THE EFFECT OF SELECTED STEROIDS

ON THE GROWTH, MORPHOLOGY, AND

STEROL CONTENTS OF CANDIDA ALBICANS

by

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A C K N O W L E D G E M E N T S

TO

MY WIFE

SUMMARY

The effect of three selected steroids: decadron, alphadolone
acetate and cholic acid salt on the growth, morphology and sterol
content of the yeast and mycelial form of Candida albicans was
studied.

The effect on growth was measured by dry weight, turbidity
and viable count. It was observed that the three steroids promoted
the growth of C. albicans.

The morphology studies showed two different types of cells:
i – cells which were able to adapt to growth in the presence of
exogenous steroids and ii – cells which were unable to so adapt.
Adapted yeast cells were longer and more elongated with a smooth
surface. These cells were observed to grow very well and to be
involved in cell division especially at high steroid concentrations.
The internal structures of these adapted cells were well preserved
with distinct intracytoplasmatic structures. The unadapted yeast
cells were smaller and with rougher cell boundaries than the adapted
ones with obscure internal structures, where intracytoplasmatic
structures could no longer be identified.

Adapted mycelial form had shorter swollen hyphae as compared
with the unadapted cells which showed constricted and collapsed
hyphae.

C. albicans treated with steroids increased their lipid contents, as compared with a decrease in sterol content.

A change in the type and quantity of sterols of C. albicans treated cells of both forms occurred. This was manifested by an increase in calciferol content, decreased in the amount of ergosterol and appearance of a new unidentified sterol.

The resistance of treated cells to antifungal polyene antibiotics was increased.

Colour differeniation using Magdala Red dye showed that cholic acid salt affected respiration, wheres, the remaining two steroids did not have any effect. However, manometric techniques using the Warburg respirometer, showed that the three steroids enhanced oxygen uptake.

C O N T E N T S

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P L A T E S

S C H E M E S

F I G U R E S

T A B L E S

CHAPTER ONE

CELL MEMBRANES

The nature of membranes has intrigued man since 1899 when Overton (1) on the basis of permeability considerations, postulated that membranes were impregnated by a fatty oil substance. In 1925 Gorter and Grendel (2) performed their first simple calculations to ascertain the amount of lipid in the red blood cell ghost and postulated enough for a bilayer. Since then, the number of models put forth to suggest the organisational basis of membranes has mounted.

MEMBRANE MODELS

The first important hypothesis of the structure of biological membranes was proposed by Davson and Danielli (3). An important feature of their hypothesis is the proposal that membranes contain a continuous hydrocarbon phase contributed by the lipid components of the membrane. Some years later, this hypothesis was modified and refined into the unit-membrane hypothesis (4) the Davson-Danielli-Robertson unit model. This model implies that the polar head groups of the phospholipids are largely not in contact with the bulk aqueous phase, but in contact with the polar and ionic groups of the proteins. However, this model has failed to account satisfactorily for the thermodynamic properties of the membranes since it necessitates the exposure of large portions of the hydrophobic groups to water. The electrostatic stabilisation results in the burial of a high percentage of ionic groups in the non-aqueous environment and this requires the input of large quantities of free energy.

Benson (5) proposed a new model to succeed the Davson-Danielli-Robertson model. Benson's model amalgamated several features but was soon ruled out since, although it did have a lower free energy requirement than Davson-Danielli-Robertson's model, the free energy level was still not low enough. The Benson model proposed that hydrophobic interactions were maximised by placing the protein, now assumed to be globular, within the membrane exterior. The principle grounds for the unacceptability of this model is that it requires non-fatty acid groups to intercalate with the polypeptide which will result in suppression of the maximum number of interpeptide hydrogen bonds (4).

The X-ray evidence accumulated by Luzzatti and Hussan (6) from observation of phospholipids have been complemented with theoretical studies. Together with these results, and the Lucy and Glauert work, there evolved the thesis that phospholipids existed in small globular micelles (7 and 8). Lucy proposed a theoretical model which considered the lipoprotein membrane to be in dynamic equilibrium between leaflet lipids and globular micelle lipids (9). This model of Lucy's is one of the forerunners of the Lipid-Globular-Protein-Mosaic model (L.G.P.M.) derived independantly by Lenard and Singer (10) and Wallack and Zahler (11). An excellent review of the unit membrane and globular (sub-unit) models is presented by Stoeckenius and Engelman (12).

Many other models were proposed by Krentz (13), Vanderkooi and Green (14). However, the most satisfactory model of membrane structure to date appears to be the fluid-mosaic model postulated by Singer and Nicholson in 1972 (15).

This model (Plate 2) postulates that the phospholipids of membranes are arranged in a bilayer to form a fluid liquid-crystalline matrix or core. In this bilayer, individual lipid molecules can move laterally, endowing the bilayer with fluidity, flexibility and a characteristically high electric resistance and relative impermeability to highly polar molecules. The fluid-mosaic model postulates that the membrane proteins are globular, to account for their high content of α-helix. Some of the proteins are partially embedded in the membrane, penetrating into the lipid phase from either side, and others completely span the membrane. To what extent a given globular protein penetrates into the lipid phase would be determined by the amino acid sequence of the protein and the location on its surface of the nonpolar amino acid R groups. Thus, the various membrane proteins would form a mosaic-like structure in the otherwise fluid phospholipid bilayer.

The fluid mosaic model, whilst not entirely satisfactory from a biophysical point of view, at least forms a fairly flexible basis for many features and properties of biological membranes. It provides for membranes with widely different protein content, depending on the number of different protein molecules per unit area of membrane, it provides for the varying thickness of different types of membranes, it can account for the asymmetry of natural membranes; since it permits proteins of different types to be arranged on the two surfaces of the lipid bilayer. It accounts for the electrical properties and permeability of membrane, and it also accounts for the observation that some protein components of all membranes move in the plane of the membrane at a rather high rate.

PLATE 1. Various membrane models (16).

(a) Goldup, Ohki, and Danielli

(b) Singer and Nicholson

(c) Lenard and Singer

(d) Lucy

(e) Krentz

(f) Vanderkooi and Green

a — Lipid / Polar pore / Protein

b — Lipid / Protein

c — Protein / Hydrophobic amino acid residues or lipids / Lipid

d — Cholesterol

e — Protein / Lipid

f — Protein subunit / Lipid
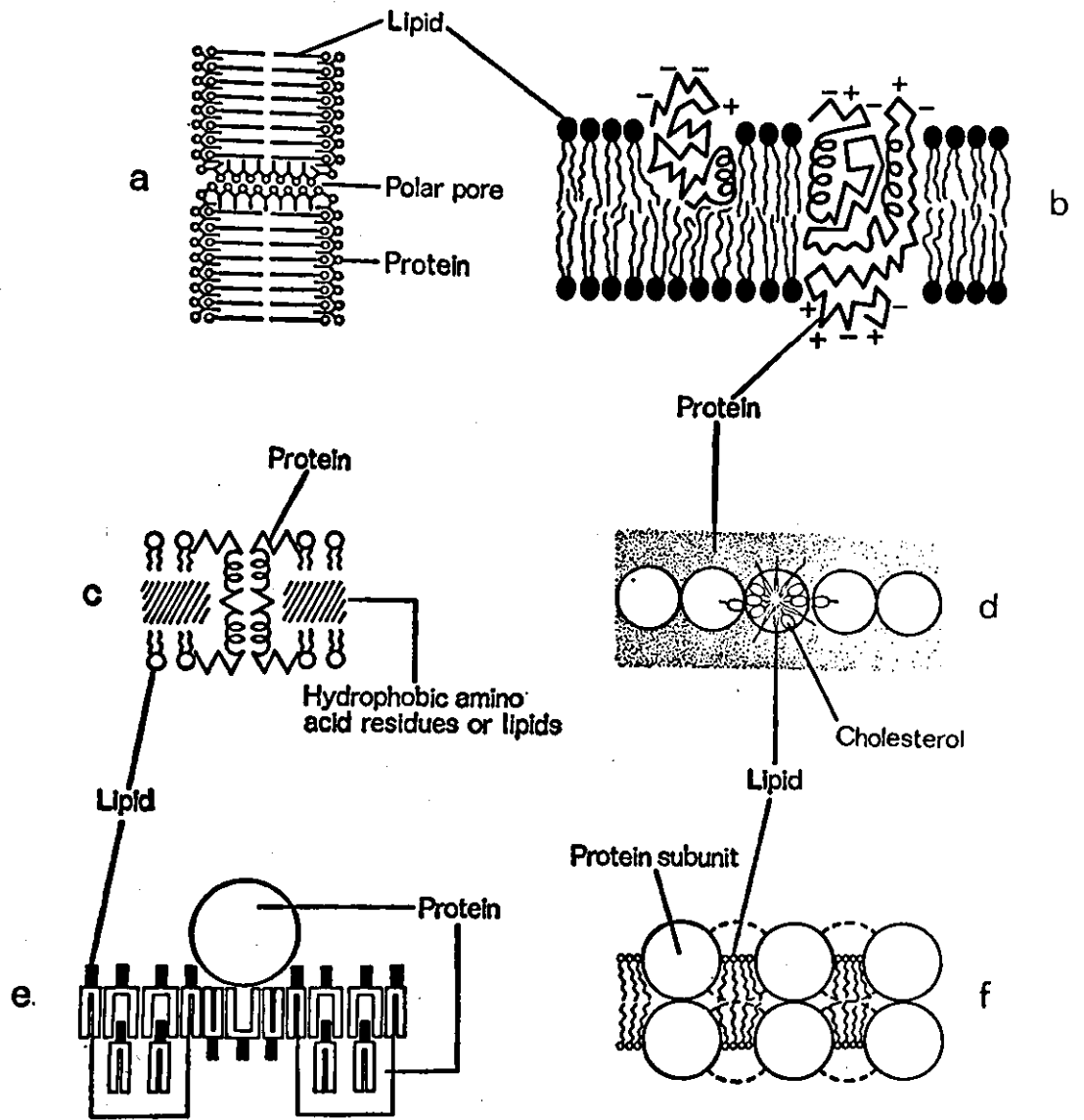
PLATE 2. The fluid mosaic model of plasma-membrane

structure as formulated by Singer and

Nicholson (15).
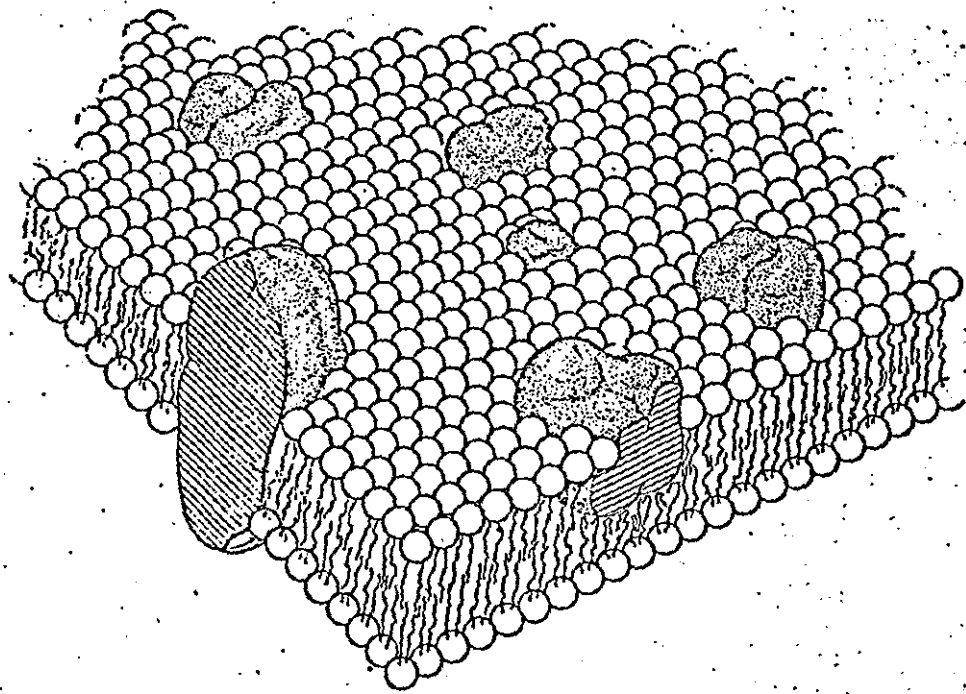
MEMBRANE FLUIDITY

The fluid mosaic model embodies a concept that the functional
membrane is a two-dimensional solution of globular integral proteins
dispersed in a fluid lipid matrix (15,17). Evidence in support of
this model comes from studies on the redistributions that lipids and
proteins can be made to undergo in the plane of the membrane, and on
the influence of lipid fluidity and phase changes on membrane functions.
Judging by the number of publications dealing with this phenomenon,
there seems little doubt that fluidity of membranes and lateral mobility
of lipids and proteins in the plane of the membrane are factors of prime
importance in membrane biology.

McConnell and his colleagues (18-20) have emphasised the difference
between first order phase transitions that occur at sharply defined
temperatures with pure phospholipids, and phase separations that occur
over a broad temperature range with binary mixtures of phospholipids.
In phase separations, solidus and liquidus phases of different charac-
teristic lipid compositions co-exist at equilibrium at given temperature,
and the lateral mobility of the lipid molecules in the bilayer is
required to achieve these separations. With bacterial mutants defective
in the synthesis of unsaturated fatty acids (21) membranes can be gener-
ated with restricted fatty acid compositions in their phospholipids,
which exhibit such lipid phase separations over temperature ranges
characteristic of the particular lipid mixtures. Arrhenius plots of
the transport rates in several independent transport systems each showed
a sharp deflection at the temperature corresponding to the onset of the
lipid phase separation (20) i.e. when very little solidus phase had yet
formed and the composition of the liquidus phase was hardly altered.

On the other hand, several enzyme activities of these membranes
showed little or no effect of lipid phase separations on their
rate (22,23).

These lipid effects no doubt reflect the different mechanisms
of transport and enzymatic activities in membranes. If the enzymes
involved are amphipathic integral proteins with their active sites
within the hydrophobic exposed segments, then little effect might
be expected of lipid phase separations on the activity.

On the other hand, if the transport components spanned the
membrane, lipid phase separations might significantly affect transport
rates. However, the possibility that in real membranes the lipids are
asymmetrically distributed in the two halves of the bilayer (see below)
may also play an as yet unanticipated role in lipid phase separation
phenomena.

Although membrane fluidity and lateral mobility of membrane com-
ponents appear to be general and functionally important phenomena, there
is clear evidence that fluidity or mobility is restricted in certain
membranes, or in regions of membrane under particular conditions.

MEMBRANE ASYMMETRY

There is a large body of evidence that the components of biological
membranes are asymmetrically disposed across the membrane. Although
membrane asymmetry has been discussed for many years, it is only
recently that direct evidence has been obtained confirming asymmetry
at the molecular level and its fuller implications in membrane function,
structure and biosynthesis have been appreciated.

Three methods have been employed in these more recent studies:-

(1)   Chemical (24) and enzymatic (25) methods have been used
      to differentially label or modify the components of the
      plasma membranes of intact cells compared to broken cells
      or organelles.

(2)   Right-side-out and inside-out vesicles can be separately
      prepared from isolated membranes under slightly different
      conditions, and such that they are largely impermeable
      to various reagents (25). They can then be compared, for
      example, in their ability to utilise an exogenously added
      and impenetrable substrate or effector of a membrane-bound
      enzyme.

(3)   The third method involves use of the electron microscope.
      Histochemical methods specifically for particular enzymes
      can be used to determine the sidedness of their active
      sites on membrane in situ.

The three methods, where they have overlapped in application,
have happily yielded the same conclusions. One result has been a
fairly detailed description of the orientations of the major proteins
and glycoproteins of the human erythrocyte membrane. These conclusions
have been reviewed by Guidotti (26), Bretscher, (16) and Steck (25).

The erythrocyte membrane is so far the most thoroughly investigated membrane with respect to the composition and asymmetrical distribution of its protein components. A beginning has been made in the distribution analysis of the electron transport chain components and of oxidative phosphorylation on the inner membrane of mitochondria (27), but so far the only conclusions are that the peripheral protein cytochrome C is exposed on the inner membrane surface that faces the outer membrane, while F1-ATPase complex, which is probably peripheral, is located on the opposite surface. The functional asymmetry of the components of the electron transport chain across the mitochondrial inner membrane is a critical element in the chemiosmotic hypothesis of Mitchell (28).

Bretscher (16) suggested that the major phospholipids of the erythrocyte membrane are asymmetrically distributed across the two halves of the bilayer by inference that is true of other biological membranes. Although this may well turn out to be correct, the evidence first presented for it (29) was not compelling. It was shown that the amino-group containing phospholipids (phosphatidyl-ethanolamine and phosphatidylserine) were essentially unreactive toward a membrane-impermeable reagent in intact erythrocytes, whereas they reacted readily in ghost preparations. Similar results were obtained with another reagent (30). This was interpreted to mean that these amino phospholipids were concentrated exclusively in the cytoplasmic half of the lipid bilayer, and by inference that the phosphatidylcholine and sphingomyelin were concentrated in the outer half.

More recently, Zwaal, Roelofsen and Colley (31) have found that the phospholipase A_2 of Naja naja venom converts 70% of the phosphatidylcholine of intact human erythrocytes to lysolecithin without much effect on sphingomyelin, phosphatidylserine, or phosphatidylethanolamine, and without lysing the cell. Furthermore, treatment with a sphingomyelinase from S. aureus degrades 80% of the sphingomyeline without lysing the cell. Neither of these treatments degrades more than 10% of the phosphatidylethanolamine. On the other hand, if the lysed cell membranes are subjected to phospholipase A_2 treatment, essentially all classes of phospholipids are extensively degraded. This evidence is consistent with the assymmetrical distribution of phospholipids in the erythrocyte membrane as formulated by Bretscher (16).

There is no evidence at present for an assymmetrical distribution of phospholipids in cell membranes other than in erythrocytes. If phospholipid asymmetry is the result of an originally asymmetric synthesis of phospholipids on the two surfaces of a membrane, then it should be a general phenomenon. On the other hand, there are many enzymatic modifications and interconversions known to occur with the lipids in intact membranes by lipases as well as by other enzymes (32). In addition exchanges of intact phospholipids between serum and erythrocyte membranes (33, 34) and between different intracellular membranes (35, 36) are known to occur. To what extent such enzymatic and exchange processes remodel the membrane lipids and affect the symmetry of their distribution in particular membranes is not known. If exchange processes are specific, that is, if only intact phospholipids with the same structure are transferred in a single exchange, they would have no effect on existing asymmetry.

If any asymmetrical distribution of phospholipids is a general phenomenon of cell membranes, then its implications for the structure and function of membranes are considerable. For example, lipid asymmetry has not previously been considered a factor in lipid phase separation experiments (20, 37). Lipid asymmetry would also play a role in the kinds of direct short range lipid-protein interactions that some integral enzymes of membranes may require to express their activity (38). Obviously, lipid asymmetry would also have to be taken into account in reconstitution experiments between isolated proteins and lipids (39).

MEMBRANE COMPOSITION

Membranes are normally described as lipoprotein structures and lipid and protein can be distinguished as common features of all membrane preparations. There is always difficulty in deciding to what extent other substances, found in membrane preparations, are intrinsic parts of the membrane or arise as a result of contamination by non-membrane materials.

It is the possession of specific membrane constituents which enables the organism to perform its characteristic functions and such specificity has been demonstrated by Esfahani, Crowfoot and Wakil (40) who used aqueous acetone to extract phospholipids from an unsaturated fatty acid auxotroph of E. coli which recovered its 70% loss of succinic-dichloroindophenol reductase activity when the phospholipids were restored.

TABLE 1 : Some examples of phospholipids detected in filamentous fungi and yeasts (50)

FUNGUS OR YEAST	PRINCIPAL PHOSPHOLIPIDS DETECTED				
PHYCOMYCETES:					
Phycomyces blakesleeanus	PC[a]	PE[b]	PS[c]		
Pythium ultimum	PC	PE	PG	DPG	
Rhizopus nigricans	PC	PE	PE		
ASCOMYCETES:					
Alternaria oleracea	PC	PE	PS		
Glomerella cingulata	PC	PE	PS		
Hansenula anomola	PC	PE	PE	PI[d]	
Lipomyces lipofer	PC	PE	PS		
Neurospora sitophila	PC	PE	PS		
Saccharomyces cerevisiae	PC	PE	PI	PS	DPG[e]
BASIDIOMYCETES:					
Agaricus bisporus (mycelium)	PC	PE	PS		
Clitocybe Illudens	PC	PE	PS		
Coprinus comatus	PC	PE	PS		
DEUTEROMYCETES:					
Candida lipolytica	PC	PE	PI	PS	
Humicola grisea var thermoidea	PC	PE	PI	DPG	
Rhodotorula graminis	PC	PE	PI	PS	

[a]PC indicates phosphatidylcholine; [b]PE, phosphatidylethanolamine; [c]PS, phosphatidylserine;

[d]PI, phosphatidylinositol; [e]DPG, diphosphatidylglycerol or cardiolipin.

TABLE 2 : <u>Composition of isolated plasma membranes from yeasts and filamentous fungi</u> (50)

ORGANISM	Content (% dry weight) of			
	Protein	Lipid	Carbohydrate	Nucleic Acid
Candida albicans (yeast form)	52.0	43.0	9.0	0.3
C. utilis	38.5	40.4	5.2	1.1
Saccharomyces cerevisiae	46.5	41.5	3.2	7.5
S. cerevisiae	65.0	29.0	3.0	4.0
S. cerevisiae	49.3	39.1	5.0	7.0
S. cerevisiae	26.6	45.5	30.8	-
S. cerevisiae	49.3	2.3	5.0	7.0
S. cerevisiae	35.0	35.0	25.0	-
<u>Filamentous Fungi</u>				
C. albicans (mycelial form)	45.0	31.0	25.0	0.5
Fusarium culmorum	25.0	40.0	30.0	-

Frerman and White (41) demonstrated that the organism must also undergo changes in lipid composition during the development of an electron transport system in S. aureus when it is shifted from anaerobic to aerobic growth which emphasises that the microbial cell membrane is not a stable structure but undergoes dynamic modification particularly during growth and cell division.

A substantial body of data on lipid chemistry has accumulated in the last two decades, and in the last few years many reviews have appeared, Kates (42) Goldfine (43) Shaw (44) and Lennarz (45) have all reviewed bacterial lipids while Smith (46) and Salton (47) have reviewed mycoplasma lipids. Brennan, Griffin, Losel and Tyrrell (48) and Magnall and Getz have reviewed fungal lipids (49).

ROLE OF STEROLS

Sterols, or in rare cases structurally similar molecules, are biosynthesised or at least required by all eucaryotic organisms, as well as certain procaryotic ones, regardless of their status as plants, animals or protista. Cholesterol is considered the major sterol in animal tissues, C_{29} sterols such as sitosterol are predominant in photosynthetic plants, and ergosterol is the major sterol in most fungi.

It would appear that three roles are possible for sterols: One being related to their effect on growth, the second involving their effect on reproduction, and the third involving their incorporation into the membranes.

The response to steroid supplementation is varied: Tetrahymena pyriformis S exhibited stimulated growth while T. pyriformis W suffered partial to total inhibition in the presence of steroids (51). The growth in liquid culture of a number of fungi is stimulated by sterols, for example, Aspergillus niger, (52) Pythium sp., and Phytophthora (53). The response in A niger (54) and Phytophthora parasitica var. nicotianae (52) is significant only in aerated cultures.

Exogenous sterols are necessary for oospore formation in the pythiaceous fungi. Hendrix (55) showed that they are necessary for zoosporangium production. Most of the Phytophthora sp. that respond in this way are homothallic, only three out of eight pairs of heterothallic species examined produced oospores when cultured on a defined medium containing sterols (56). The great difficulty in obtaining pure sterols and preventing oxidative change during experiments has made a number of structure/activity experiments in oosporogenesis of doubtful value, although it is clear that a 3β – hydroxyl, a Δ^5 – double bond, and a side chain of eight carbon atoms are essential (54).

In media which lack sterols, the growth of Pythium spp and Phytophthora spp is generally vegetative but when the media are supplemented with suitable sterols vegetative growth is increased and development of sexual stages (namely antheridia and oogonia) are enhanced, (also oospora are formed inside the oogonia) (55, 57, 58). Cholesterol (5 – cholesten – 3β -ol) and cholestanol (5⍺- cholesten – 3β -ol) both promote vegetative growth and support the development of oogonia and antheridia. However, while many oospores are produced in cultures containing cholesterol, very few of the oogonia produced in the presence of cholestanol develop oospores; most of them degenerate (59, 60).

Similar abortion of oogonia in cultures with cholestanol has been

observed in Pythium periplocum and Pythium pralatum (61).

Recently, Elliott (62) showed that abortion of oogonia of

Phytophthora cactorum grown on media containing cholestanol occurs

at or just before early meiotic prophase. He suggested that meiosis

is possibly controlled by a steroid hormone, for which cholesterol is

an effective precursor but cholestanol is not.

The role of sterols in membranes has been intensively investigated.

Two roles for sterols in membranes are important. The first is their

effect on membrane fluidity, and the second their effect on membrane

asymmetry.

The initial studies of Van Deenen, Houtsmuller, DeHaas and

Mulder (63) into cholesterol induced phospholipid condensation were

carried further by Chapman and Co-workers who used a variety of

unsaturated fatty acids (64, 65). Demel, Bruckdorpher, and Van

Deenen (66) in a thorough study of the film compression phenomenon

in mixtures of 18:1/18:0 phosphatidylcholine, with various steroids

showed a fairly precise requirement (1) a planar nucleus (2) a 3β

hydroxy group and (3) an intact side chain. Thus, cholesterol,

cholestanol, lanosterol, 7-dehydrocholesterol and B-norcholesterol

all interact in 1:1 ratio with 18:1/18:0 phosphatidylcholine so

that the molecular area of the sterol-phospholipid pair at 12 dyne

cm^{-1} and 37° is about 100 $\overset{\circ}{A}^2$.

The limiting area of the sterol under the same conditions is about 40 $\overset{o2}{A}$ and of the phospholipid, 82 $\overset{o2}{A}$, which would give an ideal area for the mixed film of about 120 $\overset{O2}{A}$. Such a gross reduction in the mean molecular area is probably explained by extensive van der Waals interactions.

Studies of pure phospholipids using a variety of physical techniques have shown that each phospholipid, on heating, exhibits a transition from a crystalline to a smectic phase (67, 68). This corresponds to a transition between highly ordered, rigid gel phase to a more random, fluid, liquid crystalline state. The transition temperature depends upon the type of fatty acid chains in the lipid (chain length, and degree of unsaturation). This property was used by many workers to shed light on the possible role of sterols in biological membranes. Ladbrooke, Williams, and Chapman (69) was among the first to observe that addition of cholesterol to lecithin dispersions results in a decrease in both lipid transition temperature (T_c) and the heat absorbed at the transition. These two parameters vary with the lecithin/ sterol ratio until, with an equimolar mixture (50-50) this phase transition disappears. They suggest that cholesterol exerts its effect by disrupting the highly ordered array of the saturated hydrocarbon chains, so that when the cholesterol and the phosphotidylcholine are present in equimolar proportions, all the hydrocarbon is in a fluid condition, even at temperatures below the normal freezing point, and there is a minimum of interaction, between like molecules. The results shown by Ladbrooke et al (69) are in agreement with the findings of Shah and Schulman (70).

They showed that cholesterol liquifies solid monolayers of dipal-mitoyl lecithin at high surface pressures. However, NMR spectro-scopic studies by Chapman and Penkett (71) using egg yolk lecithin at 33.5° showed a selective reduction in the signal due to the poly-methylene chains when cholesterol is added. One explanation given was that the presence of cholesterol caused some inhibition of the lipid chain motion. Thus, at a particular temperature (T), the pre-sence of cholesterol keeps the hydrocarbon chains of differing phos-pholipid molecules in an intermediate fluid condition. Some lipids which would normally be above their limiting transition temperature (T > Tc) may have a certain amount of inhibition of chain motion. Those lipids which would normally be in a gel condition (Tc > T) are however, given much greater fluidity.

This buffering effect of sterols is best illustrated by Rothman and Engelman (72). In their proposed model of phospholipid-cholesterol interaction they stated that "the intermediate fluid condition of the hydrocarbon chains probably corresponds to an ordered upper half" i.e. the half nearest the head groups, and a more disordered lower half. The cholesterol is proposed to insert itself nearly parallel to the chains so that its ring region is tightly packed with the upper half of the chain (steric hindrance is prominent). The tail region of chol-esterol interacts with the lower region of the hydrocarbon (steric hindrance is not as marked as for the upper half) which can then assume gauche conformations. So the effect of cholesterol is to prohibit the formation of some, but not all, gauche isomers and hence cause decrease in chain fluidity.

Presumably, those gauche isomers which are sterically forbidden
are those which involve bends in the tightly packed upper half of
the chain. Therefore, sterol will have the effect of rigidifying
a phospholipid membrane with a T_c above ambient. On the other hand,
it will make more fluid a membrane with Tc below ambient by increas-
ing the spacing of the phospholipid molecules and allowing the
formation of gauche isomers in the lower half of the fatty acid
side chain.

The buffering effect of cholesterol on the thermal properties
of hydrated phospholipids seems to be true too of the effect of
cholesterol on biological membranes. Myelin and red cell membranes
both have a high proportion or cholesterol and no phase transition
is apparent from spectral or calorimetric examination of either the
intact membranes or lipid extracts prepared from them (73, 74). In
contrast, biological membranes, having a low proportion of cholesterol
(such as plasma membranes from bacteria and mitochondrial inner mem-
branes) generally do show phase transitions (75, 76).

The existence of a non-uniform distribution of cholesterol in
membranes was first suggested by Murphy (77) and later supported by
Caspar and Kirschner (78) who found more cholesterol in the outer
half of the bilayer when analysing X-ray diffraction data obtained
from myelin. More recently, it has been proposed that cholesterol
exists in various pools within the membrane. This was thought to
be the case in pig erythrocytes, where not all the cholesterol was
found to be exchangeable (79).

This idea was supported by the analyses of d'Hollander and Chevallier
(80) who suggested that several cholesterol pools exist. One can
only speculate on how these pools may actually occur. It is unlikely
that they occur in clearly defined areas, although one might consider
the cholesterol located on the inner and outer halves of the bilayer
as distinct pools.

According to Bruckdorfer and Graham (81), cholesterol may exist
in different 'micro-environment' which distinguishes the behaviour of
one molecule from another. This may largely depend on the nature of
the neighbouring molecules e.g. another cholesterol molecule, a phos-
pholipid of a certain class, a phospholipid with saturated or unsatur-
ated acyl chains or a membrane protein. The presence of any of these
molecular species may affect the exchangeability and physical effects of
a particular cholesterol molecule in a membrane, thereby dividing
cholesterol into recognisable pools.

The effect of sterols on the fluidity and asymmetry of the membrane
has a significance on the sensitivity and resistance of organisms to
polyene antibiotics. As we mentioned before, Hsu-Chen and Feingold (82)
suggested that for a disruptive interaction between polyenes antibiotics
and membranes, the membrane should be in an ordered state. But, the
fact that sterol has an effect on the membrane fluidity shows that there
is a typical intermediate fluidity at which the organisms are most sus-
ceptable to polyenes and other intermediate fluidity at which polyenes
are less effective. Also, the asymmetric distribution will have an
effect on the sensitivity of organisms. The differences in sterol dis-
tribution and content of membranes in yeast and mycelial forms of C.
albicans may have an effect on the sensitivity of the two forms towards
polyenes.

CHAPTER TWO

POLYENE ANTIBIOTICS

The discovery in 1950 by Hazen and Brown (83) of the compound nystatin led to the isolation and characterisation of numerous antibiotics, which share certain chemical and biological properties and are now designated as the polyene antibiotics. The chief distinguishing feature of this class of antibiotics was that they inhibit the growth of a wide range of fungi, including yeasts and filamentous fungi, pathogenic or non-pathogenic, but are ineffective against bacteria.

Polyene antibiotics are characterised by a large macrolide ring containing conjugated double bond system. The macrolide ring contains 12 to 14 up to 35-37 carbon atoms and closure is effected by the formation of lactone. Both the lactone group and the conjugated double bond system are necessary for biological activity.

Another feature which is common to all polyenes is the presence of a large number of hydroxyl groups from 6-14 such groups. These hydroxyl groups are usually distributed along the macrolide ring on alternative carbon atoms. The presence of highly polar and non-polar regions within the molecule renders the polyenes amphipathic, accounting for their peculiar solubility properties.

The majority of the polyenes are amphoteric substances. The acidity of these polyenes is due to a single carboxyl groups, and the basicity to a single amino group.

