the

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creation of these characteristic changes in the morphology of the membrane due to various polyene antibiotics is a true reflection of the differences in the structures of the polyene sterol complexes within the cell membrane.

5) Changes in Plasma Membrane Function Due to Polyene Antibiotics

As stated above, obvious morphological changes in the structure of the membrane are the result of application of polyene antibiotics to the plasma membrane, and consequently there are corresponding changes in the function of the membrane [15]. That is to say, the major change is the promotion of the permeability of the membrane. Without looking at the low molecular weight polymers, leakage of /151 such things as high molecular weight polymer proteins and nucleic acid-related substances occurs; this eventually leads to cell death, and this demonstrates capability as an antifungal agent. As a mechanism of this leakage one must assume the presence of some form of a "pore." In actual experiments, by using calculations based on the many molecular-sized substances that leaked out when amphotericin B was applied to artificial lipid membranes, the size of the pores was determined to be in the range of 7-10.5 Angstroms. Many ideas have been proposed regarding the formation of the structure of the pores, and a comprehensive conclusion has not been seen yet. However, in the case of amphotericin B and naistatin, complexes of one molecule of amphotericin B and one molecule of sterol form, group together in rings of 8, and a half pore of one side of a membrane is formed. It is thought that a complete pore is formed when an identical structure on the other layer is joined with that on the first layer by means of hydrogen bonding. On the other hand, in the case of filipin, the process is completely different than this type of ring-shaped structure. The filipin and the sterol form a complex on the top and bottom in 1:1 proportions. This can be said to be a particular structure formed by two-step layering in sheets. However, compared to the above-mentioned pore structure, there is a poor foundation for backing this. At any rate, this

model of the complex of sterols and polyenes stops at the limits of conjecture, and it is necessary to have the backing of present methods of physical chemistry.

By the way, the particular interactions of polyene antibiotics and this type of membrane sterol are not the only basis for the direct affect on the variations in the membrane permeability. Recently, attention has begun to be paid to modifications in the function of various types of plasma membranes due to the loss or weakening of the function of membrane sterols based on these interactions. For example, changes in the activity of adenylic acid cyclase, acceleration of cell fusion, and other things have been reported. However, the details of such things will not be touched upon here.   .

IV. Chemical Structure of Formed Pigments and Their Function

It has been well known for a long time that various types of fungal cell produce pigmented substances. Even with respect to dermatophytes, since about 1964, a positive start in research, beginning with England and moving to the United States and Canada, has commenced. At the same time, Nozawa et al also began researching various problems such as the location of the production of pigments in dermatophytes, the chemical structure of the pigments, the function of the pigments, and the biosynthesis of the pigments.   /152
Hereinafter, two or three of these topics will be elaborated upon.

1) Site of the Production of Pigments

The separation of the intracellular organelles from Trichophyton violaceum, a strain which has a very marked production of pigments, was attempted, but due to the effort required to destroy the tough cell wall, the damage to the plasma membrane was marked, and eventually separation was deemed impossible. Initially it was determined that close to 60% of the whole pigments were

localized in a fraction obtained by centrifugation of a fraction at
20,000 X gravity for 30 minutes. Because the primary observation
was a membrane structure thought morphologically to have originated
in the mitochondria [16], it also suggested that it was also a
quinone type pigment. It was considered that there also might be
a potential correlation with the electron transfer system. Next,
based on the electron microscopic observations of pigment producing
strains and non-pigment producing strains of T. violaceum [17] and
Microsporum cookei [17], it became clear that the pigmented
substances were widely distributed on the cell surface. This led to
thinking that the major portion of the pigments were excreted
outside of the cell as the products of metabolism and then
accumulated on the surface of the cell wall. In other words, the
layer which used to be called the "dark outer coating" is thought of
as the site where the pigmentous substances are present.

2) Chemical Structure of Individual Pigments

     With respect to the pigments extracted from the cell walls of
individual dermatophytes, a large number of methods were tried, and
Nozawa et al finally carried out the extraction and purification
using thin layer chromatography and column chromatography [19,20].
By 1970, the extraction of large quantities of pigments was achieved
after making substitutions ranging from Trichophyton to Microsporum
cookei, and since then the determination of the chemical structure
has become easy [21]. When the pigments that were seen in the cell
walls and the culture medium were each extracted by means of
chloroform and acetic acid acidified chloroform and separated out by
means of thin layer chromatography, five fractions (I-V) were
obtained in the former situation and two were obtained in the latter
situation. Then, the chemical structures of the individual
fractions named aurosporin xanthomegnin, violosporin, citrosporin,
rubrosporin, luteosparin, and iridosporin were submitted to investi-
gation. Here, the chemical structures   were determined, those
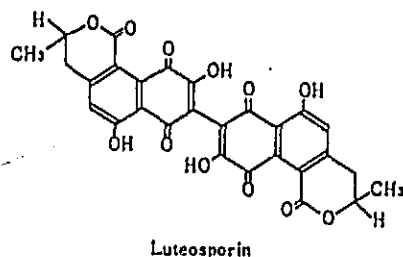of aurosporin, xanthomegnin, and luteospo..n, are shown in Figure 6.
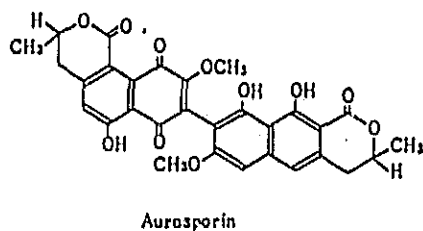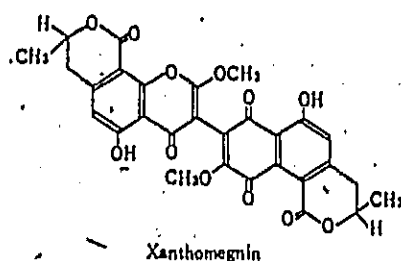