Fig. 1 . Polyene macrolides of known structure.
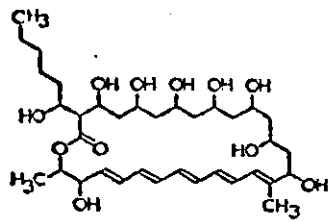
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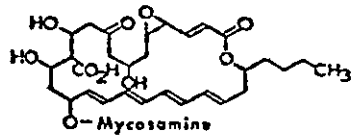
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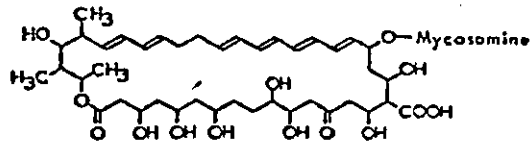
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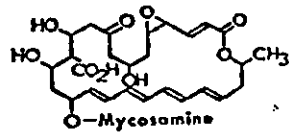
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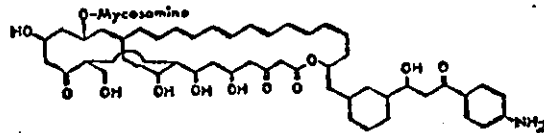
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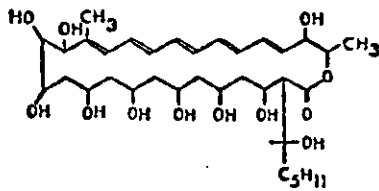
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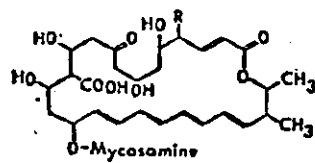
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TETRIN B   R=OH
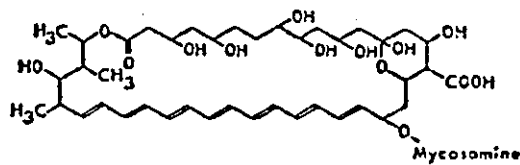
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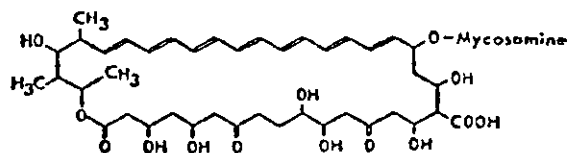
CANDIDIN

Some polyenes contain both a single amino group and a single car-
boxyl group and are therefore zwitterions. The amino group present
in some of the polyene antibiotics is associated with an amino sugar
that is connected to the macrolide ring through a glycosidic bond.

In all instances, the carbohydrate moiety has been found to
be mycosamine, a unique amino sugar first isolated from nystatin
and amphotericin B (84).

The most characteristic, and at the same time useful physical
property of the polyene antibiotics is their ultraviolet absorption
spectra. The U.V. spectra of all polyenes have a regular series of
sharp peaks of absorption, which are separated by sharp troughs
(85) all in the range of 360-280 n.m.

BIOLOGICAL PROPERTIES OF POLYENE ANTIBIOTICS

In the early 1960's several laboratories independently presented
evidence that polyene antibiotics could mediate a change in the cel-
lular permeability of a number of organisms, promoting leakage of
important cellular constituents and ultimately lysis and death of the
cell (86-88).

Kinsky (89, 90) observed that at low concentrations all the
polyenes tested against Neurospora crassa caused a marked decrease
in the dry weight of mycelial mats when incubated for short periods.

Mycelial atrophy was accompanied by the appearance of various cyto-
plasmic constituents in the medium e.g. amino acids, sugars and other
metabolites. Such atrophy was produced specifically by nystatin,
amphotericin B, and filipin. It was not obtained with any other
growth inhibiting substances e.g. (iodoacetamide, azide, p-fluoro-
phenyl alanine) or the non-polyene antibiotics (cycloheximide, and
viridin). Caltrider and Gottlieb (91), using filipin in the growth
media of Saccharomyces cerevisiae and Neurospora crassa, found that
there was a loss of vital cytoplasmic components from the cells.

The leakage of cytoplasmic components from certain organisms
implied that polyene antibiotics cause changes in the permeability
of the organisms cytoplasmic membranes. However, it was not possible
to state unequivocally that the changes in membrane permeability
resulted as a consequence of the action of the polyene at this
specific locus. Since the leakage could be the result of another
effect causing secondary permeability changes. Gottlieb, Carter,
Sloneker, Wu and Gandy (92) reported that filipin inhibited both
aerobic and anaerobic oxidation of glucose, acetate and endogenous
substrate by intact cells of the yeast, Saccharomyces cerevisiae.
High concentrations of the antibiotic had no effect on the oxidative
capabilities of cell-free homogenates (93). They also observed that filipin
caused a decrease in cellular dry weight and the leakage of components
containing nitrogen and phosphorous from the cell.

These observations suggested that the metabolic effects of the
polyenes were a consequence of an altered cellular permeability and
not to direct inhibition of glycolysis or some other critical metabolic
event.

Ultimately, the death of the fungal cell was caused by leakage of some essential cytoplasmic components.

THE SITE OF POLYENE ANTIBIOTIC ACTION : THE CELL MEMBRANE

It was shown by Lampen and Co-workers (94) that the yeast cell appears to contain two potential sites for binding nystatin and related polyenes. One is present in the cell wall, the other is in the protoplast membrane. Marini and workers (95) and Lampen (96) showed that the membrane is the site of the critical action of the polyenes and binding by the cell wall may actually prevent damage to the membrane structure. This may explain the lower sensitivity to the antibiotics of heavy-walled stationary-phase cells as compared with log-phase cells.

Lampen and co-workers (97, 98) found that nystatin absorption by cells is vital in determining sensitivity to the antibiotic. They observed that fungal cells (Saccharomyces, Candida, and Penicillium) bound significant amounts of nystatin at the minimum growth inhibitory concentrations. Bacteria, e.g. Escherichia coli and Streptococcus faecalis), whose growth is not inhibited by as much as 100 μg/ml of nystatin, absorbed negligible amounts of the antibiotic. It appeared that only cells that accumulated appreciable amounts of the polyene were sensitive to these antibiotics. These results suggested that binding of the polyenes to the fungal cell membrane in some manner interfered with its ability to function as a restraining barrier. This hypothesis is particularly attractive because it implies that the selective toxicity of these antibiotics is due to a unique binding component present in the membrane sensitive fungi, which is absent in bacteria. This unique component appears to be sterol.

STEROL-BINDING HYPOTHESIS

Insight into the possible sterol polyene antibiotic relation-
ship was first provided by Gottlieb and co-workers in a series of
studies (91, 92 and 99). They reported that addition of several
sterols (e.g. cholesterol, ergosterol, stigmasterol, sitosterol)
to the medium antagonise growth inhibition by the polyene antibiotics.
Thus, there appeared to be a competition between polyene antibiotics
and sterols. Initially it was not clear whether the reversal of
polyene inhibition mediated by exogenous sterols was due to (a)
polyene inhibiting sterol synthesis (99) or (b) the polyenes replacing the
sterols as co-factors in an essential metabolic reaction or (c)
a simple physiochemical interaction between the polyene antibiotic
and the added sterol which prevent the antibiotic from interacting with
the sterol of the organism under study. Subsequent studies have shown
that the results obtained by Gottlieb and co-workers could be explained
by simple competition between sterol and polyenes in growth media (85).
A correlation between the binding of nystatin to subcellular fractions
of the yeast Saccharomyces cerevisiae and the sterol content of the
particular fraction was reported by Lampen et al. (94). The evidence
presented gives a confirmation of the physico-chemical interaction
hypothesis between sterol and the polyene antibiotics.

More supporting evidence was obtained from the work using
digitonin. Digitonin is known to react with cholesterol to give a
non-covalently bonded tight complex of digitonin and cholesterol (100).
Lampen et al (94) showed that the binding sites for polyenes present
on the membrane of organisms containing sterol were removed by treat-
ing with digitonin.

Digitonin has also been shown to inhibit polyene binding to the fungal membrane. Kinsky (101) and Ghosh and Ghosh (102) found that digitonin inhibited the absorption of nystatin and amphotericin B by Candida albicans. Evidence for the sterol hypothesis was obtained using Mycoplasma laidlawii (Acholeplasma laidlawii) and Tetrahymena pyriformis. Feingold (103) and Weber and Kinsky (104) showed that M. laid lawii is unable to synthesise sterols de novo, but when it is grown on a medium containing sterols, it will incorporate them into the cytoplasmic membrane. These workers found that filipin had no effect on the growth of the cells raised in the sterol-deficient medium, however, when the organism was grown in a sterol-enriched medium, filipin and amphotericin B caused inhibition of growth. When T. pyriformis is grown in the presence of ergosterol it incorporates this to the exclusion of the naturally occuring equivalent, tetrahymanol. Using the ergosterol-replacement technique Conner, Mallory, Landrey, Ferguson, Kaneshiro and Ray (105) found that cells grown in the presence of ergosterol were twenty times more sensitive to polyenes than non-supplemented cells. Similarly, Nozawa, Fukushima, and Iida (106) using scanning and freeze-etch electron microscopy showed that although freeze-etched faces of filipin-treated membranes containing the native tetrahymanol showed a random distribution of 85 Å protein particles, the ergosterol-replaced membranes after the same polyene treatment revealed the marked ultrastructural alterations on the fracture faces. They showed that ergosterol-supplemented organisms were more sensitive than non-supplemented organisms.

Thus, there is much biological evidence to suggest that the interaction of polyene antibiotics with membrane bound sterols is the basis for the sensitivity of fungi to this class of compounds.

Other evidences for Polyene mode of action

Many workers utilised the ultra violet ab sorbing properties of the polyene antibiotics to carry out studies on their biological and biochemical mode of action. Kleinschmidt, Chough and Mudd (107), Schroeder, Holland and Bieber (108) and Norman, Demel, De Kruijff and Van Deenen (109) reported strking effects on the polyene U.V. absorption spectrum after addition of sterol to aqueous solutions of the antibiotic. Norman et al studied the spectral changes that occur when a variety of sterols were added to filipin. They concluded that filipin can interact with sterol in a stereochemically and stoichiometrically defined manner to produce a filipin-sterol complex. The formation of the complex was specific only for sterols, no spectral changes were observed upon addition of e.g. bovine serum albumin, lecithin, and galactose. Addition of non-ionic detergent (Triton X-100) or organic solvents which are miscible with water (methanol or dioxane) abolished the spectral changes implying dissociation of the filipin-cholesterol complex.

Addition of filipin to aqueous preparations of liposomes containing cholesterol showed similar spectral changes, whereas, in the absence of liposomally bound sterol, no spectral changes were observed (109).

Spectral change also occurred in red cell ghost membranes and in cells and membrane fractions obtained from A. laidlawii grown in the presence of cholesterol. No spectral change was observed in membranes obtained from A. laidlawii grown in the absence of cholesterol or in the presence of epi (3α -hydroxyl) cholesterol (110, 111).

All the above evidence points to the fact that polyenes spectral

changes totally depend on the presence of sterol. Whether it was free

in solution, bound in a liposomal bilayer, or present in a naturally

occuring membrane.

Normal et al (109) in a study of the sterol structural require-

ments necessary for optimal interaction between sterol and polyene

showed that this reaction was primarily hydrophobic in nature. (Due

to the fact that free sterol was in water). The presence of a

cholestane ring structure and a Δ -22 double bond produced the most

favourable interaction.

Another structural requirement, essential for sterol-polyene

interaction is the presence of a 3β - hydroxyl on the sterol nucleus.

Cholesterol with a $3-\beta$ -OH on the steroid nucleus was effective

whereas epicholesterol with a 3α -OH was unable to produce the

characteristic spectral change in filipin.

There is a correlation between the molecular size of a polyene

and the degree of membrane damage which it causes. According to

Cirillo, Harsch, and Lampen (112), polyene antibiotics can be divided

into two broad functional classes, high molecular weight polyenes

such as N-acetyl Candidin and low molecular weight polyenes such as

filipin. Filipin destroys the structural integrity of a membrane,

whereas N-acetyl candidin causes relatively minor changes in the

permeability properties of the membrane. The action of nystatin

appeared to be intermediate between these two extremes.

Low concentration of nystatin showed effects similar to N-acetyl candidin, but high concentrations the effects resembled those of filipin. (112). Cirillo et al suggested that the effect of N-acetyl candidin and filipin represent the full spectrum of effects relating to physical membrane damage caused by this group of drugs. Ranging from destruction of general structural integrity of the membrane, by filipin to the minimal change produced by N-acetyl candidin. Both high molecular weight (46 or 47-carbon atoms per molecule) and low molecular weight (34 to 37-carbon atoms per molecule) polyenes are bound to sterol components of the membrane so the molecular basis for these differences in action remain to be determined (113).

Correlation also exists between the degree of membrane damage caused by individual polyene antibiotic and observed defects seen in electron micrographs. Using negative staining techniques, Kinsky, Luse, Zopf, van Deenen and Haxby (114) showed that negative staining of erythrocytes and cholesterol containing liposomal membranes revealed that the interaction of filipin and cholesterol in the membrane results in the formation of 'pits'. They suggested that filipin prevents stabilisation of the bilayer configuration of phospholipids by cholesterol. Using similar techniques, Tillack and Kinsky (115) reported structural alterations (pits, doughnut-shaped craters and protrusions) in liposomes after incubation with filipin. The occurrence of these structural alterations was dependent on the presence of cholesterol. Examination of outer and inner surfaces, and outer and inner fracture faces, did not indicate that these structural alterations could be equated with transverse holes.

Verkleig, De Kruijff, Gerritsen, Demel, Van Deenen and Ververgaert (116) concluded from freeze-etch pictures that whereas filipin formed large aggregates in the hydrophobic core of cholesterol-containing membranes of A. laidlawii cells, lecithin-cholesterol liposomes and erythrocytes, no clusters or aggregates could be seen when these structures were treated with amphotericin B (high molecular weight).

Although the presence of sterol is a necessary criteria for polyenes'sensitivity, it is not the only factor involved. It was shown that the polyene sensitivity of a membrane may depend on phospholipid/sterol ratio and not on the presence of sterol per se (117). Hsu-Chen and Feingold (118) reported that the presence of cholesterol in liposomes derived from egg lecithin were more sensitive than those derived from dipalmitoyl or distearoyl lecithins to nystatin or amphotericin B. Thus, it appears that polyene antibiotic toxicity may also be dependent on the fatty acid composition of the phospholipids. Similar results were obtained, by Gale, Johnson, Kerridge and Koh (119). They showed that although the changes in membrane permeability are largely due to hydrophobic interactions between polyenes and membrane sterols, they can also be due to the presence, nature, and amount of other neutral lipids in the surface layers of the organisms.

However, it is apparent that the interaction between polyenes and phospholipids, fatty acids and even proteins, cannot account for the selective toxicity of polyenes because these lipids, unlike sterols, are constituents of both antibiotic-sensitive and insensitive cells.

Drabikowski, Lagwinska and Surzata (120) reported that the hydrophobic environment caused by phospholipids is a necessary pre-requisite of the binding of filipin to cholesterol. Hsu-Chen and Feingold (82, 121) reported that to have a disruptive interaction between polyenes and membranes, the membrane should be in an ordered state. From the above evidence it could be concluded that it is the overall state of the membrane organisation which determines its sensitivity or resistance to polyenes.

Molecular Model for the Transmembrane Pore

Proposals for the structure of the transmembrane pore of amphotericin B and nystatin in cholesterol containing membranes have been made (110, 111 and 122). These are based upon consideration of both membrane phenomena and the fitting of space-filling models of these molecules with cholesterol. Andreoli (123) formulated a hypothesis concerning the molecular orientation of amphotericin B cholesterol pore. A partial view of such a structure is illustrated in Fig. . A space filling model of amphotericin B shown by Andreoli indicates that the parallel C_{-1}-C_{-13} and C_{-20}-C_{-33} segments may be pictured as a rod like array 20 - 24 Å long, or approximately half the core thickness of a bilayer membrane; with the C_{-19} mycosamine group at one end of the rod.

The hydophilic substituents on C_{-15}, C_{-16} and C_{-19} are located at the water bilayer interface, and the rod is situated in the membrane interior parallel to the hydrophobic chains of phospholipid and to the planar cyclopentanophenanthrene skeleton and C_{-17} acyl residue of cholesterol.

Two types of hydrogen bonds between polar groups of amphotericin B and the 3-OH group are pictured. One of these involves a 3-OH proton and a carboxyl oxygen of a C_{-16} carboxyl group. A second is between a 3-OH oxygen and a C_{-17} hydroxyl proton on amphotericin B. It is evident that several other hydrophilic interactions between sterol and antibiotic may occur at the membrane surface, although the data presented by Andreoli indicated that the carboxyl hydroxyl group of C_{-17} and the mycosamine amino group are not involved significantly in these interactions.

It is reasonable to suppose that the heptane segment of amphotericin B will be oriented primarily toward a hydrophobic rather than a hydrophilic region. Thus, in Fig. 2, the heptane chain is situated along cholesterol and phospholipid molecules and the hydroxyl-substituted segment lines the wall of the pore. De Kruijff and Demel (124) regard the amphotericin B-cholesterol pore as a circular arrangement of eight polyene molecules interdigitated with eight sterol molecules. Taking this into consideration, the hydroxyl substituents line the cylinder, and the heptane chains contribute with cholesterol to the outer wall of the cylinder. De Kruijff and Demel (124) suggested that two such cylinders anchored to opposite sides of the bilayer are necessary to form a transmembrane pore. It is thought possible that the lone hydroxyl groups at the tail of the polyene may be involved in the anchoring of two half-pores together. The diameter of the hydrophilic pore so generated for amphotericin B is 8 Å, and which is just sufficient to accomodate a molecule having the dimensions of glucose. Filipin/cholesterol aggregates which are 150-250 Å in diameter do not form this type of pore.

Fig.2. Schematical illustration of Andreoli's

(123) hypothesis for molecular orientation in an

amphotericin B-cholesterol pore.   The dotted lines

between the hydrocarbon chains of phospholipids

represent short-range London-van-der-Waals forces.

The dashed lines represent hydrogen bonds. ·

amphotericin B | pore | cholesterol | phospholipid | water | lipid | heptaene segment
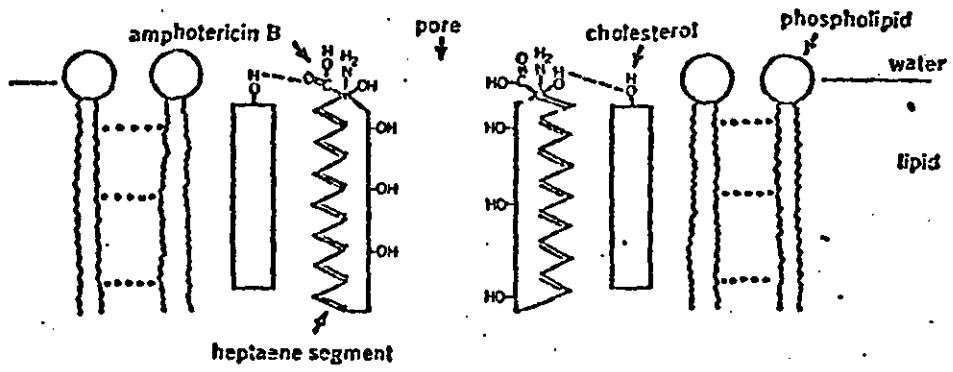
Fig. 3. Schematic representation of a well
and a complete pore formed by amphotericin B
and cholesterol in a phospholipid bilayer.
The conducting pore is formed by the end-to-end
union of two wells or half pores. From De Kruijff
and Demel (124).
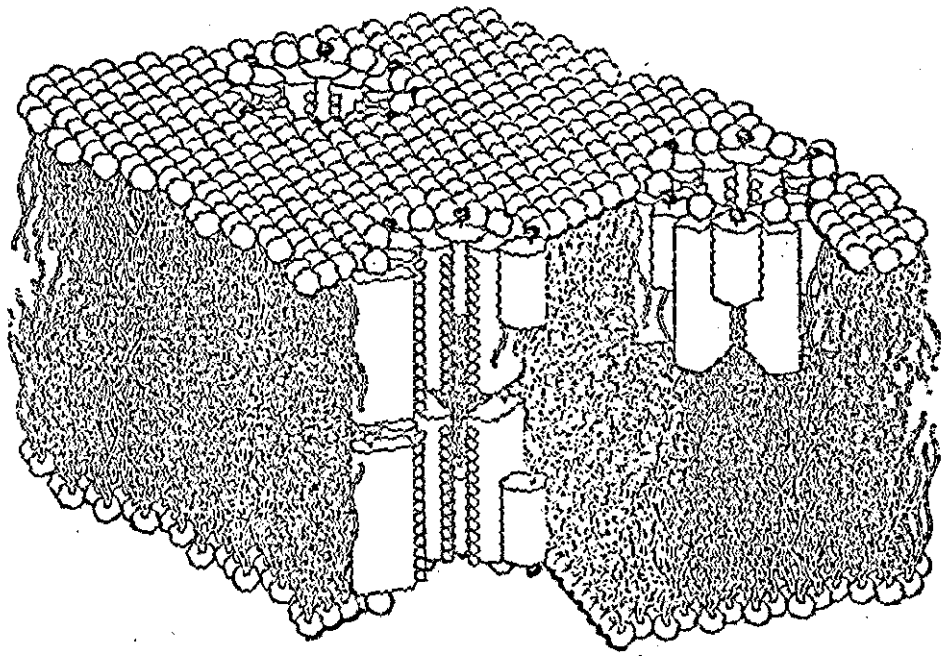
There are several interesting consequences of the model suggested by Andreoli (123). First, when cholesterol molecules interact with amphotericin B, sterol-phospholipid interaction that involves the same sterol molecules may be reduced. Second, negligible contributions of fixed changes to the anion permselectivity of porous membrane. Therefore, it is possible that relative positive changes on the hydroxyl groups of amphotericin B contribute to the anion permselectivity of the pores. Finally, it may be expected that cohesive London van der Waals forces between the short, poly unsaturated $(C_{-20}-C_{-33})$ amphotericin B segment and adjacent phospholipid molecules will be minimal). Stated in another way, the fluidity and hydration of the pore region may be considerably greater than in an unmodified region of the membrane and may approach that of bulk water. Thus, the porous bilayer membranes may be viewed as mosaic structures that contain condensed hydrophobic region and isolated aqueous regions that exhibit high permeabilities for water and small solutes.

RESISTANCE TO POLYENE ANTIBIOTICS

Resistance among yeasts to the polyene antibiotics is a rare phenomenon, (which apparently does not occur naturally) (125). During 15 years of clinical use no strains of Candida resistant to polyene antibiotics have emerged (126). However, Hejzlar and Vymola (127) reported that 31% of C. albicans strains isolated clinically failed to be inhibited by 56 units per ml. of nystatin. Bodenhoff also reported a possible development of resistance in vivo (128).

Up to now, studies on resistance to polyenes in _Candida_ species
have been confined to obtaining multistep mutants by means of serial
subculture in the presence of increasing concentrations of antibiotics
(129) by the gradient technique (125) and by one step mutation using
mutagenic agents (126). It should be noted that not all attempts to
make multi-step mutants, were successful, for example, Donorick and
Co-workers (130) and Littman _et al_ failed to obtain resistant strains
or _C. albicans_ (129).

Hamilton-Miller (131) proposed two hypotheses for the mechanisation
of resistance to yeasts. The first is the 'biochemical ' hypothesis,
it supposes that resistance arises due to changes, either quantitative
or qualitative in the sterol content of the cells. The crucial factor
in this hypothesis is that resistant cells with altered sterol content
should absorb smaller amounts of antibiotic than do sensitive cells.
The second, or 'physiological' hypothesis, suggests that resistance
arises from alterations in the surface: volume ratio of cells. While
discarding the latter hypothesis, because it cannot account for degrees
of resistance observed in four _C. albicans_ mutants, Hamilton-Miller (131)
adopted the first hypothesis and suggested three different mechanisms
for the decreased binding of antibiotics in _Candida albicans_ mutants.

(1) A decrease in the total ergosterol content of the cell,
     without concomitant changes in sterol composition.

(2) Replacement of some or all of the polyene-binding sterol
     by one which binds polyene less well e.g. substitution of
     ergosterol, cholesterol or stigmasterol by a 3- hydroxy -
     or -3 oxo sterol (109).

(3) Reorientation, or masking, of existing ergosterol, so
     that binding with polyenes is sterically or thermodynamically
     less favoured.

Since damage to cells by polyene antibiotics appears to be due
to an interaction between antibiotic and sterol, the sterol contents
of fungi have been extensively studied. According to Capek, Simek,
Bruna, Srab and Budesinsky (132) the development of inducible resis-
tance (induced by adaptation mechanism) in a given strain of C. albicans
was accompanied by a decrease in the sterol content of the cells. They
found that ergosterol formed the greater part of the cellular sterols.
They suggest that the lower ergosterol content of the cells of transient
resistant strains of C. albicans is not due to enzymatic degradation of
pre-formed ergosterol, but to inhibition of its synthesis.

Similar results were obtained by Fryberg, Oehlschlager and Unrau
(133) who reported that the principal sterols in each resistant culture
were biosynthetic intermediates in the elaboration of ergosterol in
the more sensitive cultures. Successively more resistant cultures
generally possessed principal sterols arising from blockage of the
biosynthetic sequence (leading to ergosterol) at successively earlier
stages. They reported that cultures possessing Δ^8 - sterols are more
resistant to polyenes than those possessing Δ^7 - sterols, which in turn,
are more resistant than those possessing $\Delta^{5, 7}$ - sterols. The effect
of sterol side-chain structure on culture resistance to polyenes is not
well documented.

Fryberg, Oehlschlager and Unrau (134) suggest that development

of resistance occurs by selection of naturally occuring resistant

cells, present in small numbers in the population. Thus each strain

has the ability to produce sterols of a certain structure and is able

to grow on media containing nystatin. The rate of growth is dependent

on its normal growth rate and the rate at which nystatin causes cell-

membrane damage. This latter rate is presumed to be a function of the

affinity of nystatin for the membrane sterols; the greater the nystatin-

sterol affinity, the greater the rate of membrane damage. Therefore,

the gradual loss of resistance and changes in sterol composition observed

upon culturing resistant strains on media devoid of nystatin presumably

arises from re-population of the culture by cells producing sterols

that render them more sensitive. As a corollary, one would expect each

resistant culture to exhibit a slower growth rate than its more sensitive

parent. This was in fact observed (134).

However, Athar and Winner (125) suggested that the origin of

resistance is a process of mutation rather than selection, but because

the fluctuation test (135) is considered not to be valid under all

conditions (mainly because of failure to rule out all other causes of

variability or fluctuation) the evidence is not conclusive.

The role played by the cell wall components in affecting the

interaction of membrane active antibiotics with their primary site

of action was studied extensively by Gale, Johnson, Kerridge and Koh

(119, 136).

They studied the difference between cells harvested during the exponential phase and cells harvested during the stationary phase. They found that stationary-phase cells were more resistant than exponential-phase cells. This was attributed to the fact that in the exponential-phase cells there exists a continual breakdown and resynthesis of cell-wall constituents which would result in the site of wall growth providing a ready access for antibiotic penetration to the plasma membrane. In stationary phase cells, cell wall growth has ceased, hence the increase in resistance. (136).

Recently, Gale, Johnson and Kerridge (137) suggested that amphotericin-methylester resistance can be explained by the production of a component 'R' of the cell wall and that reduction of that component decreases resistance. This 'R' component possesses a-SH group and the resistance is irreversible when the-SH group is alkylated.

Manten (138) and Garrod and O'Grady (139) considered the development of resistance in microorganisms is associated with a decrease, and sometimes a complete loss, of their virulence. Decreased virulence of C. albicans resistant to polyenes was reported by Ito, Miyamura, Yusa, and Mimiyashita (140). This may be related to their reduced growth rate, which impairs their ability to proliferate in infected tissues and give the host a greater opportunity to combat and restrict infection. In the first report on the development of resistance, the degree of acquired resistance was considered so low that it probably would not present any clinical problems (141).

However, even a slight increase in the amount of amphotericin B required to inhibit a pathogenic agent may prevent the therapeutic use of this somewhat toxic drug, and the possible development of cross-resistance to other polyenes may hinder therapy.

Nonetheless, it appears that any danger of the development of polyene-resistant isolates of Candida is more than counterbalanced by their loss of virulence. The most virulent Candida spp. known cannot be said to be very virulent by the standards of other pathogenic microorganisms. Thus, reduction of their virulence, such as that accompanying the development in vitro of polyene resistance would seem to rule out the damage of such organisms producing disease in man (125).

C H A P T E R   T H R E E

BIOSYNTHESIS OF STEROLS

The number of reports on the biosynthesis of fungal sterols is moderate and most of them involve yeasts. In many cases, these studies were not carried out with the aim of showing relevance to sterol formation by fungi but the yeast were used only as a tool for the study of analogous reactions occuring in plant and particularly mammalian systems. However, much work has been carried out on fungal sterols in an effort to explain resistance/sensitivity to polyene antibiotics.

The formation of sterols in both plant and animal systems occurs through four principal stages (a) conversion of acetate to mevalonate, (b) conversion of mevalonate to squalene, (c) cyclization of squalene, and (d) conversion of the first cyclic intermediate (lanosterol) to the 4 - des methyl sterol products.

Conversion of Acetate to Mevalonate

The enzymatic synthesis of mevalonate from acetate (Scheme 1) occurs in mammalian and avian liver (142, 143) in the insect Sarcophaga bullata (144) and in some mycoplasma species M. laidlawii and M. gallisepticum) (145). In the higher plants the synthesis of mevalonate has not yet been demonstrated. However, Modi and Patwa (146) reported the presence of mevalonate in carrot roots. In mammalian and avian liver two independent pathways are reportedly operative for the synthesis of mevalonate. One involves a soluble enzyme system, whereas the second requires soluble and microsomal components.

SCHEME 1 :

Conversion of acetate to

mevalonate.

$$3CH_3CO.CoA$$

$$\Big\downarrow\Big\uparrow \text{Thiolase}$$

$$CH_3CO.CH_2CO.CoA$$

$$+CH_3CO.CoA$$

$$\Big\downarrow\Big\uparrow \begin{array}{l}\text{3-Hydroxy-3-methyl-}\\ \text{glutaryl-CoA Synthase}\end{array}$$

$$\begin{array}{c} CH_2CO_2H \\ | \\ HO-\!\!-\!\!-C-\!\!-\!\!-CH_3 \\ | \\ CH_2CO.CoA \end{array}$$

$$\Big\downarrow \begin{array}{l}\text{3-Hydroxy-3-methyl-}\\ \text{glutaryl-CoA Reductase}\end{array}$$

$$\begin{array}{c} CH_2CO_2H \\ | \\ HO-\!\!-\!\!-C-\!\!-\!\!-CH_3 \\ | \\ CH_2CH_2OH \end{array}$$
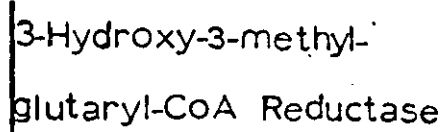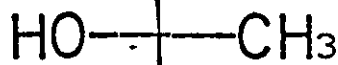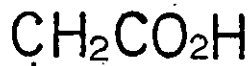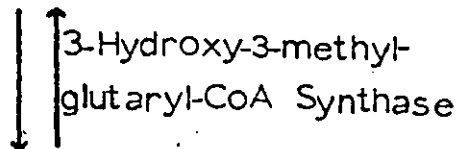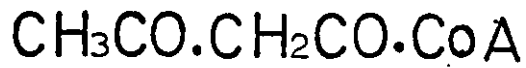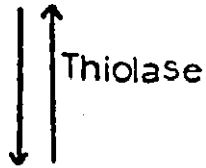
(-)mevalonic acid

The first reaction common to both of the above pathways involves activation of acetate to acetyl - Co A. This activation occurs in two distinguishable steps (147). In the first reaction 5' - phosphoacetyl adenosine enzyme and pyrophosphate are formed, and in the second the acetyl moiety is transferred to the sulphydryl group of CoA. This is not, of course, the only metabolic route for the formation of acetyl - CoA. This compound is formed by a variety of other metabolic routes, such as the oxidation of fatty acids or pyruvate, or in microorganisms through the formation of acetyl phosphate and its conversion to acetyl- CoA.

Porter et al (143) discovered the first pathway for mevalonate biosynthesis. This system synthesises 3-hydroxy-3-methylglutaryl-CoA and mevalonate. This system uses 1 mole of malonyl - CoA and 2 moles of acetyl-CoA to form a six-carbon-atom intermediate. In the absence of NADPH this enzyme system forms 3-hydroxy-3-methylglutaryl CoA or an enzyme-bound-3-hydroxy-3-methylglutarate (148,149). In the presence of NADPH, mevalonic acid is one of the products formed. This pathway must be viewed at present as either one of minor importance or one that has been artificially constituted through the removal of cell compartment barriers during homogenization.

The second, and major, pathway involves microsomal and soluble enzymes of mammalian and avian liver, and all the intermediates are bound to CoA. Two molecules of acetyl-CoA first condense to form acetoacetyl CoA. This reaction is catalyzed by thiolase, an enzyme originally reported to be present in the microsomal fraction of mammalian liver (150).

Under physiological conditions the reaction catalyzed by thiolase

favours the formation of acetyl-CoA (151). The reaction proceeds

to the right in the soluble cell fraction, however, because the

product, aceto-acetyl-CoA is removed by the next reaction, namely,

the synthesis of 3-hydroxy-3-methylglutaryl-CoA.

The synthesis of 3-hydroxy-3-methyl-CoA from acetyl and acetoacetyl-

CoA in the soluble cell fraction is accomplished by several forms of

the enzyme 3-hydroxy-3-methylglutaryl-CoA synthase. The formation of

mevalonate requires the reduction of 3-hydroxy-3-methylglutaryl-CoA.

The site of this reduction is the mitochondria in yeast (152) and the

endoplasmic reticulum in mammalian liver (153). This particular system

is being intensively investigated because the reaction catalysed by it,

is the rate - limiting step, under most conditions, in the biosynthesis

of cholesterol in mammalian liver (154, 155). The regulation of 3-

hydroxy-3-methylglutaryl CoA reductase has been reviewed recently (156).

Conversion of Mevalonate to Squalene

(1) Biosynthesis of Prenyl Pyrophosphates:-

   (i) Biosynthesis of Isopentenyl pyrophosphate:

   The biosynthesis of isopentenyl pyrophosphate is wide-

   spread in living organisms such as bacteria (157) yeast

   (158), higher plants (159) and mammals (160).

   The formation of isopentenyl pyrophosphate from mevalonate

   (Scheme 2) involves two consecutive phosphorylations at

   position 5, followed by a decarboxylation and a dehydration

   of the tertiary alcohol group.

SCHEME 2 :

Conversion of mevalonate to

isopentenyl pyrophosphate.

$$CH_2CO_2H$$
$$HO-C-CH_3 \xrightarrow[\text{kinase}]{\text{mevalonate}} HO-C-CH_3$$
$$CH_2CH_2OH \qquad\qquad CH_2CH_2O\circledP$$

$$\xrightarrow[\text{kinase}]{\text{5-phosphomevalonate}} \quad HO-\underset{CH_2CH_2O\circledP\circledP}{\overset{CH_2CO_2H}{\underset{|}{\overset{|}{C}}}}-CH_3$$

$$\xrightarrow[\text{decarboxylase}]{\text{pyrophosphomevalonate}} \quad \circledP O-\underset{CH_2CH_2O\circledP\circledP}{\overset{CH_2-CO_2^{\ominus}}{\underset{|}{\overset{|}{C}}}}-CH_3 \longrightarrow$$

$$CH_2-O\circledP\circledP \xrightarrow[\text{phate isomerase}]{\text{isopentenyl pyrophos-}}$$

$$CH_2-O\circledP\circledP$$
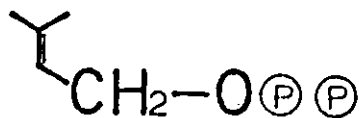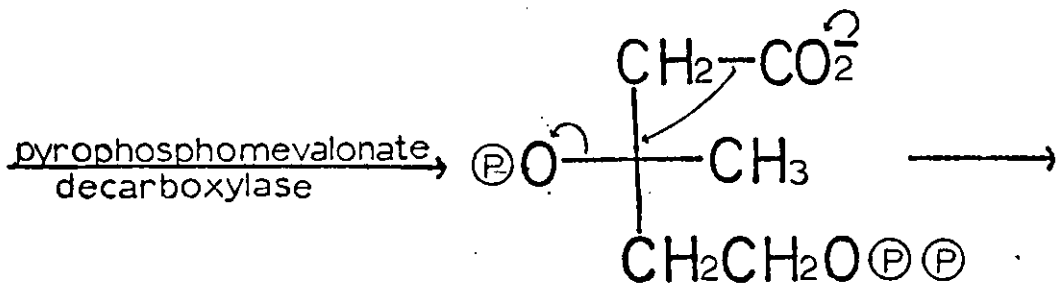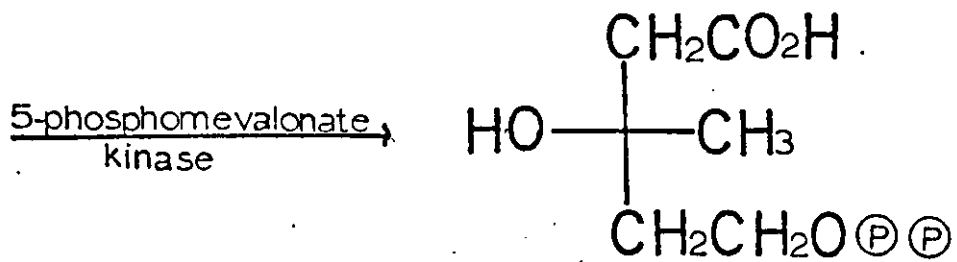
The first phosphorylation reaction converts mevalonate to mevalonate phosphate and is catalyzed by mevalonate kinase. The second phosphorylation reaction in the biosynthesis of isopentenyl pyrophosphate is that catalyzed by 5-phospho-mevalonate kinase, which catalyses the phosphorylation of 5--phosphomevalonate by Mg-ATP to produce 5-pyrophospho-mevalonate and ADP. The last step in the biosynthesis of isopentenyl pyrophosphate involves the decarboxylation and dehydration of 5-pyrophosphomevalonate. This reaction is catalyzed by pyrophosphomevalonate decarboxylase. This enzyme has been partially purified from yeast by Block, Chaykin, Phillips and de Waard (161).

(ii) Biosynthesis of Allylic Pyrophosphate

The biosynthesis of allylic pyrophosphates (Schemes 3 and 4) involves two apparently different reactions: isomeriza-tion of isopentenyl pyrophosphate to an allylic compound, dimethylallyl pyrophosphate, followed by head-to-tail conden sation with isopentenylpyrophosphate units to give, succes-sively geranyl-ornerylpyrophosphate (trans or cis C10), farnesyl pyrophosphate (C15), and geranylgeranyl pyrophos-phate (C20). Polyprenyl pyrophosphates up to C110 are built in the same way, by successive additions of isopentenyl pyrophosphate. The first enzyme in this pathway is isopen-tenyl pyrophosphate isomerase. This enzyme catalyzes the isomerization of the double bond from position 3 of isopen-tenyl pyrophosphate to position 2 of dimethylfallyl-pyro-phosphate. The formation of geranyl, farnesyl, geranylger-anyl and other polyisoprenyl pyrophosphates, involves the condensation of one molecule of dimethylallyl pyrophasphate, with one, two,

SCHEME 3 :

Conversion of isopentenyl pyrophosphate

to geranylgeranyl pyrophosphate.

geranylpyrophosphate

farnesyl pyrophosphate

geranylgeranyl pyrophosphate
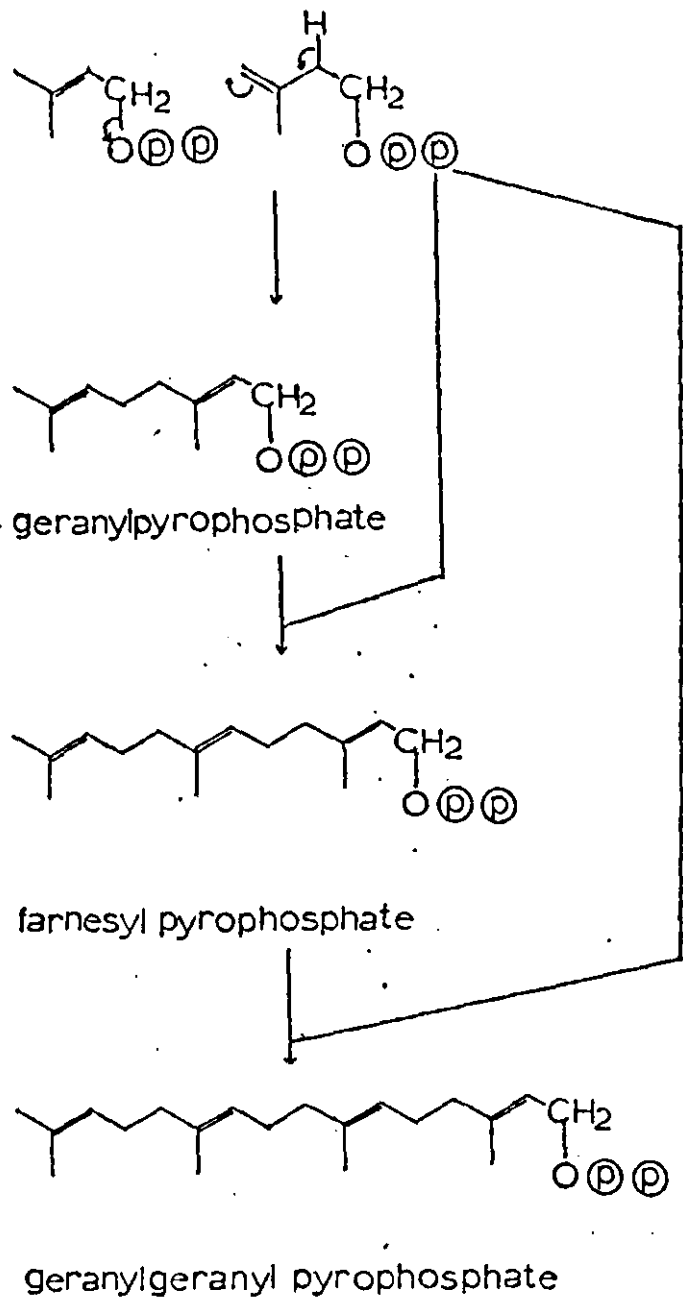
three, or N-1 molecules of isopentenyl pyrophosphate,

respectively. This reaction is catalysed by several

enzymes generically called prenyl transferases or

prenyl-pyrophosphate synthetases.

Biosynthesis of Squalene

The biosynthesis of squalene from farnesyl pyrophosphate has

been demonstrated in a wide variety of species. Lynen, Eggerer,

Henning, and Kessel (162) first reported the production of

squalene when yeast cell particles were inc,ubated with far-

nesyl pyrophosphate and NADPH. The enzyme that carries out

this reaction squalene synthetase, has been partially purified

from pig (163) beef, and rat liver (164) and from yeast (165).

Squalene synthetase catalyzes the condensation of two molecules

farnesyl pyrophosphate to yield a C_{30} pyrophosphate inter-

mediate e.g. pre-squalene pyrophosphate, which is then converted

to squalene in the presence of NADPH. Both reactions, condensation

and reduction, are carried out by the same enzyme system.

Cyclization of Squalene

The role of squalene as an obligatory precursor of the steriods

and triterpenes is firmly established and the mechanism of its

cyclization in a variety of conformation has been extensively

discussed (166-168). The process is now known to be initiated

by the formation of squalene oxide as we can see in Scheme 4 leadin

to the formation of lanosterol.

S C H E M E   4

Cyclization of Squalene
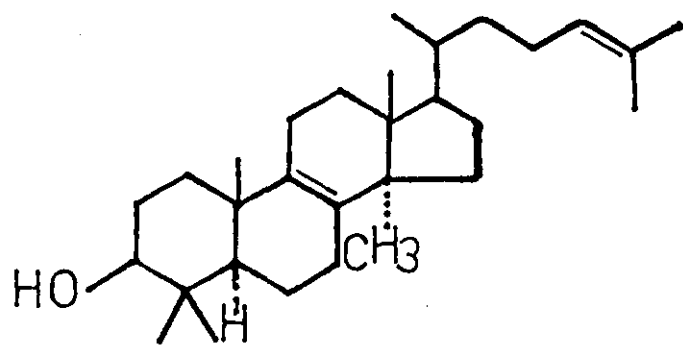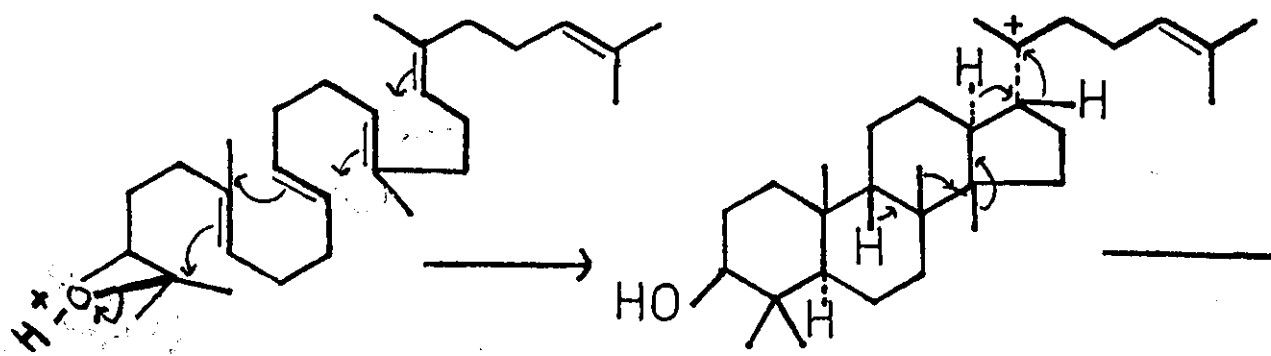
Conversion of the first cyclic intermediate (Lanosterol)
to the 4-desmethyl sterol products.

As in the case with cholesterol in animals, the first
cyclic intermediate in the formation of ergosterol by
fungi is lanosterol. It is now generally accepted,
however, that the 9β , 19-cyclopropane derivative of
lanosterol, cycloartenol, is the first cyclic intermediate
in the synthesis of phytosterols (169, 170).

Lanosterol was first identified from a fungal source
(yeast) by Wieland and Stanley (171) who called it
cryptosterol. Since that time, lanosterol has been
identified in several other fungal species.

The metabolism of lanosterol by animal systems leads
principally to the production of cholesterol, while it is
converted to ergosterol by most fungal species. During
this conversion, certain general reactions are common to
both animal and fungal systems (a) Loss of the C-4 and
C-14 methyl groups (b) Reduction of the C-24 double bond,
and (c) a series of nuclear double-bond shifts. There
are two additional reactions, however, that occur in the
formation of ergosterol which do not occur in the animal
systems. i.e. introduction of methyl group at C-24 and
formation of a Δ^{22} double bond in the sterol side-chain.

To elucidate the pathways of sterol biosynthesis, various mutant strains of S. cerevisiae have been employed. Particular use has been made of mutants resistant to polyene antibiotics.

Rathay, Schibeci, and Kidby (172) listed the possible intermediaries in the formation or ergosterol in yeast (Scheme 5).

All the sterols named in (Scheme 5) have been isolated from various S. cerevisiae strains and have been considered as possible biosynthetic intermediates by Barton, Corrie, Marshall and Widdowson (173).

The efficiency with which cell free extracts have been able to convert these postulated intermediates has been examined and the available details on some of the proposed enzyme systems have been reviewed by Weete (1974). Information, however, on the sequence of action of these enzymes is generally lacking, but evidence for a multiplicity of sterol biosynthetic pathways in yeast has been presented (175).

The initial step of lanosterol conversion involves the removal of the two methyl groups at position 4 and the methyl group at position 14. Inability to carry out the demethylation at position 14 has been found (176) to be accompanied by the accumulation of 4, 14 - dimethyl sterol derivatives.

This finding suggests that the other 4 - methyl group is subject
to earlier removal. Sterols carrying a methyl group at position 4
undergo little methylation at position 24 (177, 178). S-adenosyl-
methionine: Δ^{24} sterol methyl transferase (C 24 methyl transferase),
involved in the methylenation process, apparently shows greatest
substrate specificity for cholestra-7, 24-dien-3β -ol (179) although
zymosterol (177) is also used. This enzyme activity has been found
to be primarily located in the promitochondria or mitochondria of S.
cerevisiae (180) and to be enhanced by the presence of a fermentable
carbon source and molecular oxygen (181) Formation of cholestra- 7,
24-dien-3β-ol from zymosterol would require the activity of $\Delta^{8}:\Delta^{7}$ iso-
merase (182).

Several pathways possibly exist for the enzymic conversion of the two
C24 methylene sterols, fecosterol and episterol. The molecular events
presumably involve a 5,6 - dehydrogenase for the introduction of additional
unsaturation in ring B, a 22, 23 - deydrogenase for the dehydrogenation
of the side chain and a methylene reductase for the reduction of the
methylene group at position 24. The absence of any of these systems
would be anticipated to result in the accumulation of different sterols
whereas the presence of all three systems would permit the formation of
ergosterol. A major biosynthetic pathway from episterol to ergosterol
has been deduced (175, 183) as involving the sequential introduction of
unsaturation at position 22, 23 and then as position 5, 6 followed by
the reduction of the methylene group at position 24.

Additional knowledge on the characteristics of the enzyme

systems involved in sterol biosynthesis in yeast should

provide a better understanding of the mechanisms controlling

the process. The activity of hydroxymethyl glutaryl - CoA

reductase participating in the initial biosynthetic step,

of mevalonate formation, has been considered (184) to be

under feedback control of ergosterol or some acidic products

of ergosterol metabolism. Control of sterol biosynthesis

may occur (177) at the reaction step catalyzed by S - adenosy-

lmethionine: Δ^{24} sterol transferase which has been suggested

(185) to be competitively inhibited and possibly repressed

by ergosterol. The existence of several biosynthetic routes

of ergosterol suggests the possibility of several regulatory

mechanisms.

Various factors influencing the synthesis of sterol by yeast

have been discussed by Hamilton - Miller (186). In partic-

ular, the composition of the growth medium (187) and stage

of the growth cycle (188) have been noted to have an effect

on the amount of sterol produced. The availability of oxygen

requires special attention as it governs both the type of sterol

synthesised (189, 190) and also the quantity of sterol. The

lower levels of ergosterol occurring under anaerobic conditions

in S. cerevisiae (175, 191) and the yeast-like form of Mucor

genevenis (192) have been reported to be accompanied by

increased amounts of squalene.

The apparent accumulation of this hydrocarbon reflects,
in part at least, the observed sensitivity to lack of
oxygen of several reaction steps including oxidative
cyclization of squalene, oxidative desaturation of ring
B (193) and methylation at position 24 (177). In certain
mutants of S. cerevisiae the inability to form ergosterol
has been traced (194) to lesions in the biosynthesis of
porphyrins rather than sterols and indicates the probable
importance of an active respiratory chain in the conversion
of squalene to ergosterol.

SCHEME 5 :

Biosynthesis of sterols in yeast.

LANOSTEROL

PARKEOL

ZYMOSTEROL

FECOSTEROL

ERGOSTA-8(9),22-DIEN-3β-OL

CHOLESTA-7,24-DIEN-3β-OL

EPISTEROL

ERGOST-7-EN-3β-OL

ERGOSTA-7,22,24(28)-TRIEN-3β-OL

ERGOSTA-5,7,24(28)-TRIEN-3β-OL

ERGOSTA-7,22-DIEN-3β-OL

ERGOSTA-5,7,22,24(28)-TETRAEN-3β-OL

ERGOSTEROL

ERGOSTA-5,7-DIEN-3β-OL
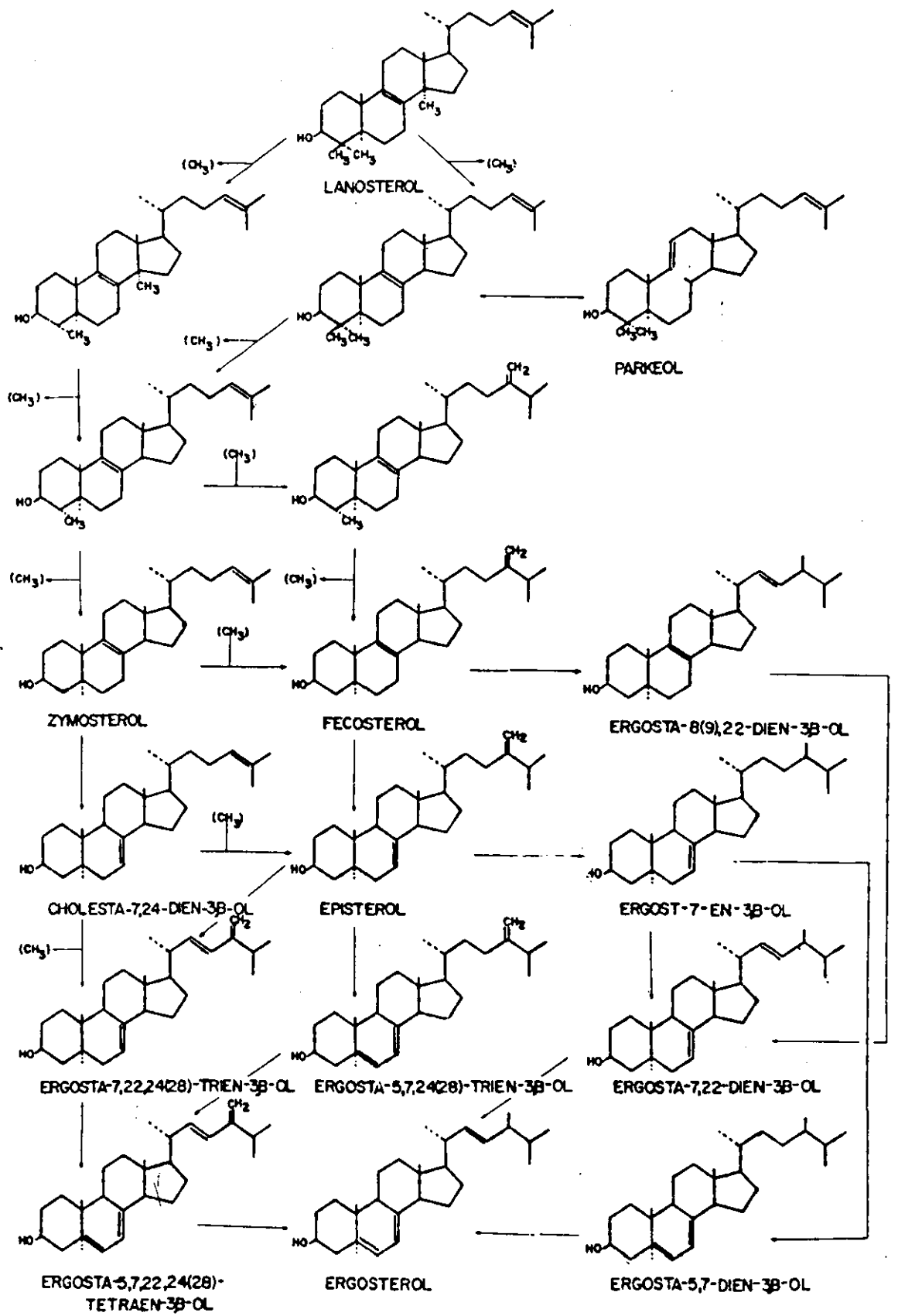
CHAPTER FOUR

POTENTIATION OF FUNGAL INFECTIONS BY DRUGS

With increase in the use of antibiotic and steroid therapy,
there has been a growing awareness of infections caused by oppor-
tunistic invaders, notably those due to fungi. Prior to the use
of these drugs, fungi posed a threat almost solely to debilitated
people.

The cases of fungal infections reported in the pre-antibiotic
and steroid era were very rare. In 1945 Downing and Conant (195,
196) wrote a comprehensive review of opportunistic infections in which
they commented that systemic infections with Candida albicans were
rare. They found a total of three fatalities due to systemic
moniliasis, and two cases of mycotic endocarditis (in drug addicts)
during the quarter century preceding their report. Winner and
Hurley (197) reported several additional cases of systemic moniliasis
and possibly four more fatalities during the pre-antibiotic and
steroid period.

The first report of invitro antibacterial potentiation of
fungi, i.e. enhancement of growth of fungi by streptomycin (198)
and increased lethality of C. albicans in penicillin-treated chick
embryos (199) appeared in 1949. Since then this problem has been
examined by many workers. In 1950 Zimmerman (200) reviewed some of
the factors which seemed to be responsible for the rising incidence
of fungal diseases.

Behrman (201) Freeman (202) Mangiaracine (203) and Reiches (204) reported in the early 1950's on the increase in oral and anogenital side effects. This was attributed to fungal overgrowth in patients receiving antibiotics, in particular the broad spectrum antibiotics such as chlor-and oxytetracycline, and chloramphenicol. Also at this time there were several cases of severe monilial infections, causing bronchopulmonary complications (205) as well as reports of an increase in endocardial (206), spinal cord (207), urinary tract (208), and systemic fungal infections (209). These fungal infections were correlated with the administration of antibiotics.

In 1956, Keye and Magee (210) analysed the clinical records, autopsy protocols, and histological and bacteriological reports relating to 15,845 consecutive autopsies from 1919 to 1955. They found a total of eighty-eight cases of fungal infections, including Candida, of which forty-seven were regarded as secondary to treatment of the primary disease. They concluded that there had been no change in the incidence of primary fungal infections since 1947, whereas there had been a striking increase in secondary fungal infections. Torak (211) related the recent increased incidence of fungus infections to the use of antibiotics, with or without corticosteroids, the more invasive forms developing in patients taking steroids. Baker (212) and Gruhn and Sanson (213) have confirmed the increased incidence of secondary mycosis in leukemic patients. Baker (212) attributed the recent increase of mycotic fatalities in patients with leukemia to the use of antileukemic drugs and corticoids rather than to antibiotics.

He reported that although no deaths were attributable to fungus
infections among the 143 leukemic patients autopsied prior to
1953, in the eight years therafter, 9 per cent of the deaths among
leukemic patients were directly due to fungus infections. Gruhn and
Sanson showed that of the total of twenty-five secondary fungal infec-
tions in the study of 103 leukemic patients five who died of disseminated
mycosis had received multiple antibiotic therapy as well as corticosteroids.

It has been generally accepted that steroids enhance ocular
fungal infections and some evidence exists to support this (214).
Mitsui and Hanabusa (215) reported four cases wherein fungal keratitis
occured de novo in patients who had been treated with cortisone topically
for a variety of ocular conditions, and showed that fungi appeared more
frequently in the conjunctival sacs of patients who had been treated
with cortisone topically (67 per cent) than in those not similarly
treated (18 per cent).

It has been shown that corticosteroids definitely enhance the
infectivity of C. albicans in the rabbit cornea. Sery and Montana
reported that prednisolone may enhance the infectivity of the organism
(217) for two weeks after injection of the steroid.

Numerous other articles have discussed predispostion of patients
to fungal infections e.g. Kaufman (218), Pheins, Suie, Van Winkle
and Havener (219), Leyden and Marples (220), Gentles and La Touche
(216), Strippoli and Simonetti (221), and Scheeta II, Carlson and
Schinitsky(222).

TABLE 3 :   Factors Predisposing to Infection

by Candida albicans (and other

Pathogenic Species of Candida) (216).

HORMONAL DISTURBANCES AND OTHER
IDIOPATHIC STATES

Diabetes

Hypoparathyroidism

Hypoadrenocorticism

Carcinoma

Leuk emia

Pernicious anaemia

Aplastic anaemia

Agranulocytosis

Bronchiectasis

Malformation of the urinary tract

Ulceration of the digestive tract

Debility

Malabsorption

Malnutrition

Moribund state

PRE-EMINENTLY RECEPTIVE STATES

Pregnancy

Infancy and old age

Carbohydrate-rich diet

Maceration of skin

Skin surface contact with carbohy-
drates

DRUG THERAPY

Antibiotics

Corticosteroids

Contraceptive drugs

INFECTIOUS DISEASE

Tuberculosis

Chronic bronchitis

Influenza

Typhoid and other enteric
Infections

Bacterial endocarditis

SURGERY

Open heart operations

Bowel resections

Colostomy

Tooth extractions

Eye operations (corneal grafts)

Ear operations (skin grafts)

ACCIDENTAL INTRODUCTION OF
CANDIDA BY INTRAVENOUS INJEC-
TION OR INDWELLING URINARY
CATHETERS

Blood transfusions

Glucose saline drips and other
supportive fluids.

Drugs, especially in addiction

ACCIDENTAL TRAUMA

Eye injury

Burns

Other factors similarly predisposing patients towards fungal infections are summarised in Table 3.

Classifications

The classification of *C. albicans* is confused, long and probably incomplete. This is apparent from the fact that Ciferri, Redaeli and Cavallero (223) in 1938, found 45 different binomial synonyms, Diddens and Lodder (224) in 1942 cite 87 and Conant and associates (225), in 1944 state that there are 172 in literature.

It was just 130 years ago, in 1847, that Robin first described a species of *Candida* (226). It is possible that Langenbeck (227) and other earlier workers had seen the fungus, but their descriptions do not appear to be sufficiently accurate to show what organism was encountered. The first Latin binomial, *Oidium albicans* was given by Robin (226). Recognising the fact that this organism did not belong to the genus *Oidium*, Quinguand (228) re-named it *Syringaspora robinii*. This new name was not accepted and other alternatives were suggested e.g. *Mycoderma vini* by Grawitz (229), but this had to be abandoned when Reess (230) showed that the fungus was distinct from *Mycoderma vini* and named the organism *Saccharomyces albicans*. Zopf (231) proposed yet another name, *Monilia albicans*. This name seemed to propagate and the credit for this must be given to Castellani (232, 233). His adoption of this name influenced the nomenclature and it is largely on his account that the name *Monilia* was retained for the organism of thrush both in the literature and in clinical practice.

In 1923, Berkout, (234) suggested the most favourable generic name for Candida albicans. She defined Candida as "few hyphae lying flat, falling apart into longer or shorter pieces. Conidia arise by budding on the hyphae or each other. Small and colourless".

Her terminology was accepted by Ciferri and Redaelli (235) and Ashford (236), but not by Shrewsbury (237). This dilemma was solved when, in September 1939, the Third International Congress for Micro-biology agreed to adopt the name Candida albicans.

Morphology

C. Albicans exists in two distinct forms: the yeast form, and the mycelial form. According to Hendry (238) the yeast form or blasto-spore are oval cells that reproduce by budding, and the mycelial form or filamentous are long, occasionally branched, septate hyphae. The existence of these forms is influenced by many factors present in their environment. These factors were reviewed by Scherr and Weaver (239), Bonner (240), Skinner (241) and McClary (242).

One of the most important factors controlling morphology is the growth temperature. Scherr and Weaver (239) observed that when fungi growing normally in a yeast form are incubated at temperatures suboptimal for growth a mycelium or pseudomycelium (elongated cells, resembling blastospores joined end to end (238) is produced. McClary (242) showed the optimum temperature for filamentation was 25-30°C. He reported that high temperature favoured the yeast form in cultures on solid media, in liquid media hardly any differences in growth due to temperature were detectable. His results were not in agreement with those obtained by Talice (243).

McClary suggests that this is due to the use of different organisms. The effect of temperature in controlling the morphology of fungi was used by Chattaway, Holmes and Barlow (244) to study the chemical composition of cell walls isolated from the yeast and mycelial form of C. albicans.

Another factor on which morphology of fungi depends is carbohydrate source (245). The use of a starch medium, free of reducing sugars favours the production of the mycelial form. This observation was employed by Marriott (246) for the production of approximately 100% filamentous forms. Recently, Shepherd and Sullivan (247) stressed the dependence of morphology on carbon source. They reported that maltose produced a mycelial morphology, whereas, with lactate, a yeast culture was obtained. With fructose or glucose as a carbon source a mixed morphology of yeast, pseudo-mycelial and mycelial forms was produced. The observation that maltose favoured the production of mycelial form supports the reports of Marriott (246), and Nickerson and Mankowski (245) that starch promotes mycelial production in batch cultures of C. albicans.

Many other factors have been reported to have an effect on dimorphism but the situation is confusing. The pH value of the medium may play a role. Talice (248) found that the maximum production of filamentous forms occurred at pH8. According to Langeron and Guerra (249) filaments were produced in an alkaline medium, yeast cells in acid. McClary (242) considered that the tolerable extremes of pH range produced yeast-like cells and found that the optimum pH for filamentation was 5-6.

However, Widra (250) denied any effect of pH on morphology.

The concentration of inorganic phosphate has also been cited as a factor involved in the control of fungal morphology (242). In particular, the dextrose inorganic phosphate ratio (250). However, Shepherd and Sullivan (247) did not find that phosphate affected the morphology. They found that a chemostat run with glucose as a carbon source but with limiting phosphate, gave a mixed morphology regardless of the dilution rate.

Finally, the nitrogen source (251) or the use of N-acetyl glucosamine as sole nitrogen and carbon source (252) can also affect morphology.

The Relation between Pathogenicity of C. albicans and Yeast/Mycelial Forms.

Mycologists have long entertained the possibility that fungi are pathogenic only in certain morphologic phases. Thus, dimorphic fungi such as Histoplasma capsulatum may be pathogenic for warm-blooded animals only, in the yeast phase, and the forced transformation to the mycelial phase might aid the host in limiting the infection (253, 239). These concepts have been applied to infections with C. albicans. In spite of the fact that both forms of C. albicans are implicated in clinical lesions, workers have incriminated one form or the other for its pathogenicity.

Some authors, including Redaelli (254), Foster (255), Ainworth (256), Rogers (257), Young (258), Gresham and Whittle (259) and Taschdjian and Kozinn (260) have insisted that the development of the mycelial phase *in vivo* indicates a change from the commensal or saprophytic to the parasitic habit. It has also been noted that in monilia granuloma, mycelia predominates to a striking degree (261). Young (258) felt that strains which did not produce mycelia *in vivo* in mice were rapidly phagocytosed and destroyed. Heineman, Yumis, Siemienski and Braude (262) noted the presence of mycelia and chlamydopores in two cases of disseminated candidiasis and came to the conclusion concerning the importance of the mycelial phase. More recently, Hurley and Stanley (263) correlated the destruction of mouse epithelial cells by C. albicans with the growth rate of the strains tested and their tendency to grow in the mycelial form.