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FIGURE 6. Chemical Structures of the Main Pigments

The thing that must be noted is that luteosporin was formed by means
of the demethylization caused by treatment of xanthomegnin with
sulfuric acid, suggesting an end point of the biosynthetic route.
Moreover, when the correlation between the breeding of fungi and the
serial pattern of the production of pigment was investigated, auro-
sporin and xanthomegnin were produced in the logarithmic
proliferation stage, and furthermore it became clear that the
quantity of violosporin, citrosporin, and rubrosporin increased in
that order. With respect to the total quantity of pigment, the
pigments which originated in the fungal cell body increased in
parallel with the breeding of the fungi, and the pigments that

originated in the culture medium showed a trend of increasing during
the quiescent stage. In order to understand the route of
biosynthesis, it became necessary to do detailed investigation with
the aid of substances labelled with isotopes. However, it is pos-
sible to obtain important information from the correlations based on
the structures among the pigments by the determination of the
chemical structures of the individual pigments, and currently other
researchers are focusing attention on determining the structures.

3) The Biophysical Significance of Pigments - Centering on the
    Effects on the Function of Mitochondria

Very few explanations have been made concerning the biophysical
significance of these pigments. According to one explanation, the
covering of the cell surface with pigment is a response to protect
the fungus from ultraviolet radiation, however the strength of this
explanation is poor. Actually, the strains of fungi in which   /153
the production of pigments is marked are said to be pathogenically
strong. In fact, the color of the fungi becomes stronger directly
after it is separated from patients, but when it is cultured
serially, the capacity to produce pigments has been observed to show
a decreasing trend. If one generalizes this to actual situations,
there seems to be important meaning in the apparent correlation
between the pathogenicity or the infectivity and the pigment,
however, no resolute basis for this has been produced by
researchers. Nonetheless, it is clear that quinone type pigments
that are extracted from fungi have shown strongly harmful effects on
the function of rat hepatic mitochondria. Initially, it was thought
that the primary pigment xanthomegnin had a strong uncoupling effect
on rat hepatic mitochondria at low concentrations (10^{-7} - 10^{-8}M)
[22,23], and it was shown that the same form of uncoupling effect
occurred to differing degrees even with other pigments such as violo-
sporin and luteosporin. Figure 7 shows the examples of xanthomegnin
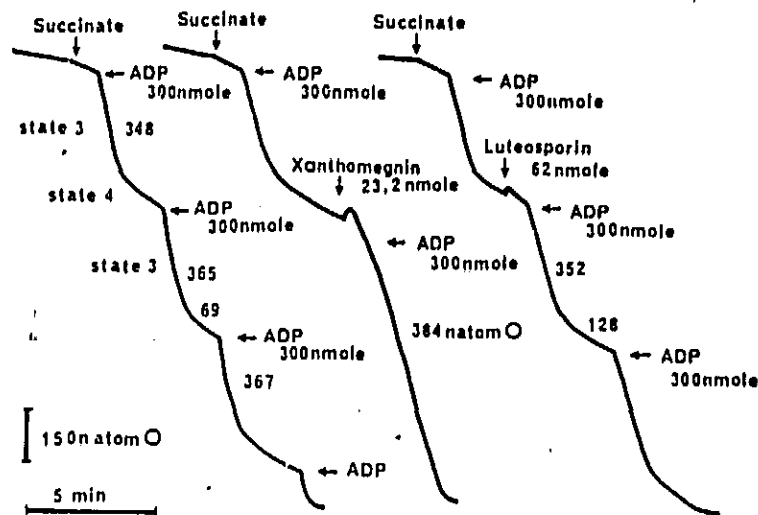and luteosporin [24].

FIGURE 7. ·Effects of Xanthomegnin and Luteosporin on the Acidified Phosphoric Acidification of Rat Hepatic Mitochondria

On the other hand, the behavior of both pigments with regard to mitochondrial swelling differs. Xanthomegnin gives rise to marked swelling of mitochondria whereas that response does not occur much at all with the luteosporin. These things suggest some sort of correlation between the strength or weakness of the uncoupling response and the affect of mitochondrial swelling, and understanding of the mechanism of uncoupling offers some kind of information.

At any rate, the experimental facts that were obtained in vitro using pigment extracts do not clarify the function of these pigments, but perhaps there is some important significance which can be thought of from the existence of quinone type structures or which cannot be predicted even with respect to the inside of fungal cells. It is hoped that further research results will deal with even these questions.

V. Conclusion

As mentioned even in the beginning of this study, it is not an exaggeration to say that research concerning the chemical and molecular level structure of the structure of the surfaces of fungal cells, particularly dermatophytes, has been relatively late in being established compared to that of bacterial cells. Moreover, the reason for this has been clarified by the fact that the isolation of the surface structure has been difficult and by other types of information. Moreover, while one can not say that great strides have been made recently, separation of the cell wall structure and chemical analysis have been performed and significant information has been obtained. In particular, the research on the structural constituents of the cell wall has been an important topic of research with respect to its relationship with the mechanism of fungal immunity and infection and with antifugicides. Active research on this topic is necessary in the future.

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16. Abstract

A review with 24 references on the biochemistry, molecular structure, and function of cell surfaces of fungi, especially dermatophytes: the chemistry and structure of the cell wall, the effect of polyene antibiotics on the morphology and function of cytoplasmic membranes, and the chemical structure and function of pigments produced by various fungi are discussed.

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