The description of the yeast form as being the invasive stage of the organism was reported by many authors. Balish and Phillips (264) fed C. albicans to 36 day old chicks and found that disseminated candidiasis was associated with the yeast phase growth in the intestinal tract and tissues. Winsten and Murray (265) found that a mycelial-growing strain of C. albicans was not pathogenic but could be made so when the addition of cysteine to the medium caused transformation to the yeast phase. Taschdjian, Reiss and Kozinn (266) induced vaginal candidiasis in mice and concluded that the yeast phase was responsible for initial tissue penetration following which, mycelial transformation and host reaction occurred.

In contrast to the above arguments, Louria, Brayton and Finkel (267) in a study of the pathogenesis of C. albicans infections in mice, found no evidence that invasiveness could be correlated specifically with either the mycelial or the yeast phase; transformation into the mycelial phase was followed by progressive infection only in the kidney. Similar results were obtained by Mackenzie (268). His results suggested that yeast-mycelial transformation is not necessarily a precursor of the invasive process of C. albicans. However, he laid down a model for the study of factors affecting pathogenesis. This model depends on the monitoring of a "pseudo germ tube stage" which is associated with parasitic growth but can also be induced in vitro.

In conclusion, it would appear reasonable to suggest that the morphology of C. albicans in vivo is determined largely by endogenous host factors such as nature of the invaded tissues, quality and quantity of growth factors and nutrients, pH and oxygen tension and the nature and degree of host cellular defences. According to Saltarelli, Gentile and Mancuso (269), this simplistic pathogenic model based on morphologic form alone should be refuted.

MECHANISMS BY WHICH ANTIBIOTICS AND STEROIDS INCREASE THE INCIDENCE AND SEVERITY OF CANDIDIASIS.

The mechanism by which antibiotics and steroids enhance the invasiveness of fungi is considered to be due to either direct effect on the organisms or the host, or both.

THE EFFECT ON THE HOST

In the early 1950's Seligmann (270) reported that the increased
lethality of Candida, given to animals with tetracycline, was due to
an effect of the antibiotic on the host, rather than an effect on
the organism itself. This observation was confirmed by the work of
De Mello and Kiser (271) and Roth and Gyler (272). Similar results
were obtained by Henry and Fahlberg (273) who reported that hydro-
cortisone increased mortality and had an additive effect when given
with tetracycline. They postulated that the enhancing of cortisone
or ·tetracycline on the infectivity of C. albicans probably resulted
from the action of the drug on the host.

Some of the possible mechanisms of host influence are direct
tissue toxicity, alteration of the gastrointestinal flora, and
alteration of the immunologic response.

Seligman (270) attributed the antibiotic/steroid enhancement of
Candida infectivity to damage caused to the host tissues at the site
of infection. Messer and Freter (274) reported that local irritation
of the tissues exposed to tetracyclines might be responsible for
decreased resistance to Candida. They showed that when gastrointestinal
irritants are used Candida infection is encouraged. They suggested
that the enhancement of C. albicans growth by antibiotic therapy is
similarly due to irritation of the host tissue. Similar results were
obtained by De Mello and Kiser (271).

Mead, Rowe, and Haslam (275) and McCoy (276), testing the effect of chemotherapeutic agents on the host flora, reported that temporary quantitative depression of bacterial flora occurs. This depression chiefly involves gram-positive organisms. Robinson (277) suggested that this reduction of bacterial forms allows "saprophytic" fungi to proliferate. Later Abrams and Bishop (278) reported that the normal intestinal flora enhanced the ability of the host to cope with infection by enhancing leukocytic mobilisation. Thus, depression of this normal flora will have an indirect effect on the defenses of the host.

The effect of antibiotics/steroids on the host defence mechanism is divided into two parts (1) Effect on antibody production (2) Effect on phagocytosis. The effect on the synthesis of antibody was mainly concerned with the suppression of antibody production which results from removal of the bacterial antigenic stimulus. Many investigators have reported a depression of antibody titre following the use of steroids (279-281).

However, many variables are apparently involved, such as the steroid used (280), the species and strain of laboratory animal tested, and the type of antigen (281).

Goreyzyca and McCarty (282) studied the effect of antibiotics on the antibody and other serum proteins of animals infected with C. albicans.

They stated that goats given oxytetracycline (60μg/day) intra-muscularly and goats given benzathine penicillin (300,000 units intra-muscularly) every other day, developed changes in their serum proteins. In addition, the alterations in serum proteins caused by C. albicans infection were influenced by antibiotics. Ambrose and Coons (283) showed that chloramphenicol at the same levels as those that suppress bacterial protein synthesis (50μg/ml), inhibits the synthesis of anti-body in tissue culture. They showed that the continuous presence of chloramphenicol in the medium throughout the 15-21 day incubation of lymph node fragments (from immunised rabbits) produced nearly complete suppression of secondary antibody response. When the antibiotic was present only during the first 6 days of the culture, the antibody response was reduced by 90%.

The mechanisms by which steroids/antibiotics exert their effect on host antibodies appears to be a depression of antibody synthesis rather than increased antibody destruction. It also appears likely that the depression of synthesis is due to a depression of lymphoid tissue mass. Indeed, Harris, Harris, and Farber (284) have demon-strated no suppression of activity in remaining lymphoid tissue. However, Thomas (285) suggested that the depression of lymphoid tissue mass, antibody synthesis and fibroblastic proliferation represent a depression of the reticuloendothelial system per se. Thus, steroids appear to alter leukocytic response to injury due either to vascular change, direct suppression or deranged hormonal control. There is also a decreased immunologic response, probably as a result of diminished lymphoid mass.

The effect on phagacytosis was studied by Munoz and Geister (286). They found that chlortetracycline depressed in the in vitro phagocytosis of S. aureus by normal human leukocytes, the percentage of cells having phagocytosed the microorganism decreasing with increasing concentrations of the antibiotic. Donomae and Kawamori (287) gave evidence for in vivo antibiotic depression of phagocytosis by comparing the phagocytic rates of cells obtained from the ascitic fluid of mice injected intraperitoneally with Candida, with and without simultaneous injection of tetracycline. They found that the phagocytic rates of the tetracycline-treated mice was lower than those of the control infected group 12,24, and 48 hours after the injection. Takahashi, Tanaka, and Tanaka (288) verified Donomae's findings. They found that the phagocytosis by histocytes and monocytes from the subcutaneous tissues of rabbits given tetracycline orally was markedly decreased, as compared with that by cells from control groups.

The impairment of phagocytic activity may be responsible for the increased susceptibility to C. albicans infections, caused by corticosteroids. Mankowski (289) reported that cortisone and estradiol depress the leukocytic response of mice to C. albicans infections.

According to O'Grady Cotton and Thompson (290) cortisone may interfere with the intracellular destruction of the engulfed organism. Allison and Adcock (291) suggested that this impaired ability may be due to the derangement of metabolic activity of phagocytes by corticosteroids.

CHAPTER FIVE

MATERIALS AND METHODS

ORGANISMS:

Two strains of Candida albicans were used throughout this work:-
Candida albicans A.39 (The Boots Drug Co. Ltd., Nottingham) and
C. albicans I.M.I. 45348.

Stock cultures were maintained on agar slopes of Neurospora maintenance
medium, stored at $4^{o}C$ and were subcultured onto fresh medium every 3-5
weeks.

The germ-tube production method of Taschdjian, Burchill, Kozinn (292)
for the i.dentification of C. albicans was used on routine basis to
check for the purity of these cultures. An inoculum of approximately
10^{4} cells was added to 5 ml. sterile horse serum in a test tube. The
cultures were incubated statically at 37° for 3-5 hours and examined
microscopically for the formation of germ-tubes.

CHEMICALS AND ANTI-FUNGAL AGENTS:

Fisons Scientific, Loughborough, supplied Cholest - 5-en - 3β -
ol (Cholesterol).

B.D.H. Limited, Poole, supplied Ergosta - 5,7,22 - trien - 3β -
ol (Ergosterol), uranyl acetate and magdala red (phloxine, C.I. 45410).

FIG. 4 : The structure of the steroids used throughout the work.
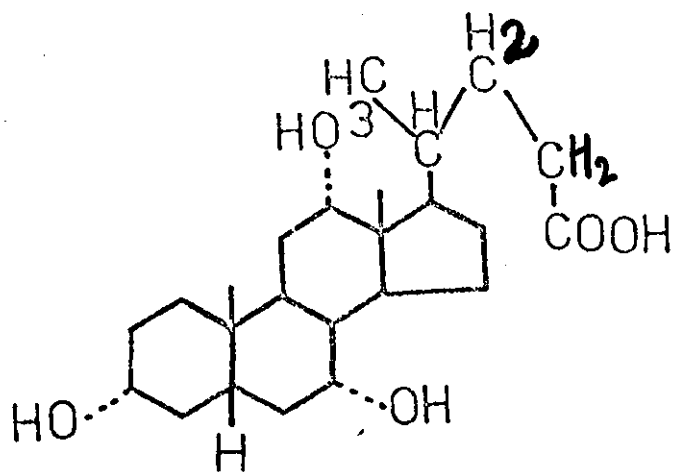
Cholic acid
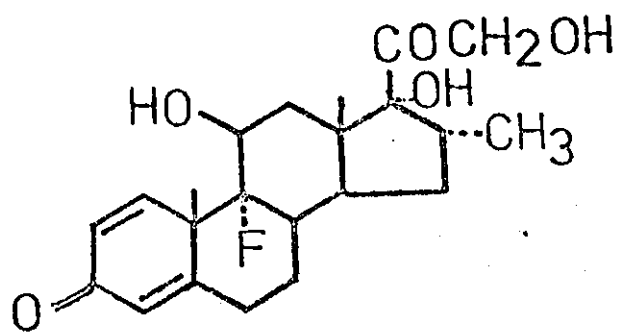
Dexamethasone
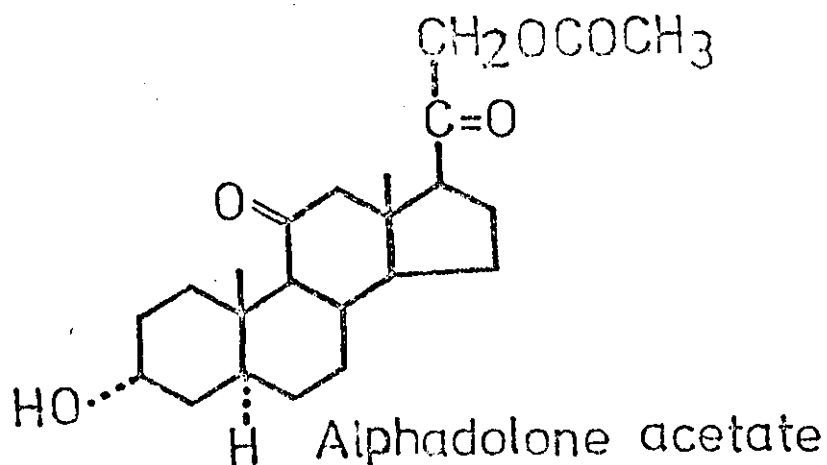
Alphadolone acetate

Sigma Chemical Co. Ltd., London, supplied $\Delta^{8,24}$ - Lanostadienol (Lanosterol) Calciferol (Ergocalciferol) Squalene (2,6,10,15,1923 - Hexamethyl - 2,6,10,14,18,22 - tetra cosahexene), Pregnenolane, 4 - Androstan - 3,11,17 - trione and Androstenolone.

Koch-Light Laboratories Limited, supplied 5α- Chlostan - 3 - β - ol, 5α- Cholestan - 3 - one, Trimethyl chlorosilane (pure), Hexamethyl - disilazane (H.M.D.S.) and N-Benzyl dimethylamine (pure).

Stigmasterol β- Sitosterol and Campesterol were a generous gift from Dr. M. S. Marriott (Cambridge University, Biochemistry Section, Sub-department of the Chemical Microbiology Department).

Nystatin (E. R. Squibb and Sons Ltd., Twickenham), Griseofulvin (May and Baker Ltd., Dagenham), Trichomycin (Fujisawas, Japan) and Amphotericin-B (Sigma Chemical Co. Ltd.).

Glaxo Laboratories, Middlesex, supplied 21-acetoxy - 3α- hydroxy - 5α- pregnane - 11,22 dione (Alphadolone acetate).

Merck, Sharp and Dohme Limited, Hoddesdon, supplied sodium 9α- Fluoro - 16α- methyl - prednisolone 21 - phosphate (Decadron).

MEDIA

The following media (g/l) were used and were sterilised at 121°C for 15 minutes (unless otherwise specified). When required, solidi-fied media were obtained by adding Oxoid No. 3 agar to give a final concentration of 2%. All media were prepared from Oxoid products.

Neurospora medium

Maltose	38g
Yeast extract	2.5g
Mycological peptone	8.0g
Malt extract	2.0g

pH adjusted to 5.0

Wolin - Bevis medium (Tween-medium)

Tween - 80	3 cm^3
Dextrose	0.25g
L-histidine HCL	0.25g
Ammonium sulphate	1.0 g
Monopotassium sulphate	1.0 g

pH adjusted to 6.0

Minimal Medium
(Without carbon source)

Ammonium sulphate	1.0 g
Monopotassium sulphate	1.0 g

pH adjusted to 6.0

Antibiotic Medium No. 1

Peptone	6.0 g
Tryptone	4.0 g
Yeast extract	3.0 g
Lab-Lemco powder	1.5 g
Dextrose	1.0 g
Phenol red	.025g

pH adjusted to 7.0

Mycelial medium (S.S.V.)[246]

Glucose	1.0 g
Starch	1.0 g
$(NH_4)_2 SO_4$	2.5 g
$KH_2 PO_4$	1.25g
$Ca\,Cl_2$	0.10g
$Mg\,SO_4\,7H_2O$	0.05g
$Mg\,Cl_2\,6H_2O$	0.16g
Vitamin solution*	10 ml/1

*Vitamin Solution

Biotin	0.01mg
Pyridoxin	2.0 mg
Calcium pantothenate	2.0 mg
Nicotinic acid	2.0 mg
Thiamin HCl	2.0 mg
Meso-inositol	10.0 mg

Sterilise by membrane filtration.

Magdala Red medium[293]

Ammonium sulphate	1.5 g
Potassium dihydrogen phosphate	1.5 g
Magnesium sulphate	1.0 g
Yeast extract	1.5 g
Peptone	1.5 g
Glucose	20.0 g

pH adjusted to 5.4

A solution of magdala red (1 mg/ml) was prepared separately and sterilised by heating at $100^{o}C$ for one hour. This solution was added to the above medium prior to pouring to give a final concentration of magdala red of 10 ppm.

Alternatively a solution of Eosin (15 mg/l), Trypanblue (15 mg/l) and tartrazine (10 mg/l) was used. This solution was treated in the same way as magdala red.

Chemically defined medium for yeast cell production[294]

	g/1.51
$(NH_4)_2 HPO_4$	3.75
$KH_2 PO_4$	3.0
$Mg SO_4 7H_2O$	1.5
Glucose	150.0
$Fe SO_4 7H_2O$	0.17
Biotin	0.00015

pH adjusted to 6.0

Biotin was sterilised separately by membrane filtration. Glucose was sterilised separately to avoid decomposition in the presence of phosphate(295).

Cultural growth of the organisms

To carry out the chemical analysis, large quantities of cells were grown. The yeast form of both strains (C. albicans A.39 and C. albicans I.M.I. 45348) was obtained by growth in the chemically defined media (page 63). Cells were harvested after 24 hours (stationary phase), washed three times with distilled water and freeze dried to be stored at $-20^{\circ}C$ prior to analysis.

Wolin—Bevis media was used for the production of a mixture of mycelial and yeast forms of C. albicans A39, whereas a predominantly mycelial form of C. albicans I.M.I. 45348 was produced by growing in S.S.V. media (page 61). The method of Yamaguchi (297) was used for getting relatively pure mycelial form (see below).

Growth Rates

The rate of growth of shake cultures was followed by determining the optical density at 420 nm (SP 500, Cambridge Instruments, Cambridge).

Viable Count

The method used in this work is based on that of Miles and Misra (296).

A modification of Yamaguchi's (297) method was used in this work to determine the proportions of yeast and mycelial phase growth. After incubation the cultures were concentrated from 1000 ml by centrifugation at 2000 r.p.m. for 10 minutes. The cells were washed with 20 ml of sterile distilled water, and then resuspended in 30 ml water. 2 ml portions were transferred onto aluminium planchets and dried to constant weight at $100^{\circ}C$ in hot air oven. Amount of growth was assessed by weighing the dried cell materials and expressed as gram dry weight of organism/liter culture.

Determination of proportions of yeast and mycelial phase growth

8ml portions of the concentrated cell suspension (stationary phase) were filtered through a 20μl metallic filter (Endecote Ltd.) which retained the filamentous cells. The filamentous cells were rinsed with water (c. 12), dried and weighed as above. The proportion of filamentous form in a given culture was calculated by dividing the weight of the filamentous cell fraction /ml by the total dry weight.

Extraction of Lipids

The method of Shaw and Dinglinger (298) was used for the extraction of total lipids. Cells were suspended in chloroform:methanol (2:1, V/V) and were shaken for 48 hours. Lipids were concentrated in vacuo at 37°C and were stored under nitrogen at -20°C prior to use.

Thin layer chromatography of lipids was performed using silica gel G (Merck Kiesllgelg (Type 60)) thin layer chromatograms (0.25 mm) which were developed in chloroform:methanol:water (65:25:4, V/V). Developed chromatograms were visualised using conc. sulphuric acid: glacial acetic acid (1:1, V/V). After the plates had been steamed for 30 seconds, they were placed in the oven at 160°C for 5 minutes.

STEROLS ANALYSIS

Extraction of Sterols

A modification of Fryberg et al (134) method was used. To 2 g. wet weight of cells, 15 g. KOH in 20 ml. distilled water and sufficient ethanol to give a total of 100 ml. were added. The solution was refluxed under nitrogen for 3 hours, diluted with an equal amount of water, and extracted with four equivalent volumes of heptane or ether. The solvent extract was washed with water (when ether was used, 5 ml. of methanol was added to break the emulsion that formed) dried over anhydrous $Na_2 SO_4$, and evaporated to give the crude sterol mixture.

The sterols were separated by thin layer chromatography using silica gel G (Merck Kieselgelg (Type 60)) thin layer chromatograms (0.25 mm.) which were developed in 40-60 pet ether:diethyl ether (3:1, v/v). The Lifschutz reagent (conc. sulphuric acid: glacial acetic acid, 1:1 v/v) was used to detect sterols as red or purple spots (299). Preparative thin layer chromatography was used to separate the crude sterols into their various components for identification using different spectroscopic methods (U.V. spectroscopy, mass-spectroscopy, I.R., and N.M.R.).

Crude sterols were separated by gas-liquid chromatography using a Pye series 104 chromatograph, fitted with polydimethylisiloxane (JXR) (Field Instruments, Richmond) and Se-30 columns (Phase Separations Ltd., Deeside).

The method of Vandenheuvel and Court (300) was followed in the preparation of the columns. Trimethylsilyl (TMS) derivatives were prepared as with Vandenheuvel et al (300). Up to 1 mg. of crude sterols were reacted in a glass-stoppered flask with 50μl. of hexamethyldisilazane and 50μl. of 10% trimethyl chlorosilane in chloroform (v/v), the reagents being added in that order. With larger amounts of crude sterols, correspondingly larger volumes of reagents were used. Brief mixing by stirring or vibration was applied after each addition; the top of the stopper was greased with silicone lubricant. The crude sterols were left in an evacuated desiccator over P_2O_5 for two hours to remove the moisture and any traces of solvent remaining after evaporation. This step was not necessary when standard compounds were involved.

The reaction mixture was left at room temperature for at least 4 hours. Excess solvent and reagents were removed using the method of Vandenheuvel, Hinderks, and Nixon, (301). CS_2 was then added to the flask contents. These, including residual ammonium chloride formed in the reaction, dissolved completely.

Individual sterols were identified by comparing their retention times with those of standards. When these were not available, tentative identification was made on the basis of data given by Vandenheuval and Court (300). The area under each component peak was calculated by multiplying peak height by the width at half the height, and expressed as a percentage of the total area (302).

FIG. 5 : Gas-liquid chromatogram on 3%

JXR   of trimethylsilyl (TMS)

derivatives of standard steroids.

Column temperature was 230°.

Stigmasterol

Ergosterol

Cholesterol

5 α-cholestan-3-one

Pregnenolone

Androstenolone
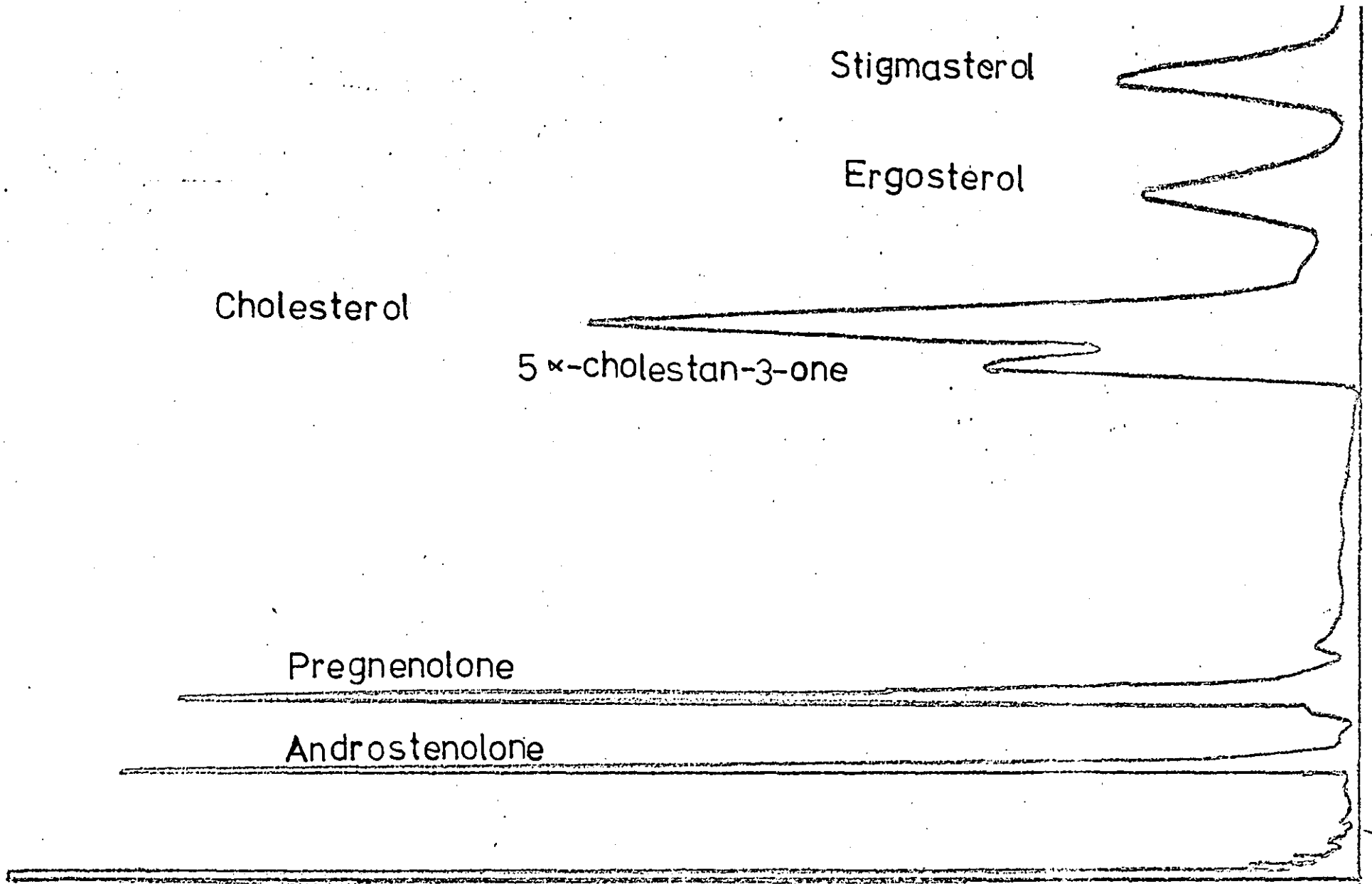
FIG. 6 : Relationship of width at half peak

height (mm), to retention time

(min.).
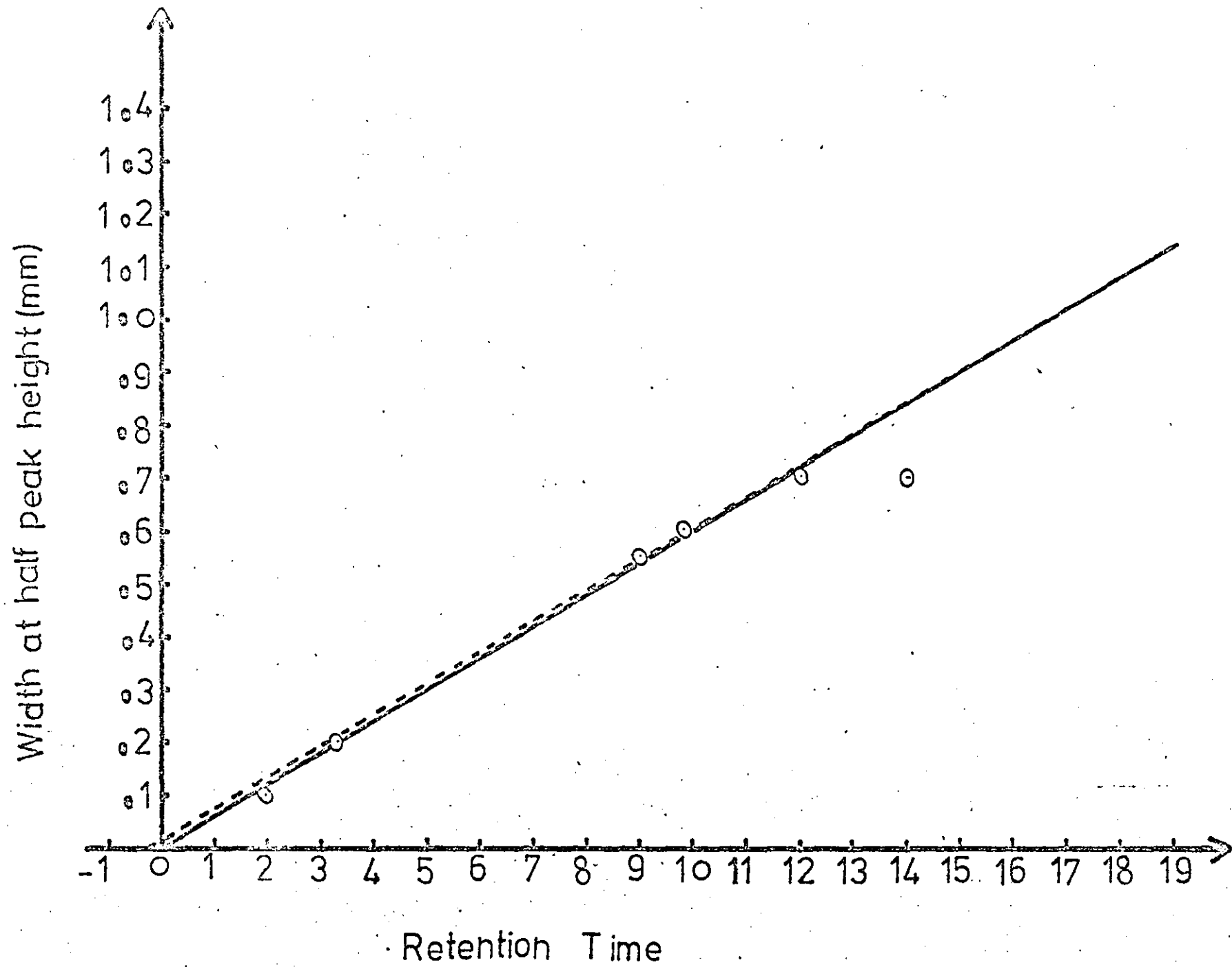
Overlapping peaks were resolved by constructing a graph of retention times versus peak width at half height (Fig. 6), which gave a straight line (303). By knowing the retention time of an overlapping peak, the width at half height could be found.

DETERMINATION OF RESPIRATION COMPETENCE

The variation in respiration competence of the fungi when cultured under conditions such as steroid supplementation of media which could influence lipid composition, was followed by the method of Horn and Wilkie (306). C. albicans was cultured on Magdala Red medium containing a range of steroids from .025% to .15%.

Coloured photographs for colour differentiation were obtained with Kodachrom - X (Kodak).

Respiration Studies

Studies on respiration were carried out using standard manometric techniques (304). Cells grown for 24 hours in neurospora medium containing no steroids and others containing steroids were harvested and washed three times with physiological saline and suspended in .2 M phosphate buffer (pH 6.0). The flasks contained a total of 2.5 ml of the fluid; the body of the cell held .5 ml of 10 mM of glucose and 1.5 ml of buffer containing steroid in amount appropriate to give the desired concentrations. A suspension of the organisms containing 60 mg of cells was added to the side arm. 0.2 ml amount of 30% potassium hydroxide was placed into the well.

Effect on Mitochondria

The method of Beck and Greenwalt (305) for determination of the number of mitochondria per cell was followed. The number of mitochondria in thin sections (T.E.M.) of the prepared material were counted. A minimum of 20 cells, representing between 125-200 mitochondria profiles, were examined for each sample and the ratio of mitochondria per cell profile were calculated.

Another way of evaluating the effect on mitochondria was by tracing over between 25-30 mitochondrial profiles cutting out and weighing them for comparison.

Determination of Minimum Inhibitory Concentration (M.I.C.) Values

Three polyene antibiotics were used: nystatin, amphotericin B, and trichomycin which were added in N,N - Dimethyl formamide: Methanol (1:1, v/v). The stock solution was prepared by dissolving the antibiotic in the above mentioned solvent mixture to a concentration of 1250 mg/ml and storing it at $-20^{\circ}C$ prior to use.

The instability of the polyenes prevented autoclaving and sterilisation was done using Sartorious-membrane filter (GmbH) which stands this organic solvent mixture. The addition of the small quantities of this solvent mixture necessary to achieve the desired concentration did not have any effect on either the growth or viability of C. albicans.

The M.I.C. values were determined in antibiotic medium No. 1
(page 61) using a tube dilution method. As soon as adequate growth
has occurred in the blank tubes, growth in the other tubes was
evaluated by colour change and turbidity. The M.I.C. is indicated
by that tube of the dilution series containing the lowest concentration
of antibiotic which does not permit growth of the test organisms.

Positive control containing organism, broth without antibiotic to check
for viability of organisms and negative controls containing broth with
anti-fungal agents with no organism to check for sterility of drug were
prepared.

MICROSCOPY

Light Microscopy

The routine examination of samples and phase contrast micro-
scopy was carried out using a Vickers M25 Series microscope. Dried
and fixed films were stained with the following:

Burdon's Sudan Black Lipid Stain

Sudan black B (0.3g.) was dissolved in 70% ethanol (100 ml.)
shaken thoroughly at repeated intervals and allowed to stand over-
night. The slides were covered entirely with Sudan black stain and
left at room temperature for 15 minutes. Excess stain was removed,
air dried, rinsed thoroughly with xylene and dried. Counter-staining
was done using safranine (0.5%). The slides were washed with tap water
and dried by blotting.

Methylene Blue Stain

Loeffler's methylene blue was used. Saturated solution of methylene blue in alcohol (300 ml.) was added to KOH (0.01%) in water (1000 ml.). The slides were flooded with methylene blue for 40 seconds, washed thoroughly with tap water and blotted dry.

Light micrographs were obtained using a Leitz photoplan. Photographs were obtained using FP4 film (Ilford).

Scanning Electron Microscopy

Scanning electron microscopy was carried out by the technique of Bulman and Stretton (307). Glutaraldehyde (G. T. Gurr, Bucks) was added to broth cultures of the strains of cell suspensions to give a final concentration of 1.5% (w/v). After 2 minutes contact, the cells were removed by centrifugation at 3,000 xg at $4^{\circ}C$, for 15 minutes and re-suspended in 2 ml. of glutaraldehyde (5% w/v) for 16 h at $4^{\circ}C$. The cells were then removed by centrifugation (3,000 xg) for 15 minutes, washed three times with distilled water and re-suspended to give the required density. One drop of this suspension was allowed to air-dry on a microscope coverslip and then dehydrated over $CaCl_2$ (anhydrous) under a partial vacuum. The samples were then coated with gold-palladium in a high vacuum unit to obtain a coating of approximately 10 nm thickness. These samples were examined in a Cambridge Stereoscan (Mark IIA Cambridge Instruments Co. Ltd., Cambridge) with a beam angle of 45°, using a voltage of 30 Kv. Photographs were obtained using FP4 film (Ilford).

Transmission Electron Microscopy

Araldite Mixture

This araldite mixture was used for all preparations except when stated. It consisted of the following components:-

Araldite (Polaron Equip. Ltd. Watford) CY 212   27 ml.

Hardener (Polaron Equip. Ltd., Watford) HY 964   23 ml.

Accelerator (N-Benzyl dimethylamine)   1 ml.

All liquids are viscous and mixing must be thorough. Because the volume of accelerator was critical, a clean pipette was used and so washed out several times with dibutylphthalate. The mixture was warmed to 50° and shaken several times before use.

Epon Mixture - Based on Luft's (308) formula

Components

1. Resin-Epon 812

2. Hardener (1) Dodecenyl succinic anhydride (D.D.S.A.)

3. Hardener (2) Methyl-endomethylenephthalic anhydride (M.N.A.)

4. Accelerator Tri-dimethylamine-methyl-phenol (D.M.P.-30).

Procedures

Two different anhydrides are employed as hardeners, their proportion in the final mixture determines the hardness of the final block.

Two stock solutions are prepared:-

Mixture A	To Make 100 g.
Epon 812	26g
Dodecenylsuccinic anhydride	41g

Mixture B	
Epon 812	18g
Methyl-endomethylenephthalic anhydride	15g

The mixture can be stored in the refrigerator for about 2 months. A and B mixtures in a ratio of 7:3 are usually employed for blocks of medium hardness. Immediately before use, the reaction accelerator, tri-dimethylamine-methyl-phenol is added to a final concentration of 1.5-2%. Exact weight should be used to ensure reproducible hardness. Mixing of the materials is also important.

Buffers

Two buffers were used in association with transmission electron microscopy. These were:-

1. Acetate-Veronal buffer of Michaelis.
2. Cacodylate-HCl buffer.

Acetate Veronal Buffer

Basic mixture:-

Sodium acetate	19.43g	9.71g
Barbitone Sodium ,	29.43g	14.71 g
Sodium Chloride	34.0 g	17.0 g
Distilled water to	1 litre	500 ml.

Mix 5.0 ml. of the basic mixture with 7.0 ml. N/10 HCl, 13.0 ml. distilled water and 0.25 ml. M Ca Cl$_2$. pH6.1.

Cacodylate - HCl. (0.2M) (309)

Solutions:

A.	Sodium Cacodylate	
	$(Na(CH_3)_2AsO_2.3H_2O)$	42.8g
	Distilled water	1,000 ml.
B.	(0.2M HCl)	
	Conc. HCl (36 to 38%)	10 ml.
	Distilled water	603 ml.

The desired pH can be obtained by adding solution B as shown below to 50 ml. of Solution A and diluting to a total volume of 200 mls.

Solution B (ml.)	pH of Buffer
18.3	6.4
13.3	6.6
9.3	6.8
6.3	7.0
4.2	7.2
2.7	7.4

These buffers were used for the preparation of fixatives, washing fluids and 2 per cent agar solution.

During the course of this work, the following techniques were used:-

I. Single Fixative Technique Method

Mix 15 ml. of suspension with 1.5 ml. of fixative and centrifuge immediately at 4,000 r.p.m. for 5 minutes. The cells were re-suspended in 1 ml. of fixative for 2-4 hours at 0-5°C. The suspension of cells in fixative was diluted with 8 ml. of veronal-acetate buffer, and centrifuged at 4,000 r.p.m. for 5 minutes. The pellets of C. albicans were re-suspended in approximately 0.03 ml. of agar, mixed well, placed on microscope slides, allowed to cool and set. These were cut into small cubes (0.5-1 mm^3), which were treated for 15-120 minutes in uranyl acetate solution. The cubes were dehydrated using a graded ethanol series. These were then transferred to xylene (link reagent between ethanol and araldite and slowly impregnated through a series of araldite/xylene proportion. The final transfer was to 100% araldite mixture in capsules and placed in an oven at 60°C for 48 hours.

The cubes were then embedded in fresh Araldite II and polymerized at 50°C for 48 hours.

Araldite I

Araldite Cy 212	10 ml.
Hardener HY 964	10 ml.

Araldite II

· This consisted of Araldite I plus 2 per cent accelerator tridimethylamino-methyl phenol (DY 064).

Primary and Secondary Fixatives Method

1.5 ml. of 2% glutaraldehyde were added to 15 ml. of cells suspension for 1 minute and centrifuged. The cells were washed three times in distilled water, then fixed in 2.5% glutaraldehyde in sodium-cacodylate buffer at 4°C for 15 minutes, then post fixed in 1% OsO_4 in buffer for 1.5 hours at 4°C. They were washed again in the same buffer for 30 minutes. The pellets were suspended in 2% agar and stained for 2 minutes in methylene blue. The agar was cut into small cubes (0.5 - 1 mm.^{3}) which were dehydrated through an ethanol series.

The cubes were washed twice in propylene oxide, each for twenty minutes. These were replaced with propylene oxide : Epon 812 (1:1, v/v) and left overnight. These were then replaced with Epon 812 for 4 hours and embedded in fresh Epon hardener for 2-3 days at 50°C.

II. Durcupan - Araldite Schedule

The method of Staubli (310) for Durcupan dehydration was tried using araldite as the embedding media. After using the same fixatives, agar embedding and washing technique as in the single fixative technique method, agar cubes were placed in Durcupan resin in set dilutions:-

Distilled Water (Per Cent)	Durcupan (Per Cent)	Time (Mins.)
50	50	15-30
30	70	15-30
10	90	15-30
-	100	30-60
-	100	30-60

The cells, water free, were processed through Durcupan/Araldite mixtures.

Durcupan (Per Cent)	Araldite I (Per Cent)	Araldite II (Per Cent)	Time (at 50°C)
70	30	-	1 hour
50	50	-	1 hour
30	70	-	several hours or overnight
-	100	-	1 hour
-	-	100	3 changes of 30 mins. each.

IV. Modification of Davison and Garland (311) Method

Cells were fixed with 2.5% (v/v) glutaraldehyde in .1 M sodium-
cacodylate buffer, pH 7.0, at 4°C for 2 hours. Cells were placed
in freshly made 2% (w/v) $KMnO_4$ solution at 4°C for 2 hours. The
cells were centrifuged at 4,000 r.p.m. for 5 minutes and placed in
a fresh solution of $KMnO_4$ for 2 hours. They were washed five times
with distilled water. The cells were then placed in a solution
containing 1% (w/v) potassium dichromate and 1% (w/v) uranyl acetate
for 2 hours at 4°C. These were washed several times with distilled
water and embedded in agar, left to set and cut into small cubes
(.5 - 1 mm^3.) which were dehydrated through an ethanol series. The
100% ethanol was replaced with propylene oxide twice for 20 minutes
and embedded in araldite by graded impregnation.

V. Modification of Gealt, Sheir-neiss and Morris (312) Method

Cells were fixed with 1.5% (w/v) p-formaldehyde and 1% (v/v)
glutaraldehyde in veronal - acetate buffer. The pellets were washed
with distilled water and post-fixed with 1% (w/v) osmium tetroxide
in Veronal-acetate buffer, pH 6.2. The cells were embedded in agar
(2%) then cut into cubes and washed with 0.5% (w/v) uranyl acetate
(Tabb Laboratories, Reading) in the same buffer. The cubes were
dehydrated through a series of graded ethanol and embedded in Epon.

All samples are embedded in beam capsules (Taab Laboratories,
Reading), sectioned using Cambridge Huxley microtome with glass knives
(angle 55°) and collected in a water trough using New 200 Athene
grids.

Sections were post-stained with uranyl acetate (dissolved in 70%

ethanol) for 20 minutes and lead citrate (Taab Laboratories, Reading),

(.04%) in solution with sodium hydroxide, 0.IN, carbonate free for

5 minutes. Extensive surface washing was done, first with sodium

hydroxide, carbonate free and then with distilled water. These were

dried using millipore filters. Electron micrographs were recorded

with an AEI EM 6G electron microsope operated at 60 or 80 Kv. accelera-

ting voltage. Photographs were taken on EM4 electron microscope square

plates $3\frac{1}{4}$ x $3\frac{1}{4}$ (Ilford).

CHAPTER SIX

FIG. 7 : The dry weight of C. albicans

cultivated in media containing

different concentrations of

steroids:-

cholic acid (O-O), decadron

(Δ——Δ), and alphadolone acetate

($\bullet$ - $\bullet$) on the 24 h. of cultivation.
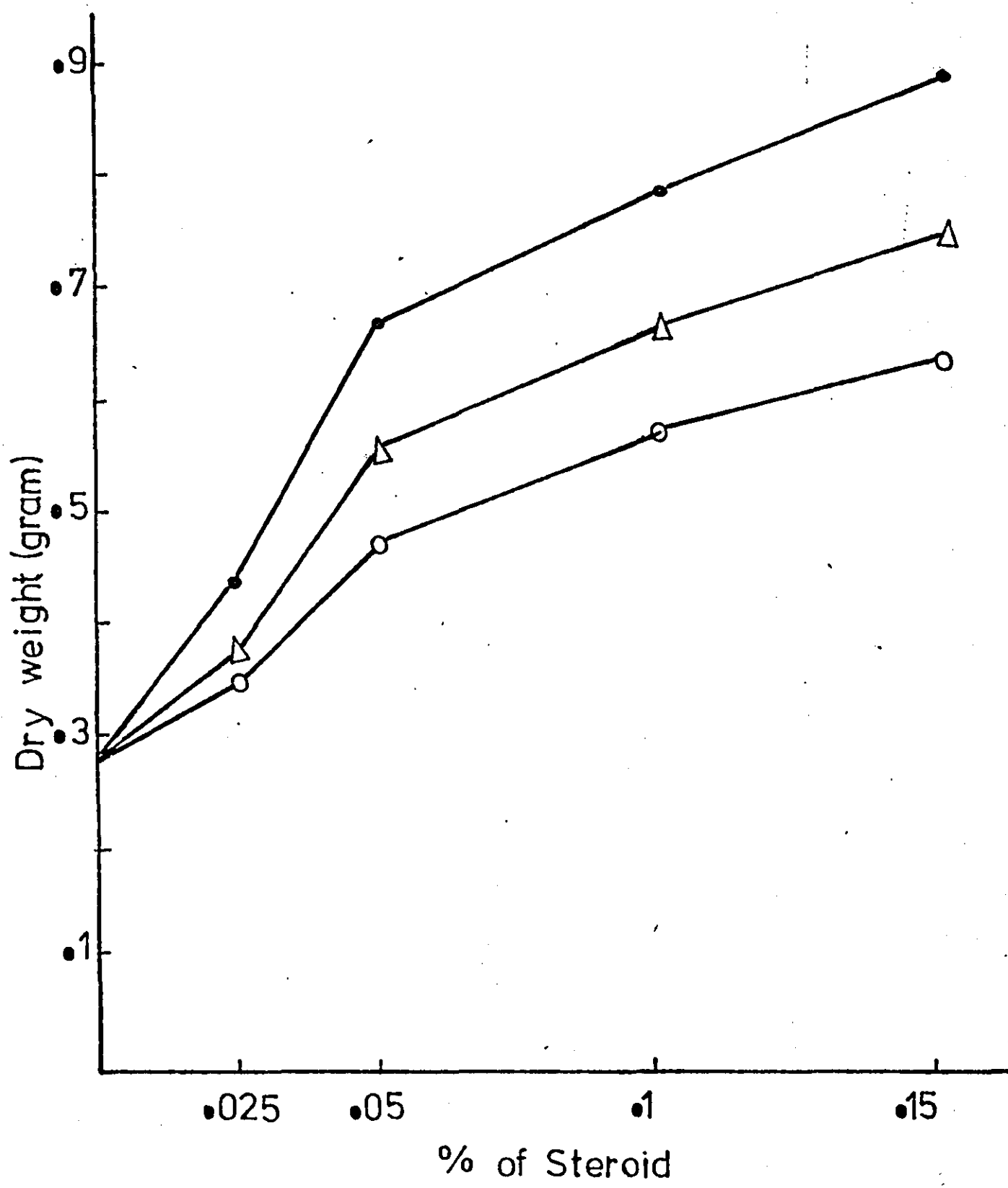
FIG. 8 : Growth curves of C. albicans grown

at 37°C as a shake culture in a

minimal medium containing:- glucose

(● - ●), 0.15% cholic acid salt (O - O),

and 0.15% cholic acid salt plus glucose

(x - x).
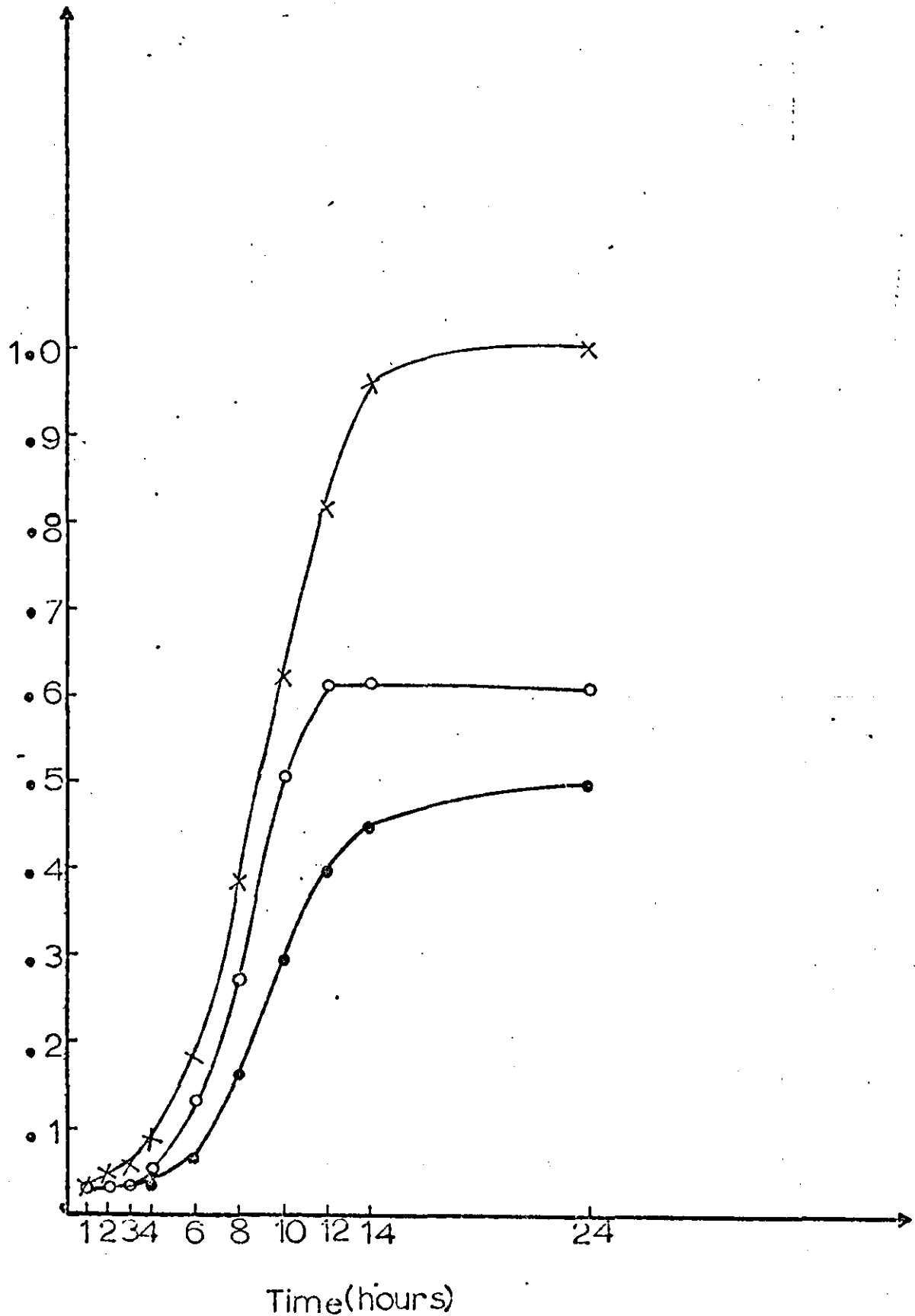
FIG. 9 : Growth curves of C. albicans grown
at 37°C as a shake culture in a
minimal medium containing:- glucose
(□ - □), 0.15% cholic acid salt
(0 - 0), and acetate (Δ - Δ).
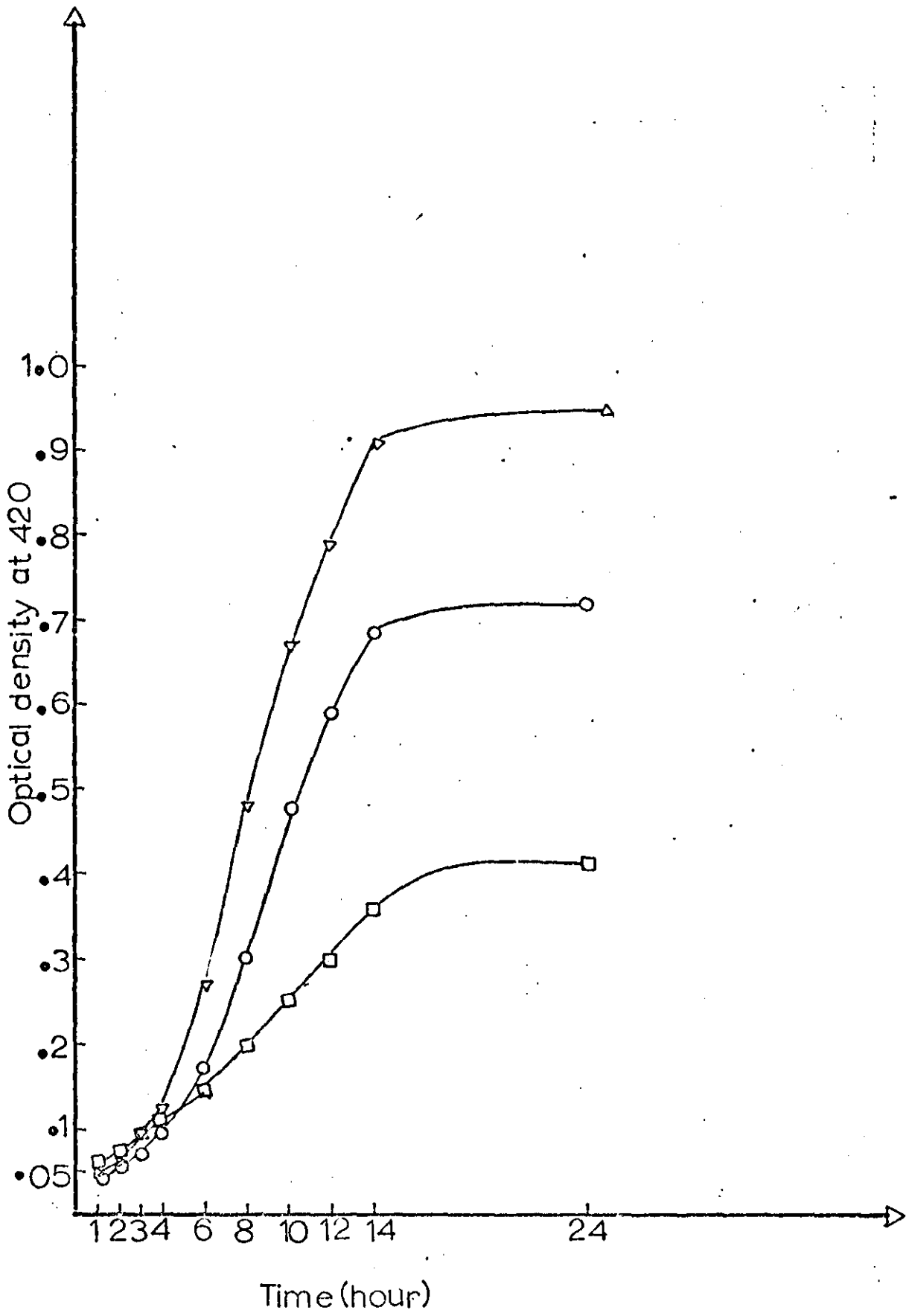
FIG. 10 : Log viable count versus time of C. albicans grown at 37°C as a shake culture in a minimal medium containing:-

glucose (0 - 0), 0.15% cholic acid salt (x - x), and acetate (Δ - Δ).
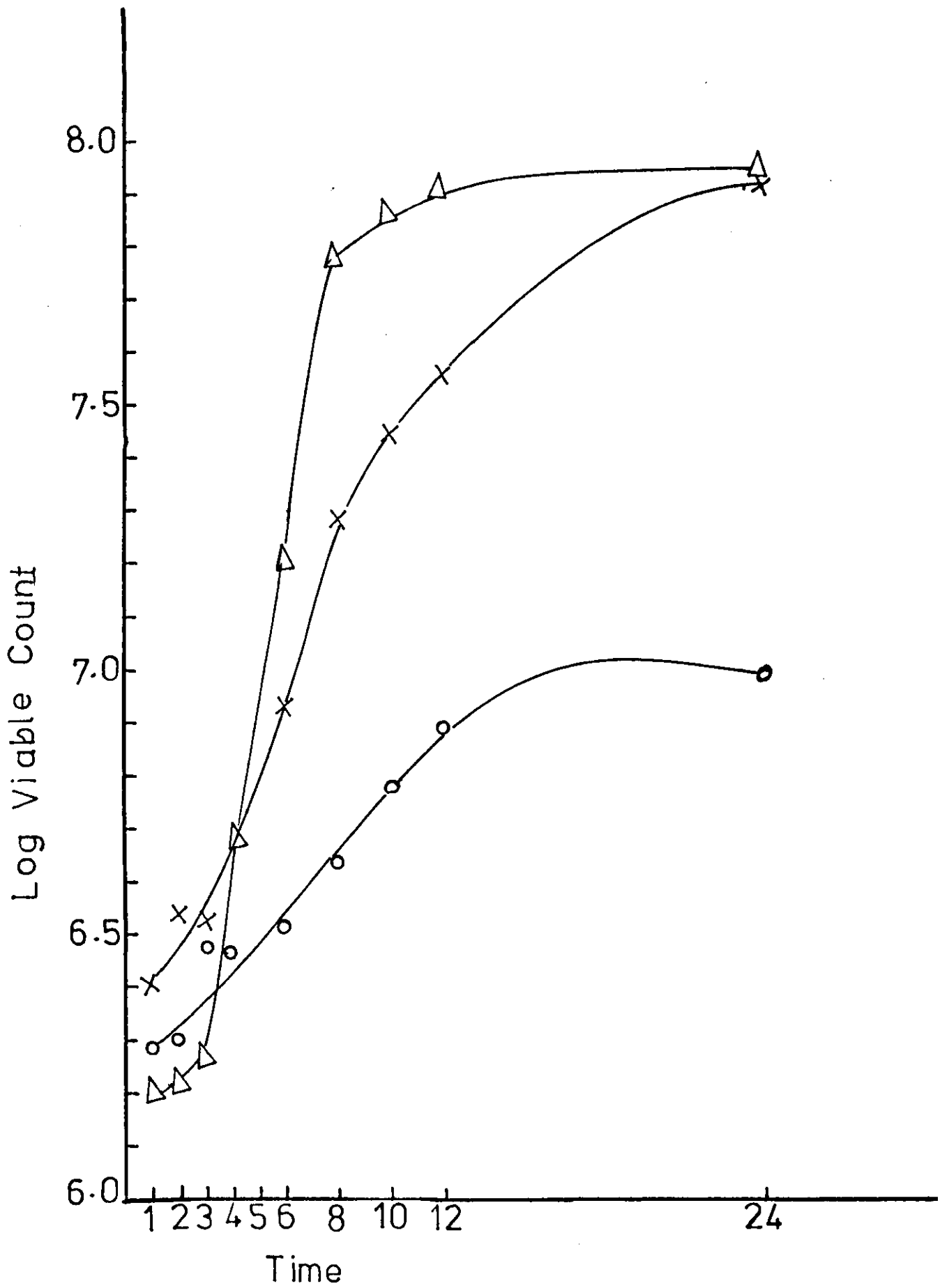
FIG. 11 :   The effect of steroids on the percen-

tage of mycelial to yeast form. Medium

containing: no steroids (----), 0.15%

cholic acid salt (● - ●), 0.05% decadron

(0 - 0), and 0.05% alphadolone acetate

(x - x).
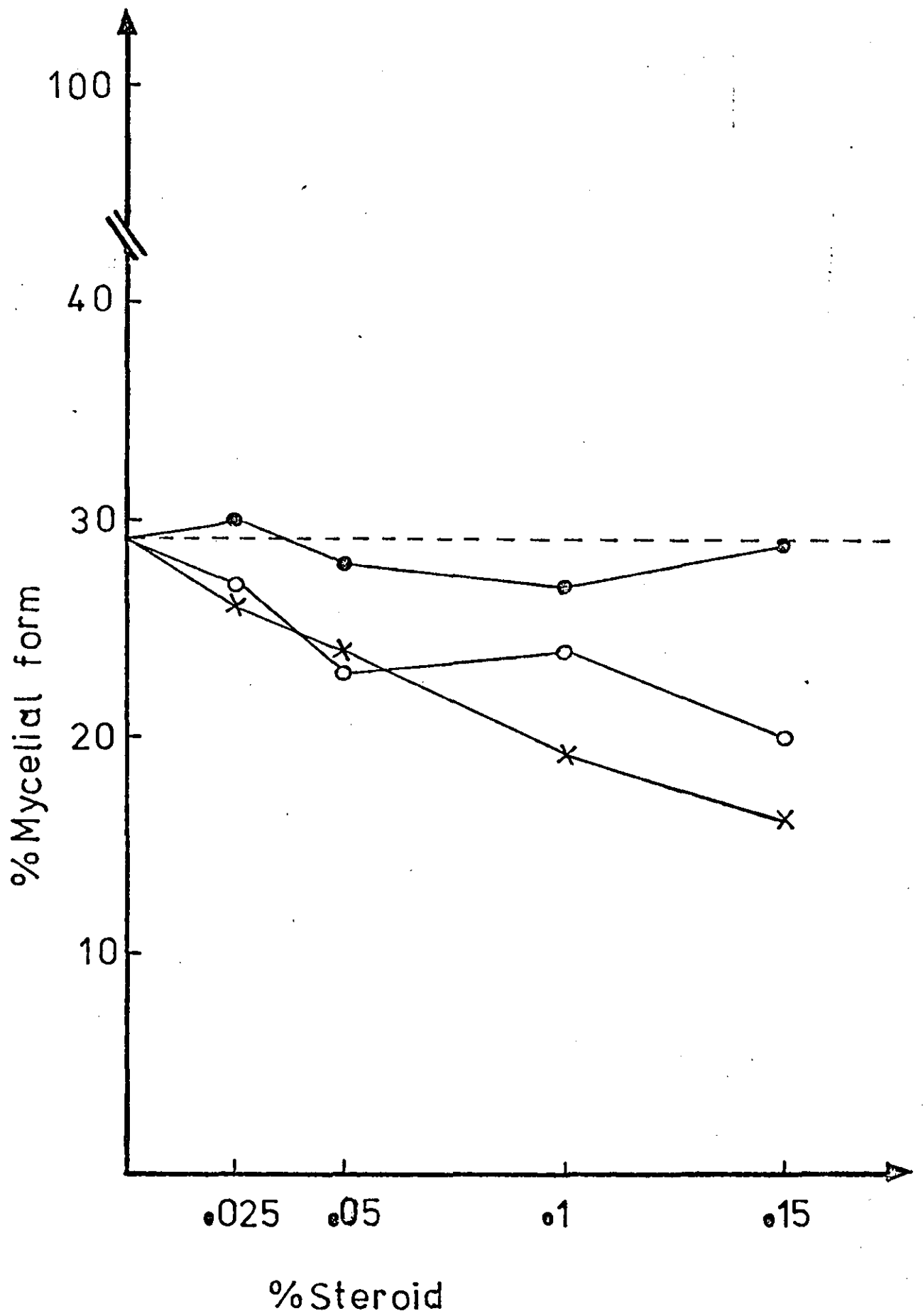
EFFECT ON GROWTH

As the concentration of the steroids in the media was raised
C. albicans showed progressive increase in growth rates as shown
by dry weight studies. This was more noted in the range of 0.05%
to 0.15% of the steroids. Under similar experimental conditions,
alphadolone acetate showed higher enhancement in growth rate,
followed by decadron and the least was cholic acid salt (Fig. 7).
When the growth rate was estimated by following the O.D. at 420
nm. and viable count the same trends were observed. (Fig. 10).

Cholic acid salt was shown to be a better carbon source than
glucose (Fig. 8) whilst acetate was consumed even faster than cholic
acid salt (Fig. 9).

The medium for the maximum production of mycelial form varied
with the strain C. albicans A39 gave 28% mycelial form when grown
in Wolin Bevis medium but produced very low percentage when grown
in Marriotts (246) medium (Page 61). However, C. albicans IMI 45348
produced 70% mycelial form when grown in Marriotts medium and very
low percentage (<5%) of mycelial form when grown in Wolin Bevis
medium.

As the concentration of steroids increased the percentage of
mycelial form in the culture decreased (Fig. 11). This decrease was
most prominent with alphadolone acetate followed by decadron and the
least was cholic acid salt.

EFFECT ON MORPHOLOGY

Yeast Form

Scanning electron microscopy studies showed two different types
of cells when steroids were added to the growth medium, both were
different from the control cells (no steroid) (Plate 6). One type
were larger and more elongated with a smooth surface, the other were
smaller and rougher.

This phenomenon appeared at concentrations as low as 0.025% of
the steroids (Plates 7, 11 and 15). The three steroids: cholic acid
salt, decadron and alphadolone acetate showed similar affects but to a
varying degree. At low concentrations of alphadolone acetate, the
majority of cells were of the small and rough type although some
elongate smooth cells were present (Plate 15). Whereas at high con-
centration more smooth elongated cells were observed (Plate 17).

The response to steroids addition was less with decadron (Plates
11 - 14) and even less with cholic acid salt (Plates 7 - 10).

When examining the effect of added steroids on the morphology
of C. albicans the major problem was to preserve the ultra structure
in untreated cells. Several different techniques were examined
before a method was found. The single fixative technique (glutaraldehyde)
method (page 75) (Plate 18) preserved the interior of the cell where
the expected intra cellular structures were observed. However, sharp
contrast partially obscured the cytoplasmic organelles.

The disadvantage of this method was in preserving the cell wall which was manifested by a weakening of the boundary between the wall and cell membrane. Other fixatives, Osmium tetroxide, potassium permanganate or p-formaldehyde were not noticeably better than glutaraldehyde. In all cases, cytoplasmic organelles were likewise partially obscured.

When the method of durcupan dehydration followed by araldite embedding was used (Page 77) this proved to be completely unsatisfactory (Plate 19). Here the major disadvantage was that it was very difficult to obtain good sections.

The material was elastic with different areas of hardness causing rippling and piling up. Furthermore, on examining electron micrographs of such sections it was noted that there was poor impregnation of the embedding material.

More satisfactory results were obtained with a primary and secondary fixative method (Page 78). The cytoplasmic organelles were prominent and well preserved, particularly the nucleus containing the nucleolus and chromatin (Plate 20). The only disadvantage with this method was that the cytoplasm became densely stained with scattered electron thin areas. Also, modification of the method of Gealt et al (312) (Page 76) gave similar results but had the disadvantage that the cell wall (Plate 21) was poorly defined.

The method which gave optimum results and was used throughout was a modification of the technique of Davison et al (311) (Page 78). With this technique all the cytoplasmic organelles could be seen, the cell membrane appeared as a three layered structure, the cell wall structure showed two electron dense boundaries separated by an electron thin zone in the middle (Plate 22) and the mitochondria exhibited cristae (Plate 23). Permanganate fixation proved to be the best for membranous structures.

Using transmission electron microscopy two different types of yeast cells were observed. Large cells with smooth envelopes containing distinct intractoplasmatic organelles, and small cells with convaluted envelopes and obscure internal structures.

In the presence of cholic acid salt large cells with well preserved organelles were obtained (Plates 25 and 27). Cell division was detected especially at high steroid concentration (Plate 31). The small cells showed a convoluted cell envelope (Plate 28) and electron dense areas with scattered electron thin areas where intra cytoplasmatic organelles and plasmalemma can no longer be identified (Plates 26, 28 and 30).

The addition of alphadolone acetate and decadron had similar affects to cholic acid. Large cells were present with the intra cytoplasmatic organelles and plasmalemma well identified (Plates 33, 35 and 39). Cells were actively involved in cell division (Plates 36 and 41). Whereas the small cells had a disorganised cytoplasm (Plates 34 and 37) with numerous lytic and electron dense areas with scattered electron thin areas (Plates 37 and 40) and a disintegrating cell wall (Plate 34).

Plate 3   S.E.M. of C. albicans, A39, grown as a shake

culture in synthetic medium. Cells were grown

for 8 hours at 37°C. Micrograph shows yeast

form cells' .x 10,000.

Plate 4   S.E.M. of C. albicans, A39, grown as a shake

culture in synthetic medium. Cells were grown

for 5 hours at 37°C. The micrograph shows yeast

form cells .x 10,000.
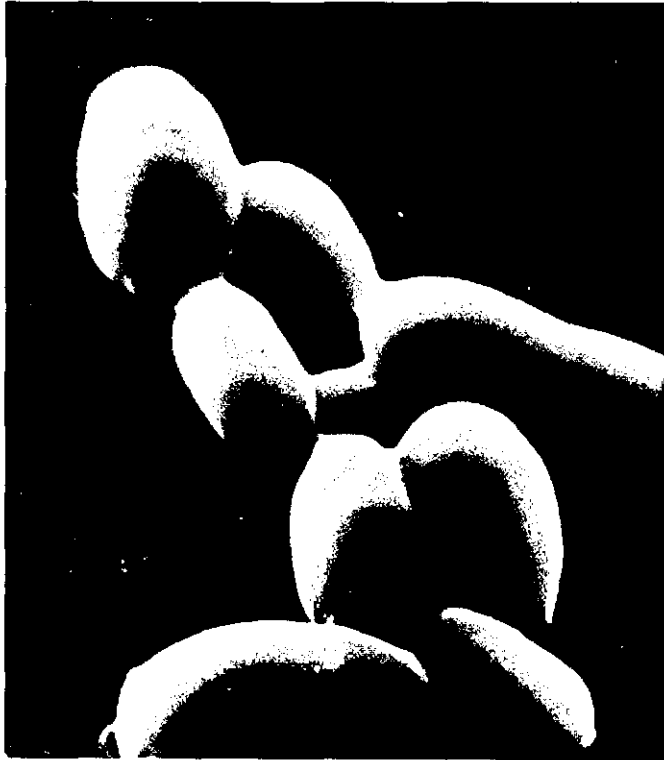
Plate 5   S.E.M. of C. albicans, A39, grown as a shake culture

in synthetic medium. Cells were grown for 12 hours

at 37°C. Micrograph shows yeast form and budding

cells .x 5,000.

Plate 6   S.E.M. of C. albicans, A39, grown as a shake culture

in synthetic medium. Cells were grown for 24 hours

at 37°C. Micrograph shows yeast form and budding

cells. .x 10,000.

Plate 7   S.E.M. of $\underline{C}$. $\underline{albicans}$, A39, grown as a shake culture

in synthetic medium containing 0.025% cholic acid

salt. Cells were grown for 24 hours at 37°C.

Micrograph shows both elongated smooth and rough

surface yeast cells. .x 10,000.

Plate 8   S.E.M. of $\underline{C}$. $\underline{albicans}$, A39, grown as a shake culture

in synthetic medium containing 0.05% cholic acid

salt. Cells were grown for 24 hours at 37°C.

Micrograph shows cells with a rough surface and

budding yeast cells. .x 10,000.
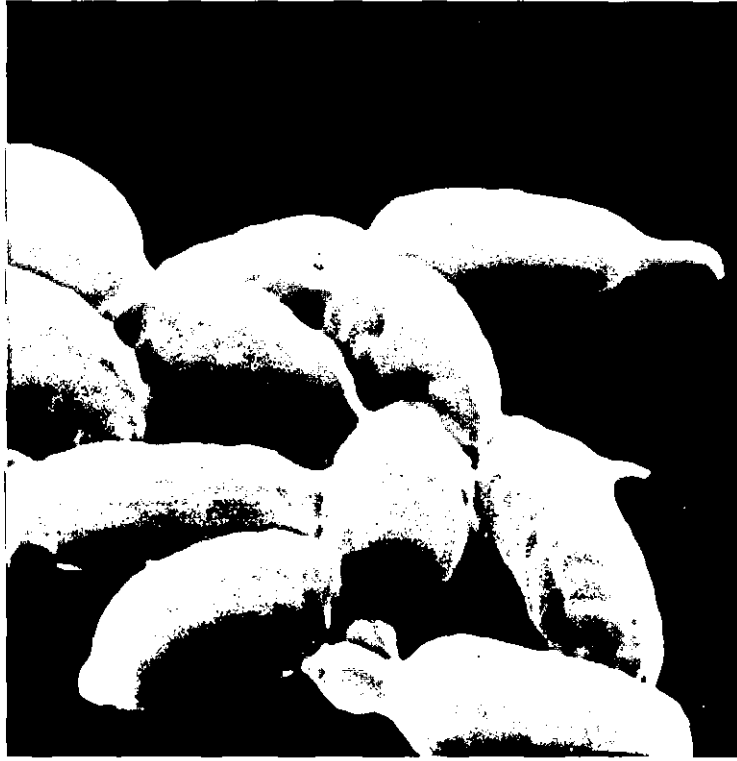
Plate 9   S.E.M. of C. albicans, A39, grown as a shake culture

in synthetic medium containing 0.01% cholic acid

salt. The cells were grown for 24 hours at 37°C.

Micrograph shows two types of cells elongated

smooth and small rough cells. .x 10,000.

Plate 10   S.E.M. of C. albicans, A39, grown as a shake culture

in synthetic medium containing 0.15% cholic acid

salt. The cells were grown for 24 hours at 37°C.

Micrograph shows two types of yeast cells elongated

smooth and small rough cells. .x 10,000.
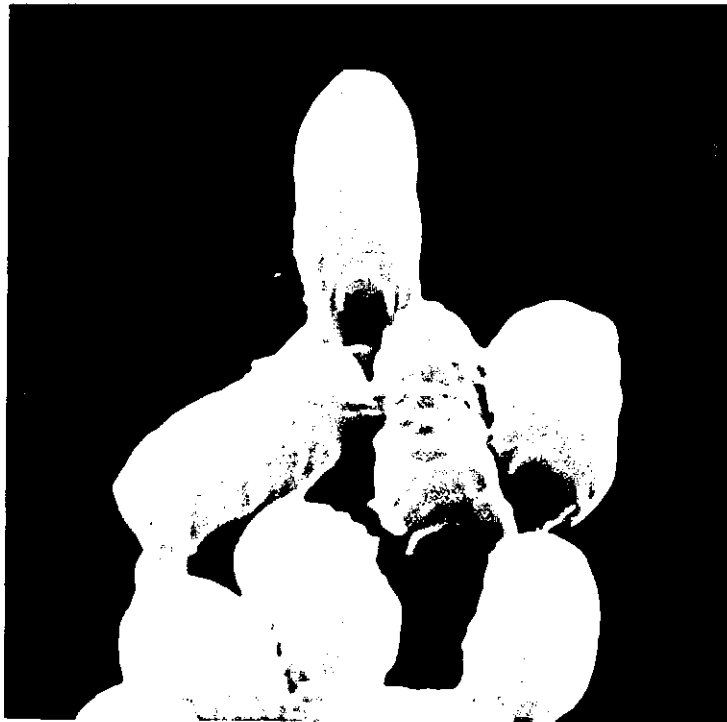
Plate 11   S.E.M. of C. albicans, A39, grown as a shake culture

in synthetic medium containing 0.025% decadron. Cells

were grown for 24 hours at 37°C. Micrograph shows

smooth elongated yeast cells. .x 10,000.

Plate 12   S.E.M. of C. albicans, A39, grown as a shake culture

in synthetic medium containing 0.05% Decadron. Cells

were grown for 24 hours at 37°C. Micrograph shows

elongated yeast cells. .x 10,000.
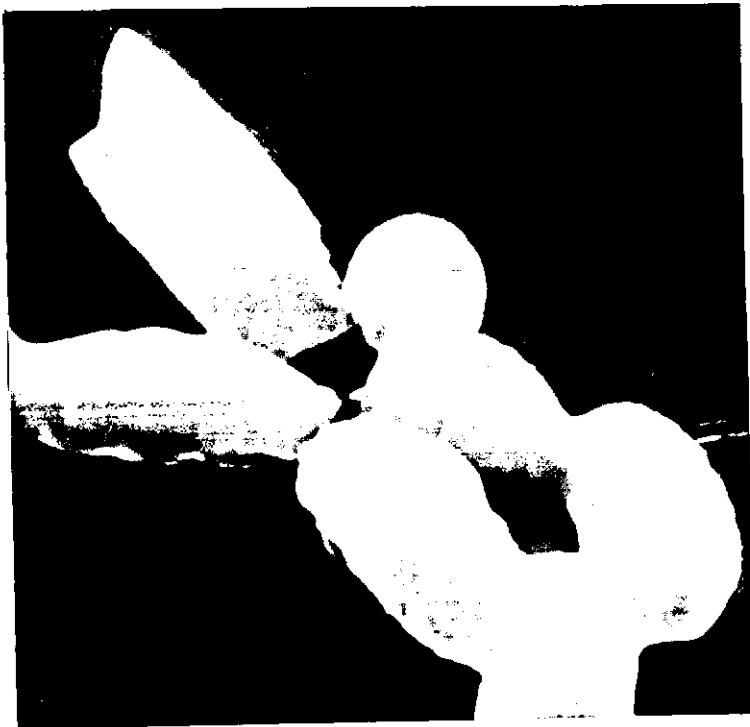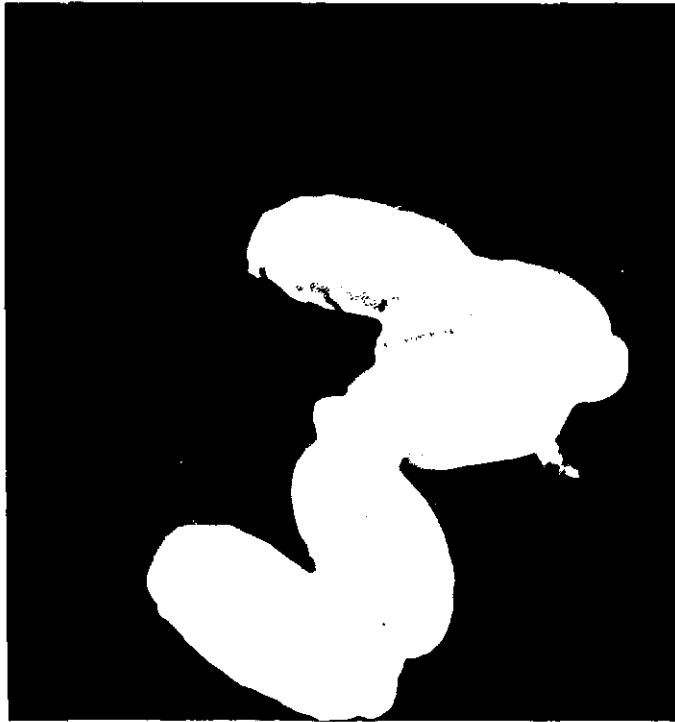
Plate 13   S.E.M. of C. albicans, A39, grown as a shake culture in

symthetic medium containing 0.1% decadron. Cells were

grown for 24 hours, at 37°C. Micrograph shows elongated

rough yeast cells. .x 10,000.

Plate 14   S.E.M. of C. albicans, A39, grown as a shake culture

in synthetic medium containing 0.15% decadron. Cells

were grown for 24 hours at 37°C. Micrograph shows

elongated yeast cells. .x 10,000.

Plate 15   S.E.M. of C. albicans, A39, grown as a shake culture
            in synthetic medium containing 0.025% alphadolone
            acetate. Cells were grown for 24 hours at 37°C.
            Micrograph shows small rough yeast cells .x 10,000.

Plate 16   S.E.M. of C. albicans, A39, grown as a shake culture
            in synthetic medium containing 0.05% alphadolone ace-
            tate. Cells were grown for 24 hours at 37°C.
            Micrograph shows elongated yeast cells with either
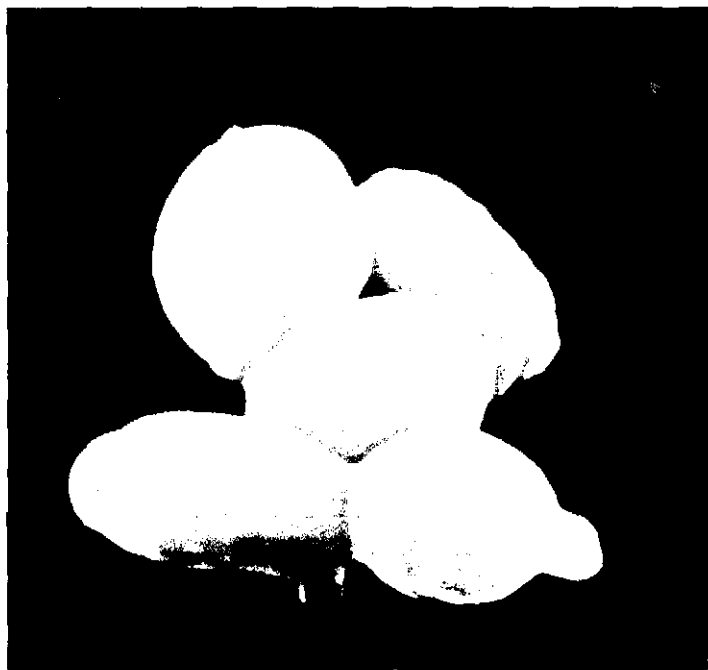   smooth or rough surfaces. .x 10,000.

Plate 17   S.E.M. of C. albicans A38 grown as a shake

culture in synthetic medium containing 0.1%

alphadolone acetate. Cells were grown for

24 hours at 37°C. Micrograph shows elongated

yeast cells with either smooth or rough sur-

faces. .x 10,000.

Plate 18   Electron micrograph of a section of <u>C</u>. <u>albicans</u>

fixed by glutaroldehyde using the single fixative

technique method. Cells were grown for 24 hours

at 37°C. Micrograph shows poor embedding, electron

dense areas, and electron thin areas. The nucleus,

nuclear membrane and nucleolus are distinctly demon-

strated .x.

Plate 19   Electron micrograph of a section of <u>C</u>. <u>albicans</u>

fixed by glutaraldehyde using the durcupan-araldite

schedule. Note: poor impregnation, large electron

thin area, and electron dense areas .x 19,500.

Plate 20   Electron micrograph of a section of C. albicans
            fixed by glutaraldehyde and OsO_4 using the primary
            and secondary fixatives method. Cells were grown for
            24 hours at $37^{\circ}C$. Micrograph shows cell wall mitochon-
            dria, nuclear membrane, nucleus and nucleolus. Note
            the electron thin and electron dense areas scattered
            across the cytoplasm .x 24,000.

Plate 21   Electron micrograph of a section of C. albicans
            fixed by p-formaldehyde, glutaraldehyde and OsO_4
            using a modification of Gealt et al method. Cells
            were grown for 24 hours at $37^{\circ}C$. Micrograph shows
            cell wall, cell membrane, mitochondria, nucleus
            and nucleolus. Note the electron thin and electron
            dense scattered across the cytoplasm. .x 30,000.

Plate 22   Electron micrograph of a section of C. albicans
            fixed by glutaraldehyde, potassium permanganate,
            and potassium dichromate using a modification of
            Davison's method. Cells were grown for 24 hours
            at 37°C. Micrograph shows the cell wall, cell
            membrane, mitochondria with cristae, nucleus, nuclear
            membrane, vacuole and storage granules. Note the
            absence of electron dense and electron thin areas.
            .x 19,500.

Plate 23   Electron micrograph of a section of C. albicans
            fixed by glutaraldehyde, potassium permanganate,
            and potassium dichromate using a modification of
            Davison's method showing cell wall, cell membrane.
            mitochondria with cristae, and storage granules.
            .x 19,500.

Plate 24   Electron micrograph of $\underline{C}$. $\underline{albicans}$

grown as a shake culture in synthetic

medium containing no steroid (control).

Cells were grown for 24 hours at $37^{\circ}C$.

Note the cell wall, cell membrane,

mitochondria, nuclear membrane, nucleus,

vacuole and storage granules. .x 24,000..

Plate 25   Electron micrograph of a section of C. albicans

grown as a shake culture within synthetic medium

containing 0.025% cholic acid salt. Cells were

grown for 24 hours at 37°C. Micrograph shows

yeast cell with cell wall, cell membrane, mito-

chondria, mucleus and storage granules. .x 19,500.

Plate 26   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium con-

taining 0.025% cholic acid salt. Cells were grown

for 24 hours at 37°C. Micrograph shows yeast cell

with lomasome in the cell wall and electron dense

area with scattered electron thin areas. Intracto-

plasmatic organelles and plasmalemma can no longer

be identified. .x 30,000,

Plate 27   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.05% cholic acid salt. Cells grown

for 24 hours at 37°C. Micrograph shows a yeast cell with

cell wall, cell membrane, mitochondria with cristae,

storage granules, and nucleus. .x 19,500.

Plate 28   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium con-

taining 0.05% cholic acid salt. Cells grown for 24

hours at 37°C. Micrograph shows two types of yeast

cells: cells with the intracytoplasmatic organelles,

and plasmalemma distinctly identified, and smaller

cells where the intracytoplasmatic organelles and

plasmalemma can no longer be identified. Note the

convoluted edge of the smaller cells. .x 15,000.

Plate 29   Electron micrograph of a section of $\underline{C}$. albicans

grown as a shake culture in synthetic medium con-

taining 0.1% cholic acid salt. Cells grown for 24

hours at 37°C. Micrograph shows yeast cell with

cell wall, cell membrane, mitochondria with cristae,

vacuole, and storage granules. .x 24,000.

Plate 30   Electron micrograph of a section of $\underline{C}$. albicans.

grown as a shake culture in synthetic medium

containing 0.1% cholic acid salt. Cells grown

for 24 hours at 37°C. Micrograph shows yeast cells

with electron dense and electron thin areas. .x 24,000.

Plate 31   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.15% cholic acid salt. Cells grown

for 24 hours at 37°C. Micrograph shows two

types of yeast cells: cells with numerous lytic

areas in the cytoplasm with accumulated electron

dense substances, and cells involved in cell

division with preserved internal structures:

nucleus, cell wall, cell membrane and storage

granules. .x 12,000.

Plate 32   Electron micrograph of a section of C. albicans

            grown as a shake culture in synthetic medium

            containing 0.025% decadron. Cells grown for

            24 hours at 37°C. Micrograph shows yeast cell

            with cell wall, mitochondria, nucleus and nucleolus.

            Note the cell with numerous lytic areas at the bottom

            left hand corner of the micrograph. .x 24,000.
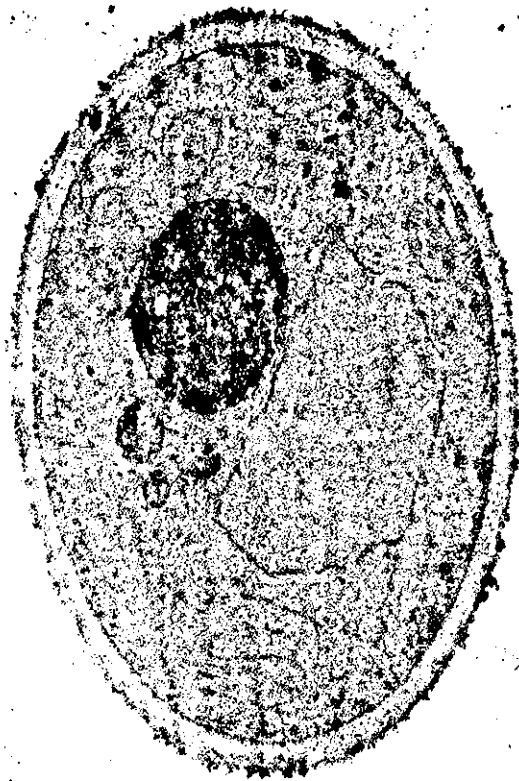
Plate 33   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.05% decadron. Cells grown for 24 hours

at 37°C. Micrograph shows yeast cell with cell wall,

cell membrane, mitochondria with cristae and storage

granules. Note the elongated mitochondria. .x 24,000.
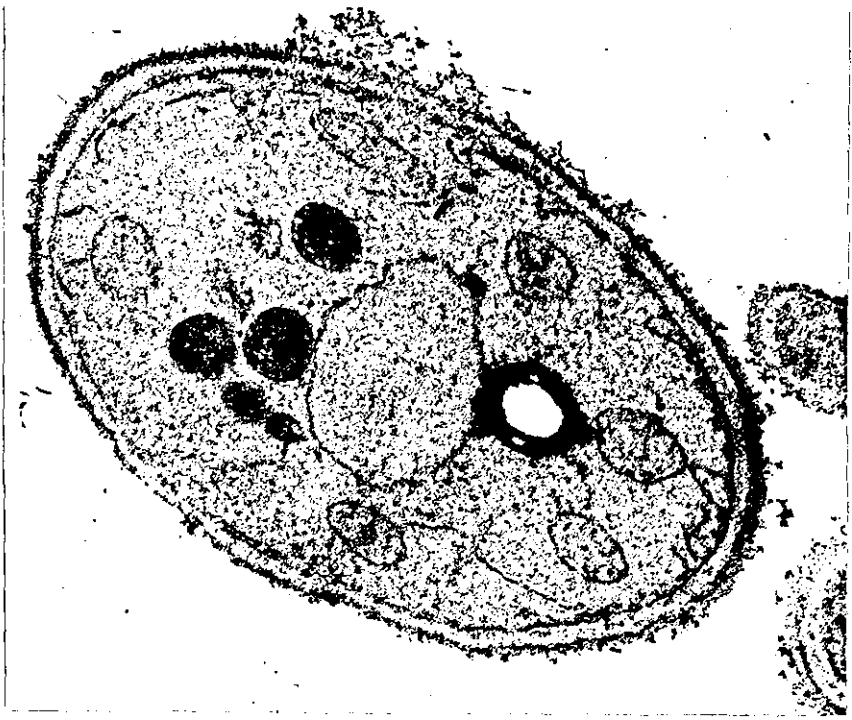

Plate 34   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.05% decadron. Cells grown for 24

hours at 37°C. Micrograph shows yeast cell with

extensive damage. Note the disintegration of the

cell wall disappearance of cell membrane and the

lysis of the cytoplasm. .x 24,000.

Plate 35   Electron micrograph of a section of $\underline{C}$. albicans

grown as a shake culture in synthetic medium

containing 0.1% decadron. Cells grown for 24

hours, at 37°C. Micrograph shows yeast cell with

cell wall, cell membrane, mitochondria, nuclear

membrane, nucleus, vacuole, and storage granules.

.x 19,500.

Plate 36   Electron micrograph of a sectionof $\underline{C}$. albicans

grown as a shake culture in synthetic medium

containing 0.1% decadron. Cells grown for 24

hours at 37°C. Micrograph shows a group of yeast

cells involved in cell division. .x 19,500.

Plate 37   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.15% decadron. Cells grown for 24

hours at 37°C. Micrograph shows yeast cell with

numerous lytic areas in the cytoplasm. Intra-

cytoplasmatic organelles and plasmalemma can no

longer be identified. .x 30,000.
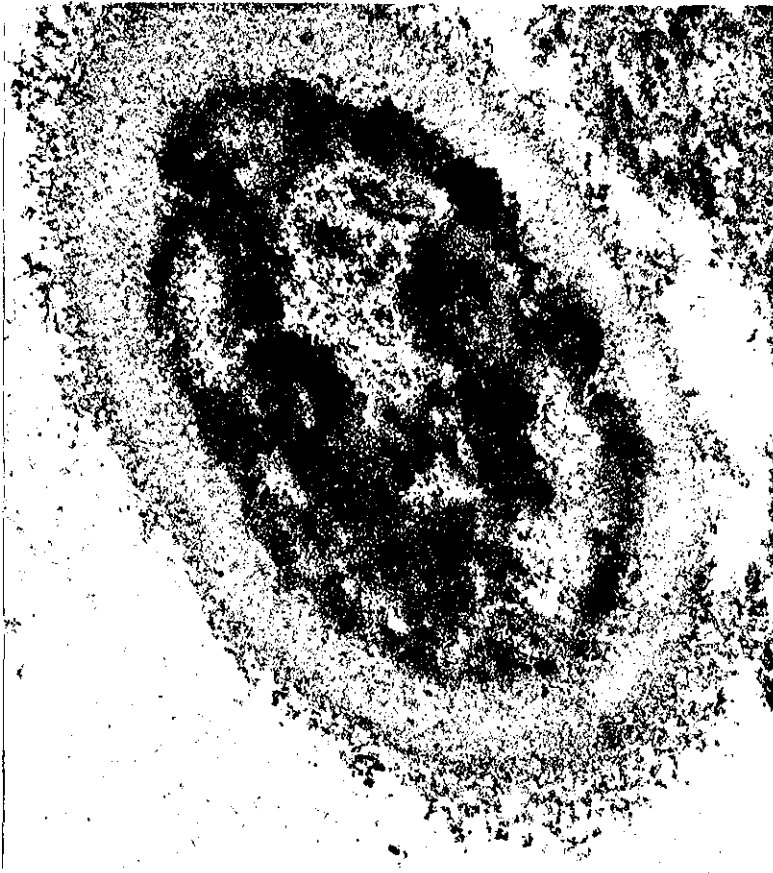
Plate 38   Electron micrograph of a section of $\underline{C}$. albicans

grown as a shake culture in synthetic medium

containing 0.025% alphadolone acetate. Cells

grown for 24 hours at 37°C. Micrograph shows

yeast cell with cell wall, cell membrane mito-

chondria with cristae, nucleus and storage gran-
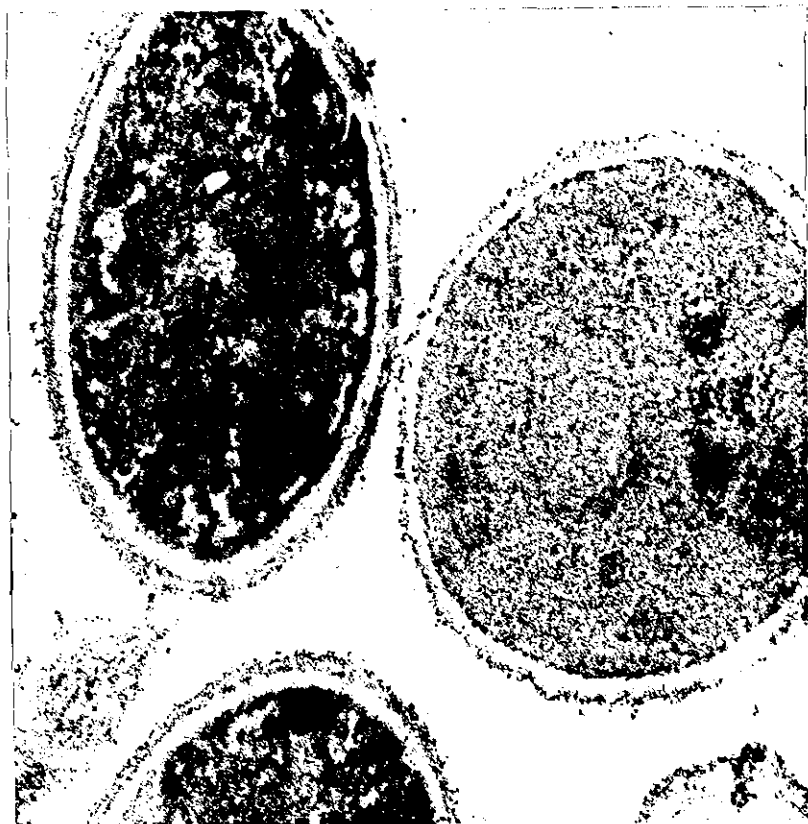
ules. .x 19,500.
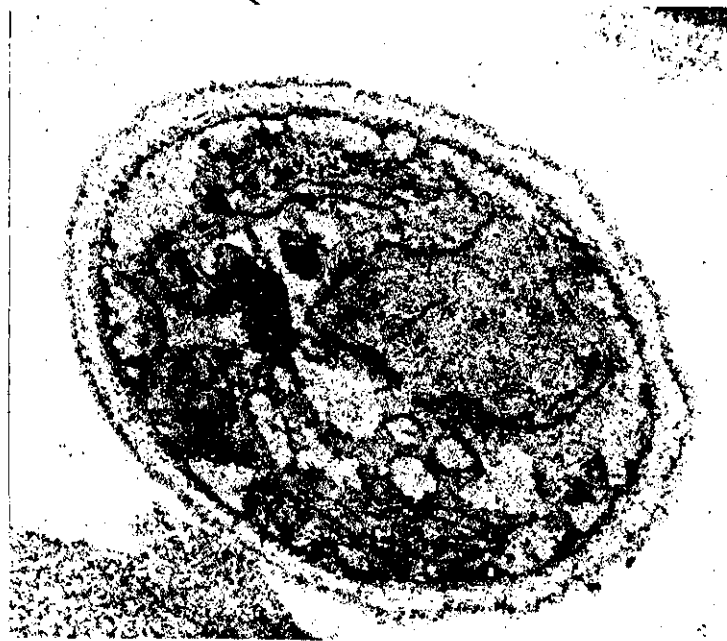
Plate 39   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.05% alphadolone acetate. Cells grown

for 24 hours, at 37°C. Micrograph shows yeast

cell with cell wall, cell membrane, mitochondria

with cristae, nucleus and storage granules. Black

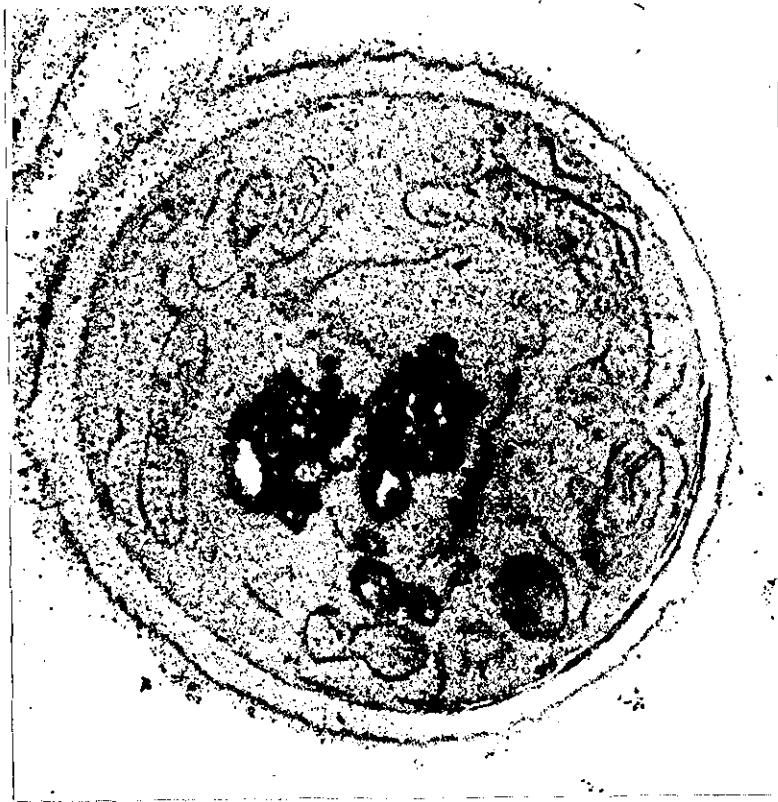spots are due to lead citrate stain. .x 15,100.

Plate 40   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.05% alphadolone acetate. Cells

grown for 24 hours at 37°C. Micrograph shows

two types of yeast cells: cell with numerous lytic

and electron dense areas, and cell with cell wall,

cell membrane, mitochondria and a faint nucleus

.x 24,000.
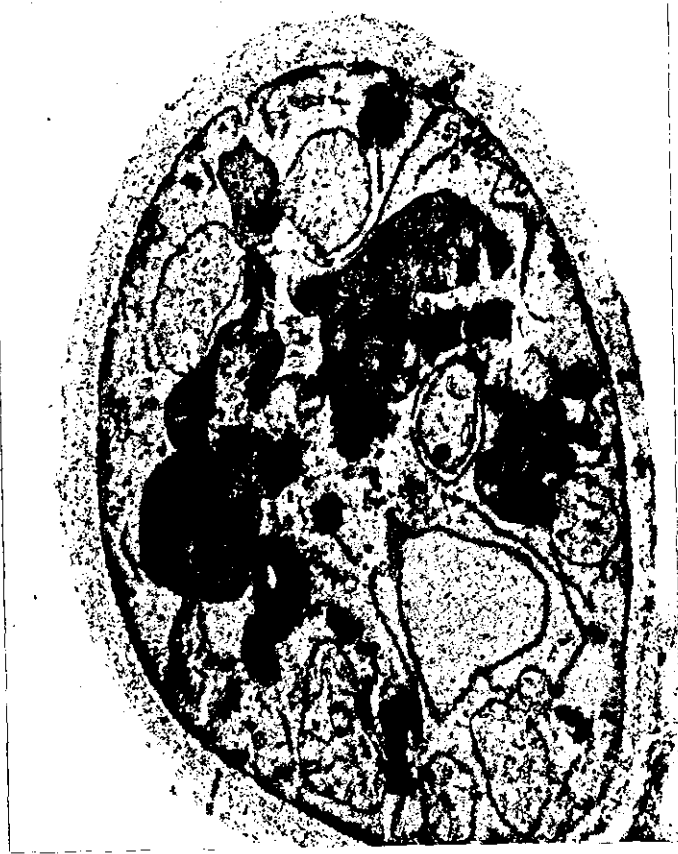
Plate 41   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.1% alphadolone acetate. Cells grown

for 24 hours at 37°C. Micrograph shows yeast cell

involved in cell division with well preserved

internal structures: cell wall, cell membrane,

mitochondria with cristae, nucleus vacüöle and
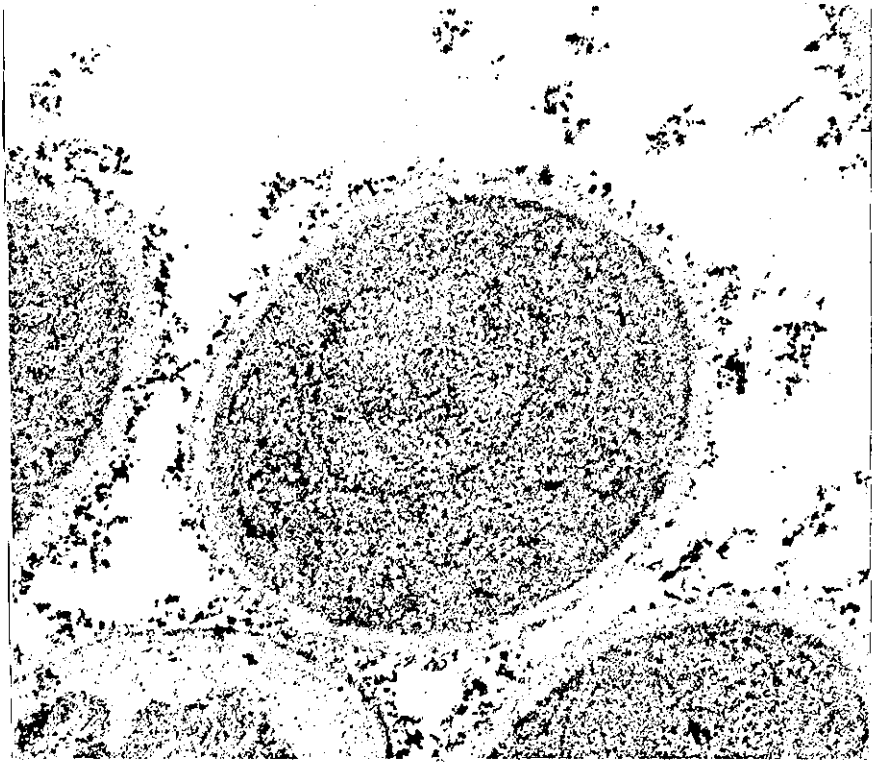
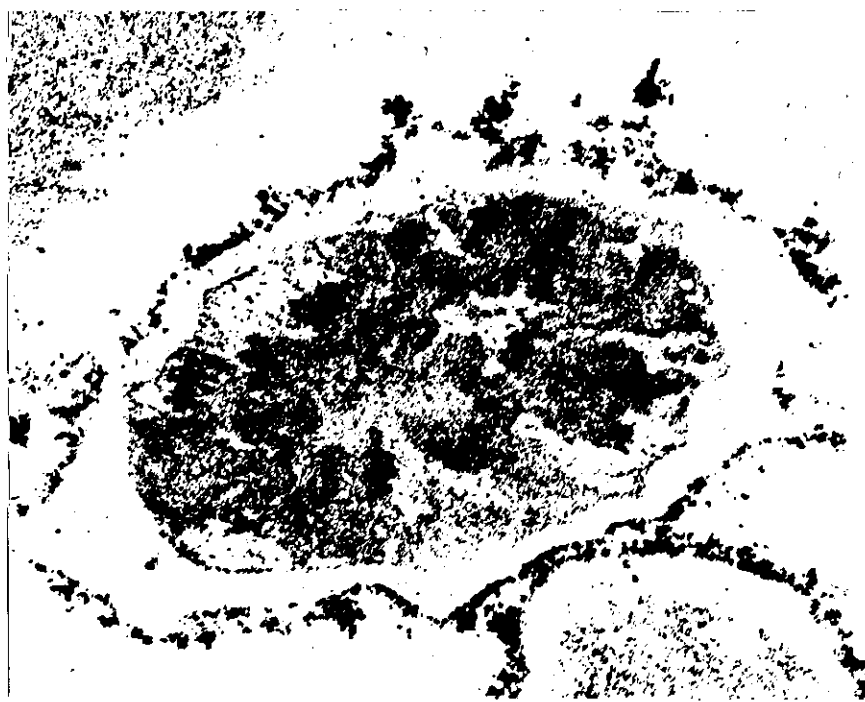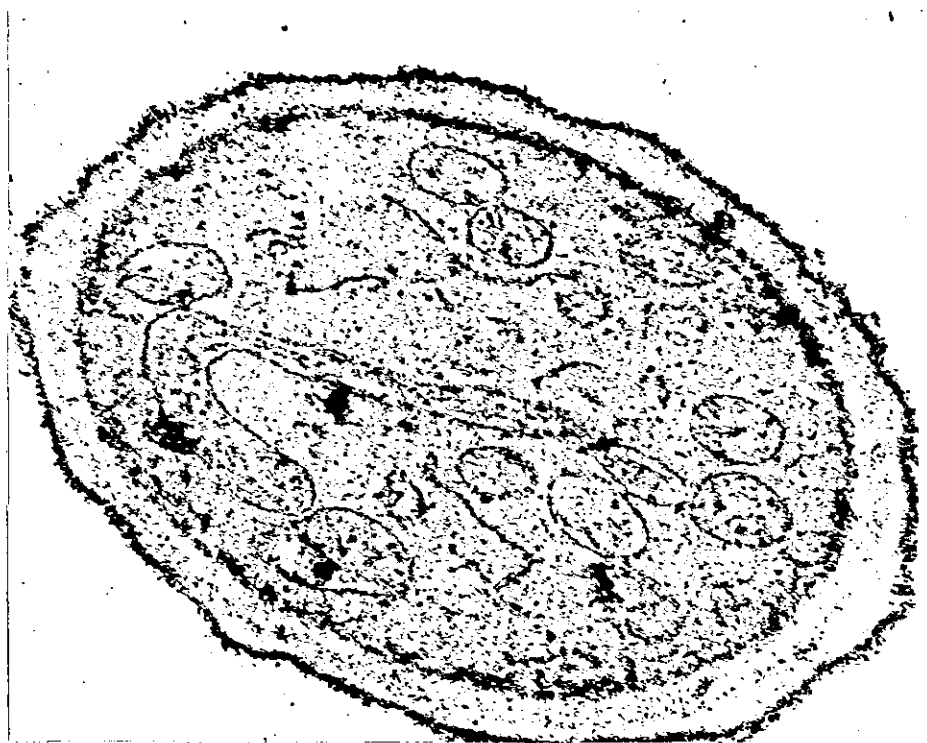septum. . x 19,500.

Plate 42   Electron micrograph of a section of C. albicans

grown as a shake culture in synthetic medium

containing 0.1% alphadolone acetate. Cells grown

for 24 hours at 37°C. Micrograph shows yeast cell

with disintegration in the cell wall, the absence

of cell membrane, and numerous lytic areas in the

ctyoplasm with accumulated electron dense substances.

.x 19,500.
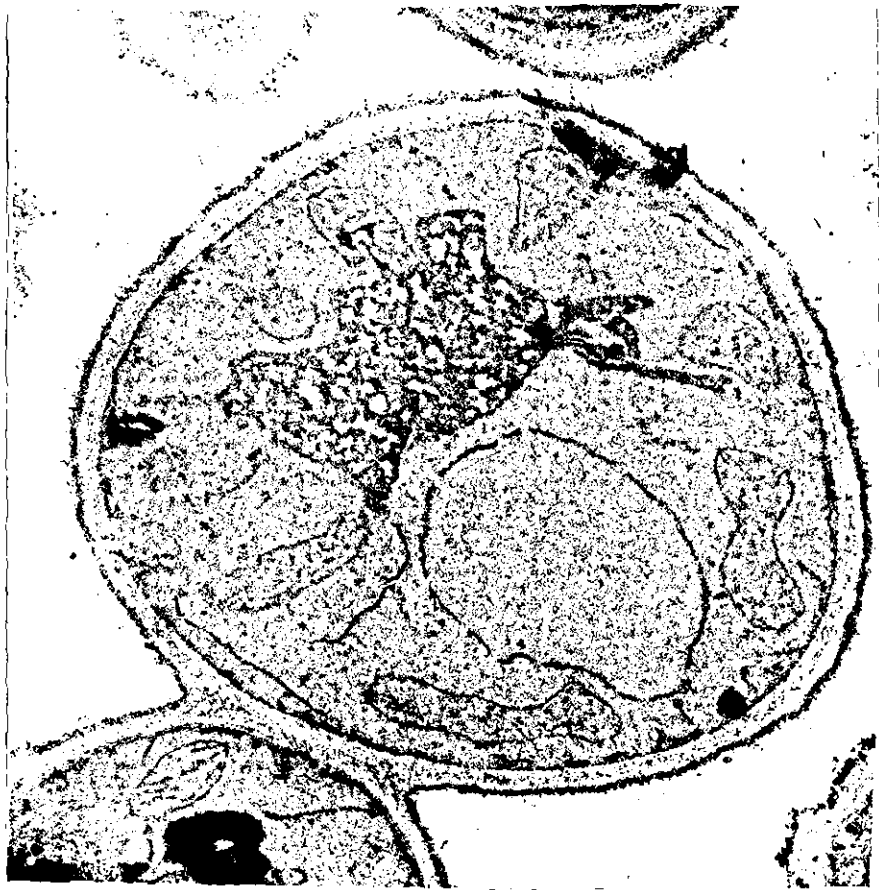
Intracytoplasmatic organelles and plasmalemma could no longer be identified. As with cholic acid salt, convoluted cell envelopes were noticed with the small cells (Plate 42).

Mycelial Form

Light microscopy showed that C. albicans grown in the presence of steroids had shorter deformed hyphae when compared with the control cells (Plate 43). In the presence of 0.15% cholic acid salt the hyphae were short and swollen (Plate 44). Shorter hyphae were observed with cells grown in the presence of decadron (Plate 45) and alphadolone acetate (Plates 46 and 47).

More evidence of the effect of steroids on the morphology of the mycelial form of C. albicans was obtained using scanning electron microscopy. At alow concentrations, cholic acid salt showed (Plate 53) no apparent difference from the control (Plate 52) but as the concentration was increased the effect became more obvious (Plate 54). At high concentrations the occurence of smooth surfaced cells becomes more prominent (Plate 55).

The effect on morphology in the presence of decadron is manifested at low concentration (Plate 56) where constriction in the hyphae becomes apparent. This constriction becomes more prominent at high concentrations (Plates 58 and 59). In addition to constricted-hyphae collapsed mycelial cells were observed (Plate 57).

At concentrations as low as 0.025% of alphadolone acetate the hyphae were damaged (Plate 60). Both constriction (Plate 62) and collapsed hyphae (Plates 62 and 63) occurred.

Plate 43   Phase contrast microscopy of C. albicans grown in Tween

medium without steroid. Incubated for 24 hours at

37°C. The micrograph shows filamentous form with long

hyphae. .x 1,000.

Plate 44   Phase contrast microscopy of C. albicans grown in Tween

medium containing 0.15% cholic acid salt. Incubated for

24 hours at 37°C. Micrograph shows filamentous form

with short swollen hyphae. .x 1,000.

Plate 45   Phase contrast microscopy of C. albicans grown in Tween

medium containg   0.15% decadron. Incubated for 24 hours

at 37°C. Micrograph shows filamentous form. Note the

short hyphae. .x 1,000.

43
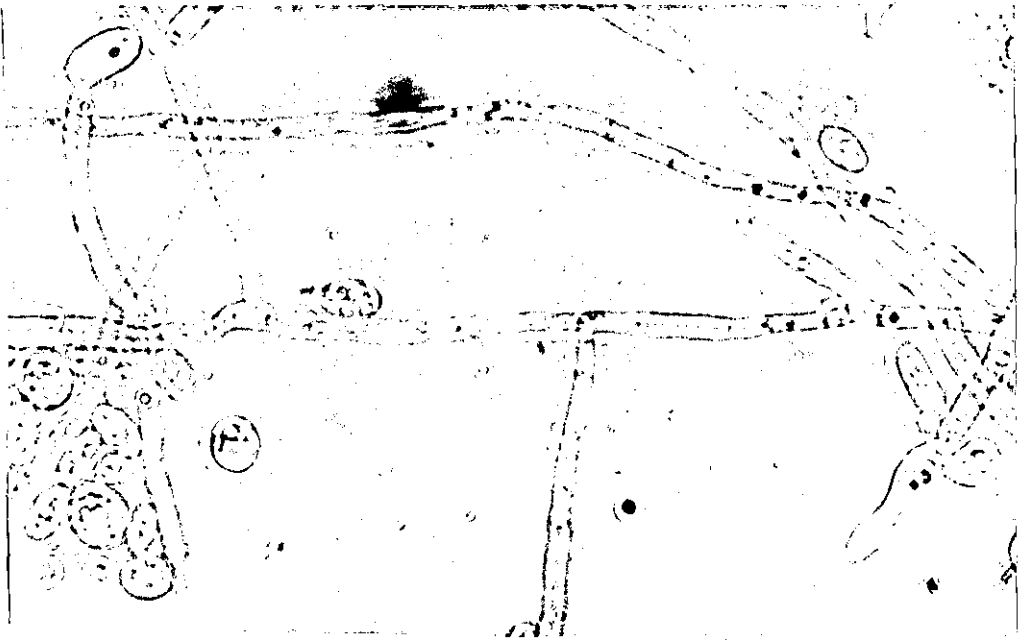
44
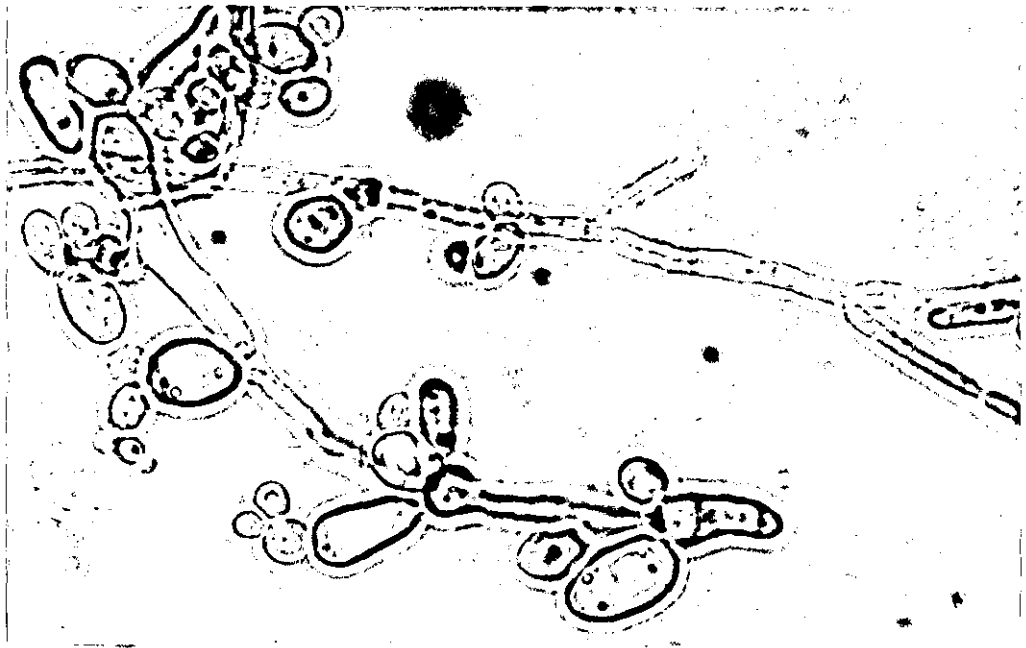
45
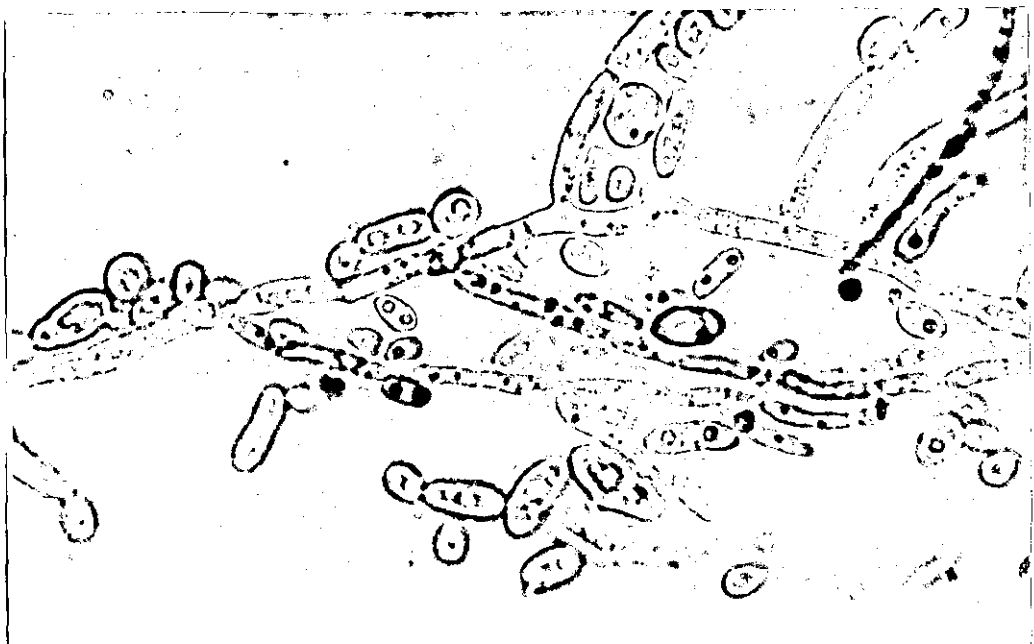
Plate 46   Phase contrast microscopy of C. albicans grown on

Tween medium containing 0.05% alphadolone acetate.

Incubated for 24 hours, at 37°C. Micrograph shows

filamentous form with short hyphae. .x 1,000.

Plate 47   Phase contrast microscopy of C. albicans grown on

Tween medium containing 0.15% alphadolone acetate.

Incubated for 24 hours at 37°C. Micrograph shows

filamentous form with short hyphae. .x 1,000.
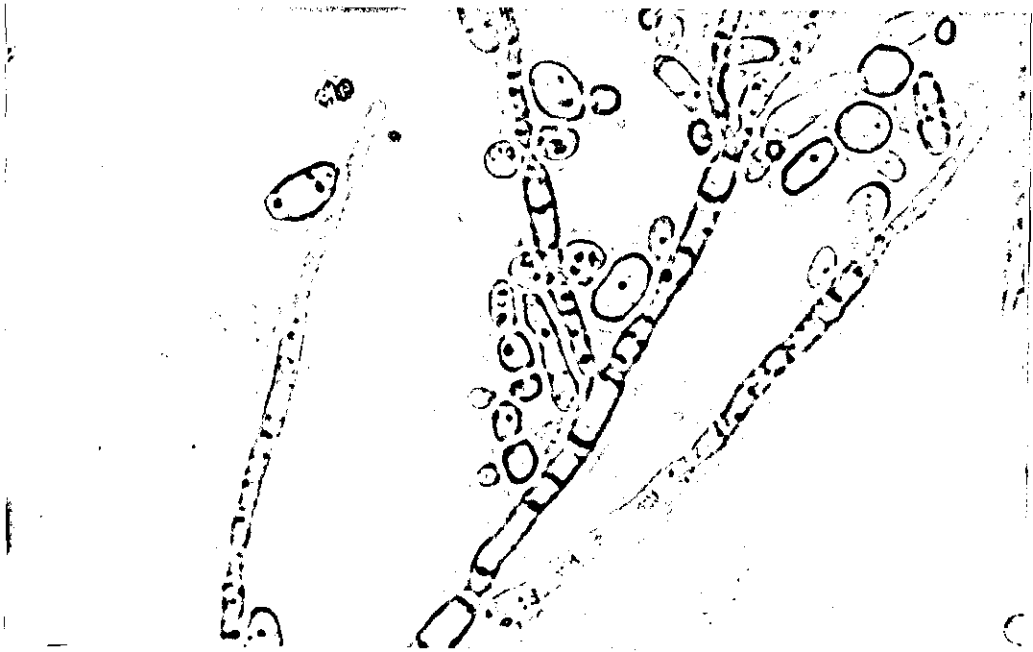
Plate 48   S.E.M. of C. albicans, A39, grown as a stationary

culture in Tween medium.   Incubated for 5 hours,

at 37°C.   The micrograph shows the filamentous form

.x 10,000.

Plate 49   S.E.M. of C. albicans A39, grown as a stationary

culture in Tween medium.   Incubated for 8 hours at

37°C.   Micrograph shows the filamentous form. .x 10,000.

Plate 50   S.E.M. of C. albicans, A39, grown as a stationary culture in Tween medium. Incubated for 12 hours at 37°C. Micrograph shows filamentous form. .x 10,000.

Plate 51   S.E.M. of C. albicans, A39, grown as a stationary culture in Tween medium. Incubated for 24 hours at 37°C. Micrograph shows filamentous form and some yeast form cells. .x 10,000.

Plate 53   S.E.M. of C. albicans, A39, grown as a stationary
            culture in Tween medium containing 0.025% cholic
            acid salt. Incubated for 24 hours, at 37°C.
            Micrograph shows two types of filamentous form:
            smooth surfaced cells and collapsed ones. .x 10,000.

Plate 52   S.E.M. of C. albicans, A39, grown as a stationary
            culture in Tween medium without steroid.   Incubated
            for 24 hours, at 37°C. Micrograph shows all smooth
            surface filamentous form .x 10,000.
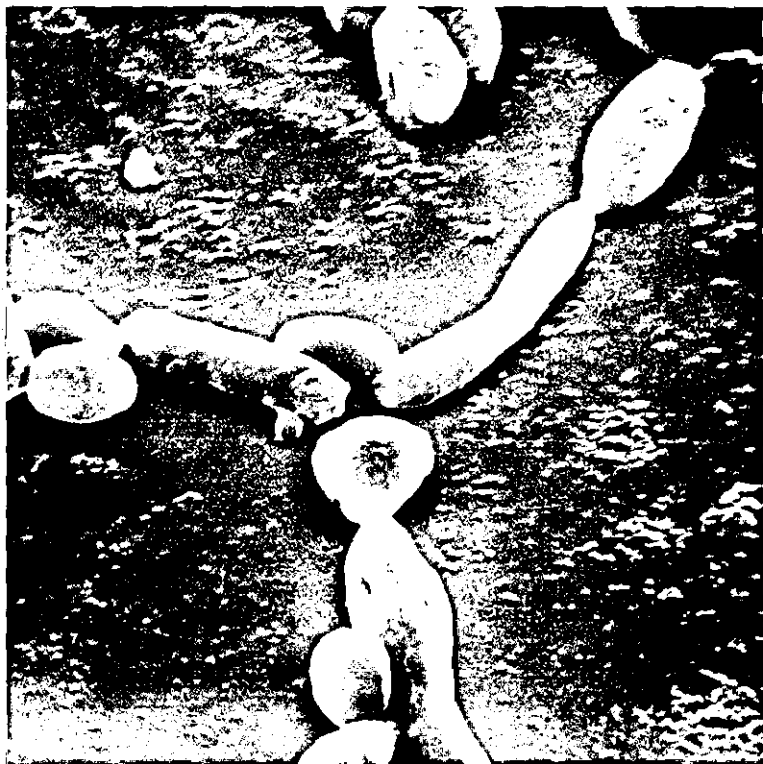
Plate 55   S.E.M. of C. albicans, A39, grown as a stationary

culture in Tween medium containing 0.15% cholic

acid salt. Incubated for 24 hours, at 37°C.

Micrograph shows smooth surface with no collapse

filamentous form. .x 10,000.

Plate 54   S.E.M. of C. albicans, A39, grown as a stationary

culture in Tween medium containing 0.1% cholic

acid salt. Incubated for 24 hours, at 37°C. Micro-

graph shows filamentous and yeast form cells. .x 10,000.
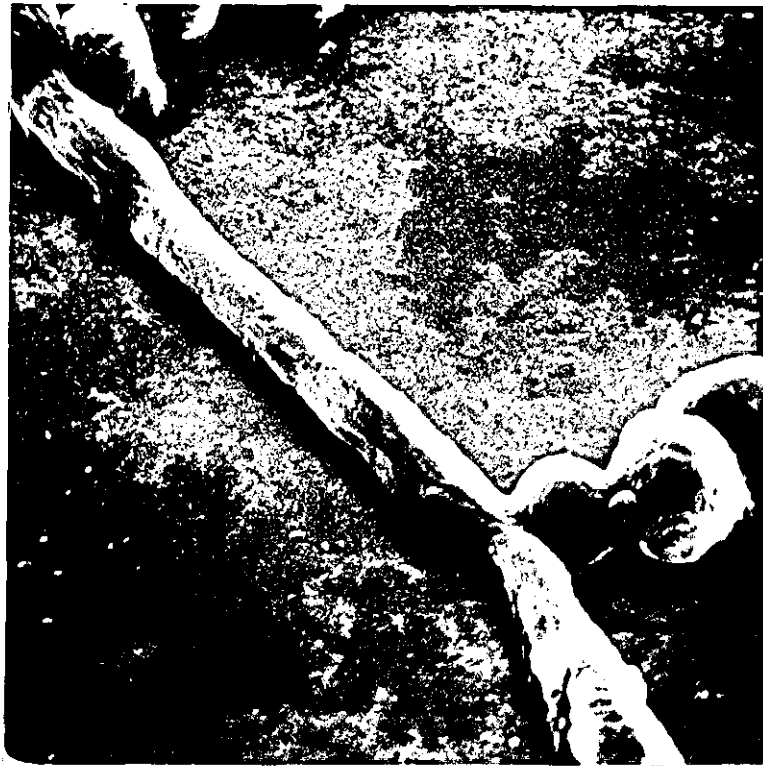
Plate 56   S.E.M. of C. albicans A39, grown as a stationary

culture in Tween medium containing 0.025% decadron.

Incubated for 24 hours, at 37°C. Micrograph shows

filamentous forms with constriction across the hypha.

.x 10,000.

Plate 57   S.E.M. of C. albicans, A39, grown as a stationary

culture in Tween medium containing 0.05% decadron.

Incubated for 24 hours, at 37°C. Micrograph shows

collapsed, filamentous forms and yeast form. .x 10,000.
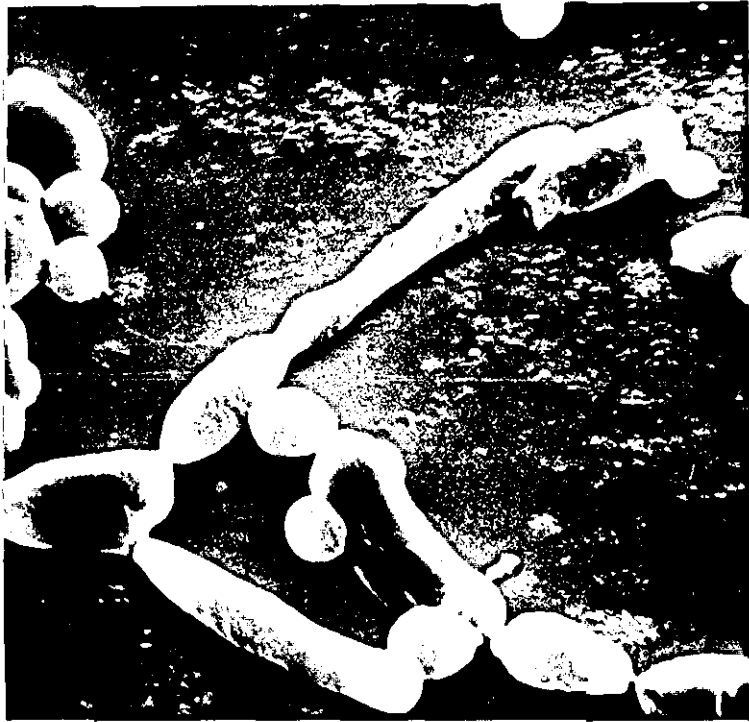
Plate 58   S.E.M. of C. albicans, A39, grown as a stationary

            culture in Tween medium containing 0.1% decadron.

            Incubated for 24 hours, at 37°C. Micrograph shows

            smooth surfaced filamentous forms, also breaks in

            the hyphae. .x 10,000.

Plate 59   S.E.M. of C. albicans A39, grown as a stationary

            culture in Tween medium containing 0.15% decadron.

            Incubated for 24 hours, at 37°C. Micrograph shows

            filamentous forms with constrictions across the

            hyphae. .x 2,500.

Plate 60   S.E.M. of C. albicans, A39, grown as a stationary

            culture in Tween medium containing 0.025% alphadolone

            acetate. Incubated for 24 hours, at 37°C. Micrograph

            shows collapsed filamentous and yeast forms. .x 10,000.

Plate 61   S.E.M. of C. albicans, A39, grown as a stationary

            culture in Tween medium containing 0.05% alphadolone

            acetate. Incubated for 24 hours at 37°C. Micrograph

            shows filamentous and yeast forms. .x 10,000.
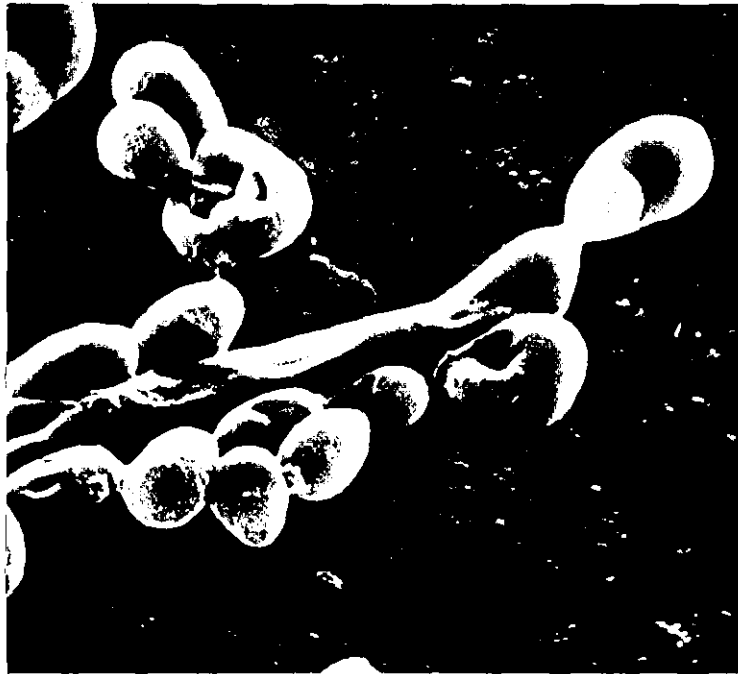
Plate 62   S.E.M. of C. albicans, A39, grown as a stationary

culture in Tween medium containing 0.1% alphadolone

acetate. Incubated for 24 hours, at 37°C. Micro-

graph shows filamentous forms with constrictions and

others collapsed. .x 10,000.

Plate 63   S.E.M. of C. albicans, A39, grown as a stationary

culture in Tween medium containing 0.15% alphadolone

acetate. Incubated for 24 hours, at 37°C. Micro-
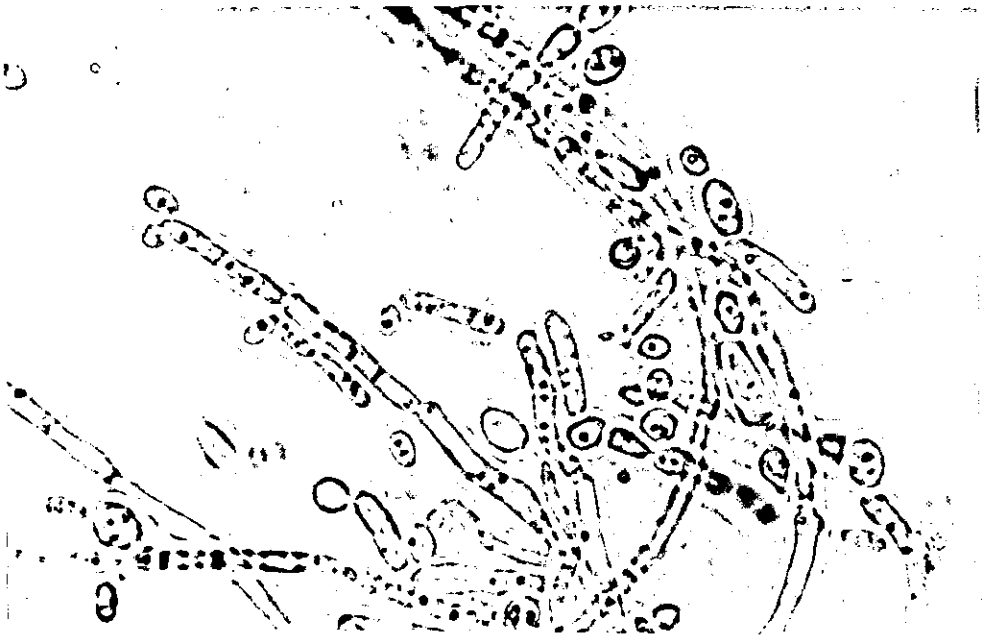
graph shows collapse of filamentous form. .x 10,000.

IDENTIFICATION OF STEROL

The crude sterols were extracted and isolated. The various fractions were separated by preparative t.l.c. (Silica; 33% diethylether in petroleum ether (40-60%)) in the increasing order of Rf.

(a) Ergosta-5,7, 22-trien-3β-OL (Ergosterol((5)

m.p. 159 - 61°C (lit. 163°C) (311a) (Ethanol)

1 Hnmr $\widetilde{\ }$ (CDCl$_3$) 4.34 - 4.83 (m;5H); 5.2 - 9.5 (m;37H)

6.1 - 6.82 (m;broad hump; 1H) 7.4 - 9.5 (m;37H)

V max (KBr) 3430, 3040, 2960, 2870, 1655, 1603, 1460, 1382
1370, 1325, 1240, 1155, 1055, 1038, 980, 965, 945,
835, 800, and 725 cm^{-1}.

λ max (CHCl$_3$) 291, 279.5, 269.5, 259 nm (Mass Spectroscopy): 396 M$^+$ (Mass Spectroscopy): 396.

(b) Calciferol (Ergocalciferol) (C)

V max (KBr) 3300, 3080, 2960, 2930, 2870, 1625, 1455, 1440
1370, 1350, 1265, 1160, 1055, 965, 905, 890,
830, and 720 cm^{-1}.

λ max (Ethanol): 262 n.m.

NMR spectra could not be carried out because of insufficient sample. Spectra of the compound was characterised by comparing the sample with standard compounds.

(c) $\Delta^{8,24}$ - Lanostadienol (Lanosterol) (8)

1 Hnmr $\widetilde{\uparrow}$ (CDCl$_3$): 4.75 - 5.02 (m;1H) 6.55 - 6.94 (m;1H) and

7.5 - 9.52 (m;48H).

V max (KBr) 3320, 2950, 2930, 2875, 1670, 1465, 1445, 1370, 1240,

1205, 1155, 1100, 1028, 930, 882, and 760 cm^{-1}.

M$^+$ (Mass Spectroscopy): 426.

(d) 2, 6, 10, 15, 19, 23 - Hexamethyl - 2, 6, 10, 14, 18, 22 - tetra -

cosahexene (Squalene) (1)

1 Hnmr : $\widetilde{\uparrow}$ (CDCl$_3$) : 4.7 - 5.0 (m;broad hump, 6H) 7.74 - 8.14

(S;20H) and 8.20 - 8.50 (d;24H).

V max (oil) :   2965, 2920, 2850, 1665, 1445, 1380, 1150, 1105,

980 and 835 cm^{-1}.

λmax (EtoH) :   208 n.m.

M$^+$ (Mass Spectroscopy): 410.

All the compounds were finally characterised by comparison with
the authentic standards. U.V., I.R., and N.M.R. spectra are listed
in Appendix I. More complete separation and identification was carried
out using gas-liquid chromatography.

Only ergosterol, lanosterol, calciferol and squalene standards
were available. Breakdown products of ergosterol were positively
identified from a solution of ergosterol kept at room temperature for
periods of up to a week (246).

By using the data of Vandenheuval and Court (300) it was possible to calculate the retention times of these components. By expressing the retention times of other sterols relative to ergosterol, it was possible to identify tentatively the other sterols present in the crude sterol. 24, 28 - dehydroergosterol was identified by U.V., using the techniques of Marriott (246).

The gas-liquid chromatograms obtained are listed in the Appendix(II).

EFFECT ON STEROLS CONTENT

C. albicans grown in the presence of exogenous steroids showed
an increase in total lipid content compared to a decrease in sterol
content (Table 4). The maximum effect was seen when cells were
grown in the presence of alphadolone acetate then decadron with
cholic acid having the least effect.

Growth of C. albicans, yeast form, in the presence of steroids
cause an alteration in the type and quantity of the sterol components.
As the concentration of cholic acid salt increased, three important
features were observed on the gas-liquid chromatograms (Table 9).
Firstly, a decrease in ergosterol content, secondly the emergence
of two new peaks (refer to Appendix II), one corresponding to calciferol
and the second corresponding to an unidentified sterol and thirdly,
there was an increase in the percentage of 4,4' dimethyl zymosterol.
As the concentration of steroid increased, the above three changes·
continued to be observed, a maximum value was reached at 0.2% cholic
acid salt.

Alphadolone acetate and decadron had similar effects to cholic
acid salt, but the effect was more marked (Table 10). Under similar
conditions but when the cells were grown in the dark, the same effect
was obtained.

Growth in C. albicans, mycelial form, in the presence of exogenous
steroids caused an increase in the quantity of calciferol and a
decrease in ergosterol (Table 11).

Using thin layer chromatography, it was not possible to detect any of these changes in sterol content in the yeast or mycelial form of C. albicans. However, thin layer chromatography (Plates 69 and 70) was useful only for obtaining a qualitative picture of the major sterol fractions present.

Plate 64   Mobility tests of sterol standards and non-sapon-

ifiable heptane extracts of the yeast form of

C. albicans grown in a medium containing no

steroids (control). Incubated for 24 hours at

37°C.

40-60 P. ETHER   : 3

DIETHYLETHER : 1

PLATE: MERCK KIESELGEL G(TYPE 60)

crudesterol   Ergosterol   Cholesterol   Lanosterol   Calciferol   Squalene
yeast
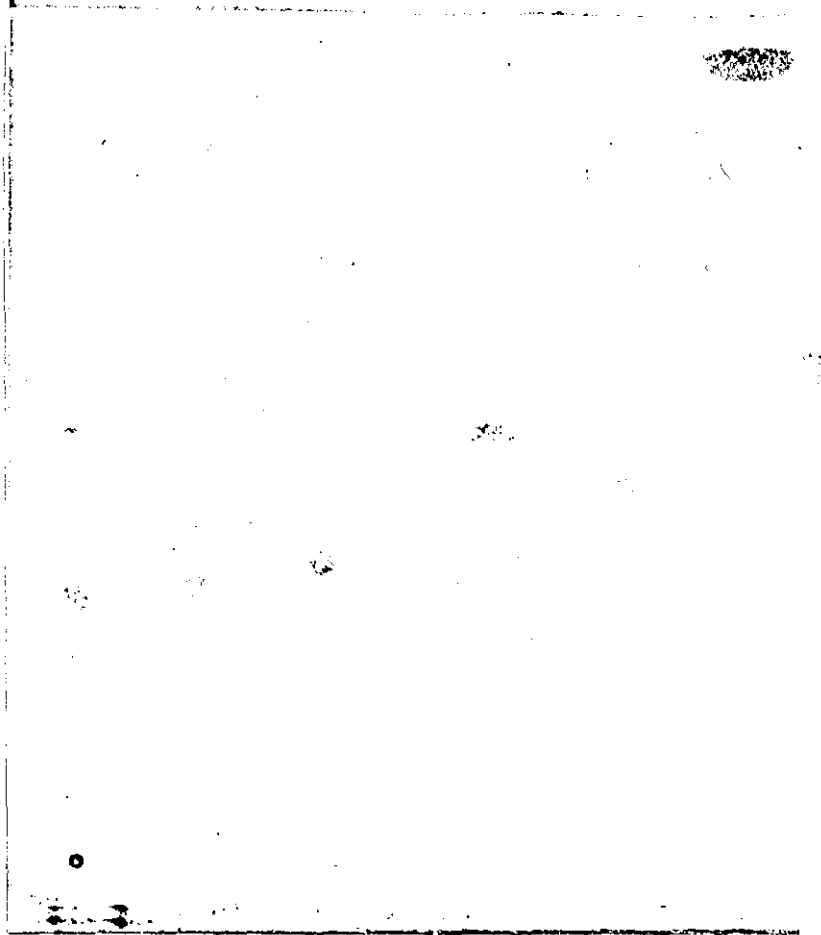
Plate 65   Thin layer chromatogram of sterols extracted from

the yeast form of C. albicans I.M.I. 45348 grown

in a medium containing no steroids (control).

Incubated for 24 hours at 37°C.

THIN LAYER CHROMATOGRAPH OF STEROLS:

C. Albicans I.M.I.45348

SOLVENT SYSTEM

40-60 P.ETHER :3

DIETHYLETHER : 1

PLATE: MERCK KIESELGELG(TYPE 60)

Crudesterol
yeast
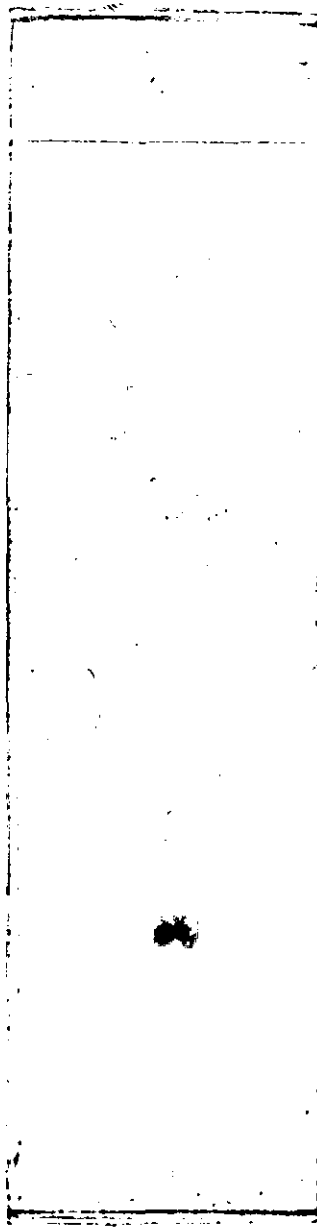
Plate 66   Thin layer chromatogram of sterols extracted from

the mycelial form of C. albicans A39 grown in a

medium containing no steroids (control). Incu-

bated for 24 hours at $37\,^{\circ}C$.

THIN LAYER CHROMATOGRAPH OF STEROLS:

C. Albicans (A39)

SOLVENT SYSTEM

40-60 P.ETHER : 3

DIETHYLETHER : 1

PLATE: MERCK KIESELGEIG (TYPE 60)
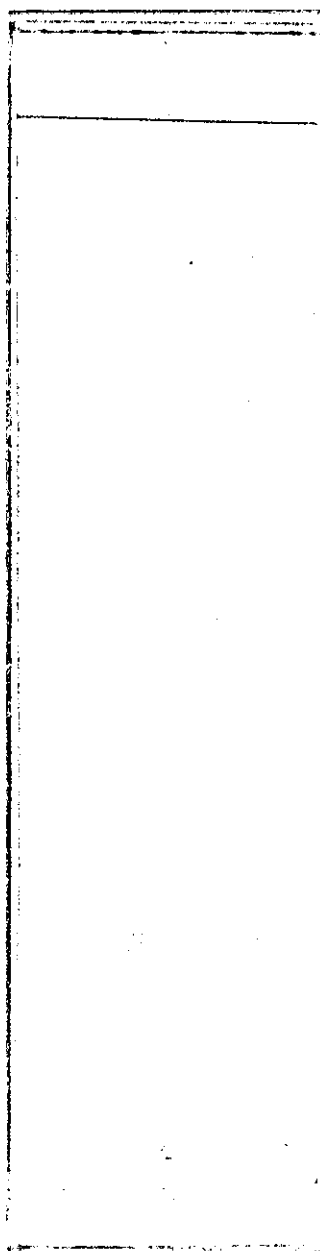
Mycelial

form

Plate 67   Thin layer chromatogram of sterols extracted from

the mycelial form of C. albicans I.M.I. 45348

grown in a medium containing no steroids (control).

Incubated for 24 hours at 37°C.

THIN LAYER CHROMATOGRAPH OF STEROLS:

C. Albicans I.M.I.45348

SOLVENT SYSTEM

40-60 P.ETHER :3

DIETHYLETHER : 1

PLATE: MERCK KIESELGEIG (TYPE 60)

Mycelial
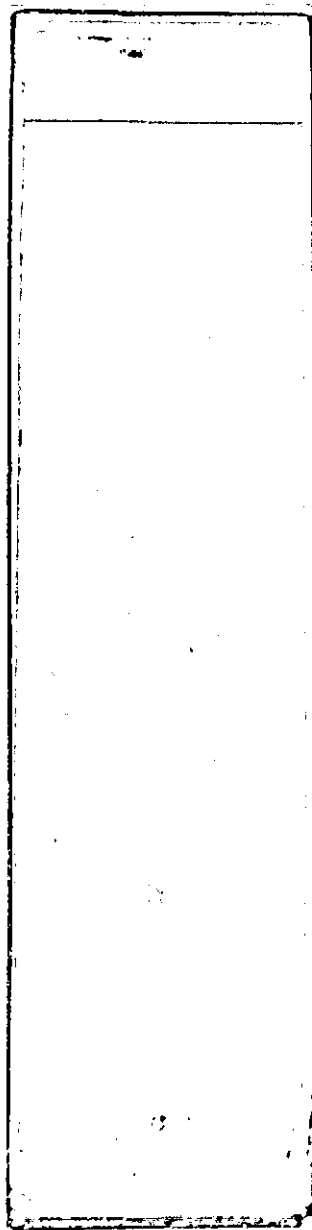
form

67

plate 68   Thin layer chromatograms of lipid extracted from

yeast form of C. albicans showing the presence

of squalene.

THIN LAYER CHROMATOGRAPH OF LIPID:

SOLVENT SYSTEM:

40-60 P. ETHER   : 3

DIETHYLETHER : 1

PLATE: MERCK KIESELGEL G(TYPE 60)

LIPID   SQUALENE
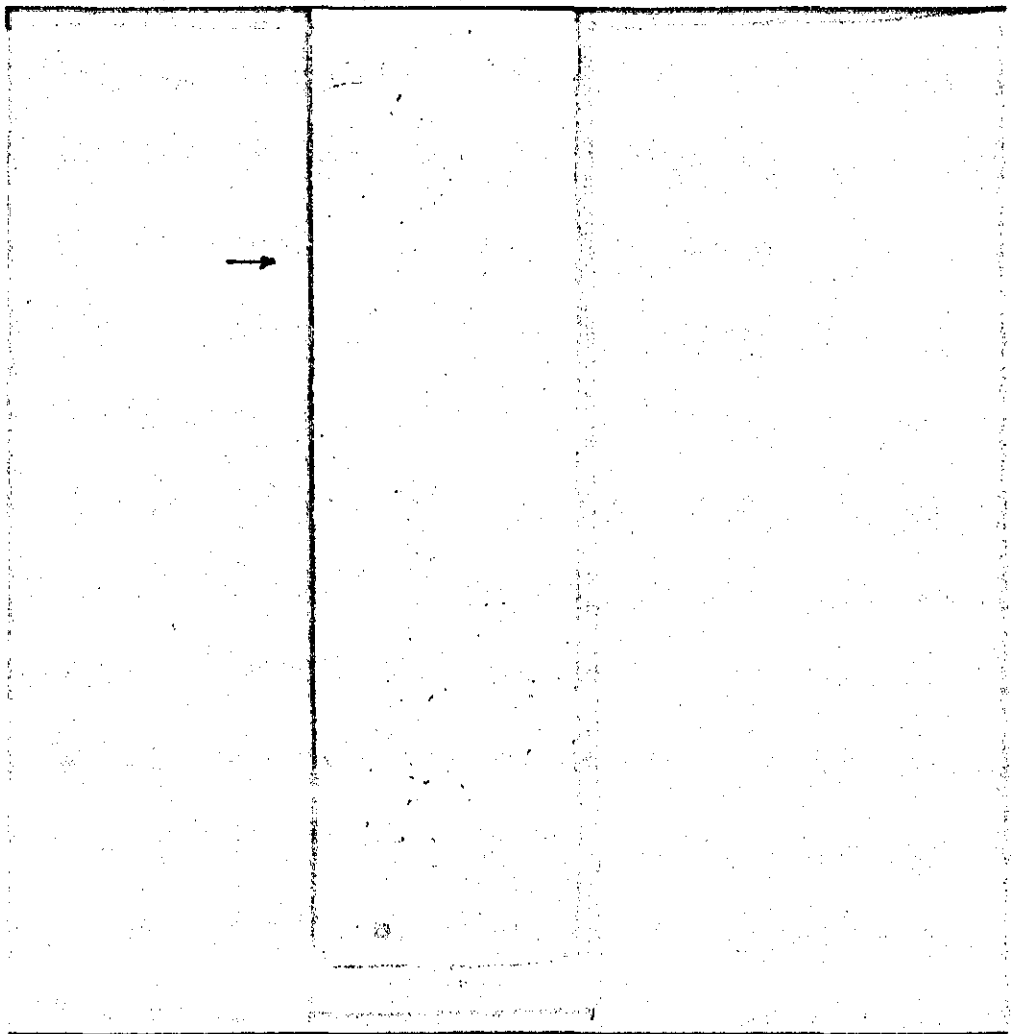
TABLE 4

Effect of steroids on total lipid and sterol content of C. albicans

% steroid	% lipid *			% sterol +		
	Cholic Acid Salt	Decadron	Alphadolone acetate	Cholic Acid Salt	Decadron	Alphadolone Acetate
0.0%	5.09	5.09	5.09	26.05	26.05	26.05
0.05%	6.05	7.22	7.90	20.0	18.29	17.57
0.1%	9.62	14.7	14.0	18.29	12.19	13.05
0.15%	12.51	18.6	19.05	18.01	9.44	8.04

* percentage lipids are expressed as part of dry weight.

\+ percentage sterols are expressed as part of total lipids.

TABLE 5

Total sterol (free and esterified) composition of the yeast form

of C. albicans A39

Component	Relative retention time *	Percentage
Squalene	$0.345 \pm .006$	$5.025 \pm .17$
Breakdown products of ergosterol	$0.46 \pm .006$ $0.532 \pm .01$	$0.94 \pm .11$ $1.7 \pm .07$
Calciferol	$0.82 \pm .008$	ND
Zymosterol	$0.934 \pm .004$	$10.17 \pm .45$
Ergosterol	1.00	$37.12 \pm .8$
24,28 - dehydro - ergosterol +	$1.088 \pm .03$	$8.6 \pm .14$
(4&Me zymosterol)	$1.17 \pm .005$	$15.55 \pm .4$
Lanosterol	$1.29 \pm .01$	$16.77 \pm .3$
(4,4' dimethyl zymosterol)	$1.48 \pm .01$	$3.55 \pm .07$

* Retention time calculated relative to ergosterol which is considered to be = 1. Components between brackets have been identified depending on their retention time using the data of Vandenheuval and Court (300).

ND Not detected.

+ Identified on the basis of U.V. spectrum.

TABLE 6

Total sterols (free and esterified) composition of the mycelial

form of C. albicans A39

Component	Percentage
Squalene	$10.479 \pm .5$
Breakdown products of Ergosterol	$1.114 \pm .26$ $1.605 \pm .4$
Calciferol	$.869 \pm .007$
Zymosterol	$9.397 \pm .1$
Ergosterol	$43.812 \pm .1$
24,28 – dehydro – ergosterol	$5.518 \pm .26$
(4 ∝ Me zymosterol)	$11,08 \pm .07$
Lanosterol	$12.095 \pm .12$
(4,4' dimethyl zymosterol)	$4.013 \pm .16$

TABLE 7

Total sterols (free and esterified) composition of C. albicans

IMI 45348 yeast form

Component	Relative retention Time	Percentage
Squalene	0.345	$1.8 \pm .6$
Breakdown products of ergosterol	0.46 0.532	$3.57 \pm .9$ $2.4 \pm .2$
Calciferol	0.8	$1.37 \pm .1$
Zymosterol	0.934	$11.02 \pm .8$
Ergosterol	1.00	$37.1 \pm .9$
24,28 - dehydro - ergosterol	1.088	$9.5 \pm .4$
(4 α Me zymosterol)	1.17	$15.9 \pm .5$
Lanosterol	1.29	$11.9 \pm .03$
(4,4' dimethyl zymosterol)	1.48	Not detected

TABLE 8

Total sterols (free and esterified) composition of C. albicans
IMI 45348 Mycelial form

Component	Percentage
Squalene	$12.847 \pm .8$
Breakdown products of ergosterol	$0.407 \pm .19$ $0.939 \pm .08$
Calciferol	$0.678 \pm .09$
Zymosterol	$8.429 \pm .09$
Ergosterol	$38.78 \pm .65$
24,28 - dehydro - ergosterol	$8.393 \pm .23$
(4 ∝ Me zymosterol)	$19.215 \pm .29$
Lanosterol	$9.130 \pm .07$
4,4' dimethyl zymosterol	$1.173 \pm .02$

TABLE 9

Effect of cholic acid salt on the total sterol (free and esterified) composition of the yeast form of C. albicans A39

Component	Relative Retention Time	Control 0.0%CAS	0.05%CAS	0.1%CAS	0.15%CAS	0.2%CAS
Squalene	0.345	5.025	3.01	2.8	3.39	2.5
Breakdown products of Ergosterol	0.46 0.532	0.099 1.70	0.66 1.41	0.85 1.1	0.96 2.88	0.9 2.4
Unidentified	0.734	ND	1.59	5.9	7.65	5.6
Calciferol	0.82	ND	2.95	4.6	6.47	6.2
Zymosterol	0.934	10.17	12.52	9.6	9.23	8.6
Ergosterol	1.00	37.12	36.4	29.8	21.75	23.2
24,28 dehydro - Ergosterol	1.088	8.6	10.53	9.1	8.35	8.0
(4α Me zymosterol)	1.17	15.55	14.48	14.9	14.5	15.3
Lanosterol	1.29	16.77	12.4	15.9	17.9	19.1
(4,4' dimethyl-zymosterol)	1.48	3.55	3.54	4.9	5.53	7.7

CAS = Cholic acid salt

TABLE 10

Effect of cholic acid salt, Decadron and Alphadolone Acetate on the total sterol (free and esterified)

composition of the yeast form of C. albicans A39

	PERCENTAGE STEROLS			
Component	Control (0.0% steroid)	0.05% cholic acid salt	0.05% decadron	0.05% alphadolone acetate
Squalene	5.025	3.01	6.64	6.11
Breakdown products of Ergosterol	0.99 1.70	0.66 1.41	0.71 2.91	0.68 2.97
Unidentified	ND	1.59	8.08	7.59
Calciferol	ND	2.95	9.72	9.32
Zymosterol	10.17	12.52	12.58	13.58
Ergosterol	37.12	36.4	15.76	15.00
24,28 dehydro-ergosterol	8.6	10.53	9.4	9.74
(4 ∝Me zymosterol)	15.55	14.48	11.8	11.38
Lanosterol	16.77	12.4	16.2	16.83
(4,4' dimethyl-zymosterol)	3.55	3.54	6.1	6.75

TABLE 11

Effect of cholic acid salt, Decadron and Alphadolone Acetate on the total sterol (free and esterified) composition of the mycelial form of C. albicans A39

	PERCENTAGE STEROLS			
Component	Control (0.0% steroid)	0.05% cholic acid salt	0.05% decadron	0.05% alphadolone acetate
Squalene	10.479	7.695	8.96	9.383
Breakdown products of Ergosterol	1.114 1.605	4.87 3.896	6.36 4.889	6.899 5.004
Calciferol	0.869	4.558	3.70	3.827
Zymosterol	9.397	15.602	10.30	10.34
Ergosterol	43.812	23.768	14.91	14.59
24,28 dehydro - ergosterol	5.518	14.465	11.429	10.92
(4αMe zymosterol)	11.08	11.065	14.46	14.3
Lanosterol	12.095	10.569	18.54	18.0
4.4' dimethyl zymosterol	4.013	3.506	6.417	6.6

PLATE 69   Thin layer chromatogram of sterols extracted

from the yeast form of C. albicans grown in a

medium containing 0.15% cholic acid salt.

Grown for 24 hours at 37°C. For the purpose

of comparison sterols obtained from a control

sample were run at the same time.

THIN LAYER CHROMATOGRAPH OF STEROLS:

SOLVENT SYSTEM:

40-60 P. ETHER · : 3

DIETHYLETHER : 1

PLATE: MERCK KIESELGEL G(TYPE 60)

Crudesterol   .15% C.A.S.
yeast

PLATE 70   Thin layer chromatogram of sterols extracted from

the yeast form of C. albicans grown in the presence

of 0.05% decadron. Grown for 24 hours at $37^{\circ}C$.

For the purpose of comparison sterols obtained from

a control sample were run at the same time.

THIN LAYER CHROMATOGRAPH OF STEROLS:

SOLVENT SYSTEM:

40-60 P. ETHER   : 3

DIETHYLETHER : 1

PLATE: MERCK KIESELGEL G(TYPE 60)

Crudesterol   .05% Decadron
   yeast

Resistance to polyene antibiotics

The resistance of C. albicans A39 towards polyene antifungal agents increased in the presence of steroids. No change in resistance was observed with non-polyene antibiotics (Tables 12 and 13).

In the presence of 0.05% alphadolone acetate the resistance of C. albicans yeast form (Table 12) to amphotericin B and trichomycin was increased 16 fold and to nystatin twice. With decadron, the resistance to trichomycin was the greatest followed by amphotericin B and the least was nystatin. The presence of cholic acid salt increased the resistance two fold for both nystatin and trichomycin, and four fold to amphotericin B.

All three steroids increased the resistance of the mycelial form to nystatin by a factor of four. Trichomycin rendered cells grown in the presence of cholic acid salt two fold more resistant than cells grown in the absence of steroids but those grown in the presence of alphadolone acetate and decadron were four times as resistant (Table 13).

When C. albicans IMI 45348 was similarly tested higher resistance to polyene but not to non-polyene antibiotics was obtained (Table 13a).

Effect on Respiration

When C. albicans was cultured on Magdala Red medium supplemented with cholic acid salt, there was a change in the colour of the colonies from tinted light red in the control (Plate 71) to a deep-red sheen colonies in the supplemented medium (Plates 72 and 73).

No change in the colour of colonies on Magdala Red medium supplemented with either alphadolone acetate or decadron was observed. It would appear that cholic acid salt did induce impairment of respiratory enzymes in C. albicans, but the other two steroids had no effect.

C. albicans supplemented with cholic acid salt had an increase in the number of mitochondria per cell. The number of mitochondria increased with the concentration particularly in the range of 0.05% to 0.15% of the steroid. However, no significant change in the number of mitochondria per cell was detected with the increase in concentration of both alphadolone acetate and decadron (Fig. 12).

The uptake of oxygen by C. albicans as measured by Warburg constant volume respirometry was enhanced in the presence of cholic acid salt (Fig. 13). Comparison of the uptake of oxygen by the three steroids (Fig. 14) showed that this uptake was highest in the presence of alphadolone acetate followed by decadron and was the least with cholic acid salt.

Inocula were prepared of overnight cultures of C. albicans grown in the presence and absence of cholic acid salt. The uptake of oxygen by C. albicans was measured by Warburg manometry on a glucose substrate with and without cholic acid salt. Fig. 15 shows that in the presence of cholic acid salt the enhancement of oxygen uptake occurred for both types of inocula, although a greater effect was found for the inoculum grown in a medium containing cholic acid. However, the uptake of oxygen was similar for both types of inocula when only glucose substrate was used.

TABLE 12

Minimum inhibitory concentrations (M.I.C.) of some polyene and non-polyene antifungal agents towards the yeast form of C. albicans A39 in the absence and presence of steroids

Antifungal Agent	M.I.C. $\left(\mu g/ml\right)$			
	Control	0.15% cholic acid salt	0.05% decadron	0.05% alphadolone acetate
Nystatin	3.15	6.25	6.25	6.25
Amphotericin B	0.78	3.125	6.25	12.5
Trichomycin	0.048	0.09	0.78	0.78
Griseofulvin	62.5	62.5	62.5	62.5
4,7, -phenanthroline-5,6 - quinone	12.5	12.5	12.5	12.5

TABLE 13

Minimum Inhibitory concentration (M.I.C.) of some polyene and non-polyene antifungal agents

towards the mycelial form of C. abicans A39 in the absence and presence of steroids

	M.I.C. (μg/ml)			
Antifungal Agent	Control	0.15% cholic acid salt	0.05% decadron	0.05% alphadolone acetate
Nystatin	.78	3.125	3.125	3.125
Trichomycin	.38	.78	1.56	1.56
Griseoflulvin	7.8	7.8	7.8	7.8

TABLE 13a

Minimum inhibitory concentrations (M.I.C.) of some polyene and non-polyene antifungal agents
towards the yeast and mycelial form of two strain of C. albicans

Antifungal Agent	Yeast Form		Mycelial Form	
	A39	IMI 45348	A39	IMI 45348
Nystatin	3.15	6.25	0.78	1.58
Trichomycin	0.048	0.09	0.38	0.78
Amphotericin B	0.78	1.56	-	-
Griseofulvin	62.5	62.5	7.8	7.8
4,7 - phenanthroline 5,6 - quinone	12.5	18.75	-	-

PLATE 71   C. albicans grown on Magdala red medium containing

                 no steroids. Incubated for 24 hours at 37°C. Note

                 the tinted light red colonies.

PLATE 72   C. albicans grown on Magdala Red medium containing

                 0.025% cholic acid salt. Incubated for 24 hours

                 at 37°C. Note the slight change in colonies colour

                 as compared to the control ones.

PLATE 73   C. albicans grown on Magdala Red medium containing

                 0.05% cholic acid salt. Incubated for 24 hours at

                 37°C. Note the deep red sheen colonies in contrast

                 to the control ones.
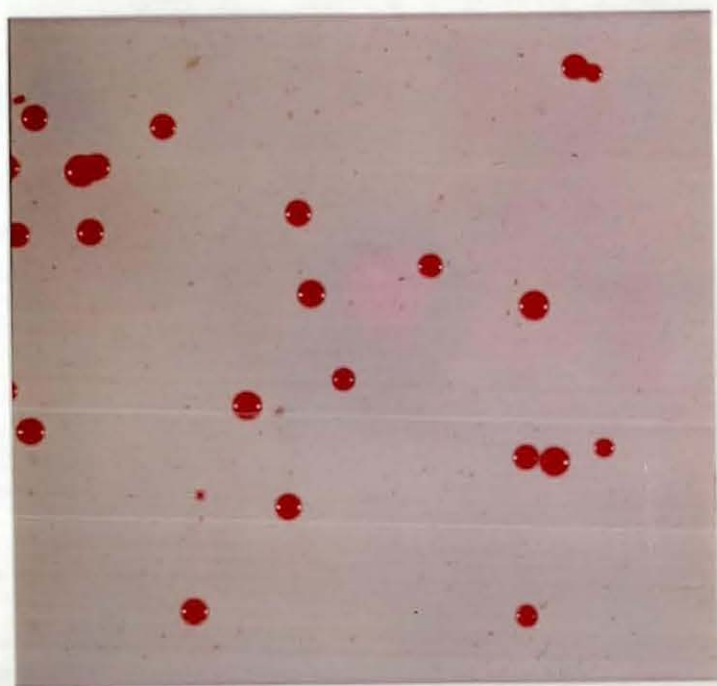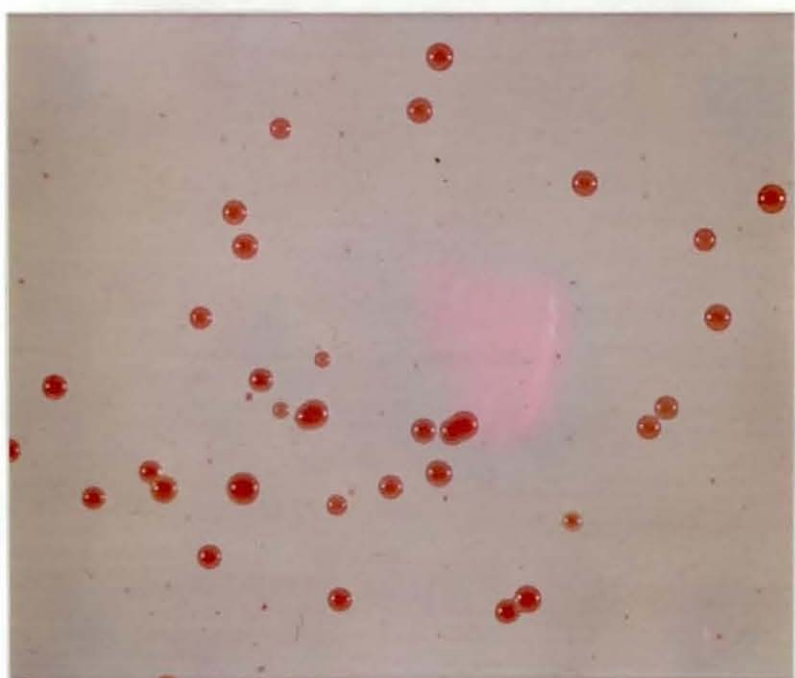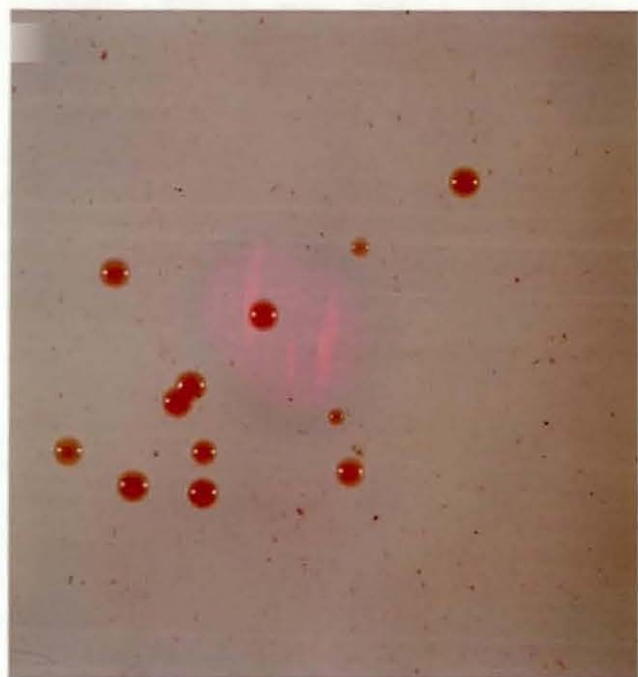
FIG. 12 : The effect of steroids on mitochondrial

formation. The number of mitochondria in

cells grown in medium containing: cholic

acid (O-O), decadron (●-●), or alphadolone

acetate (Δ - Δ) were counted. Values

plotted represent the averages obtained

from 20 to 25 cells at each concentration.
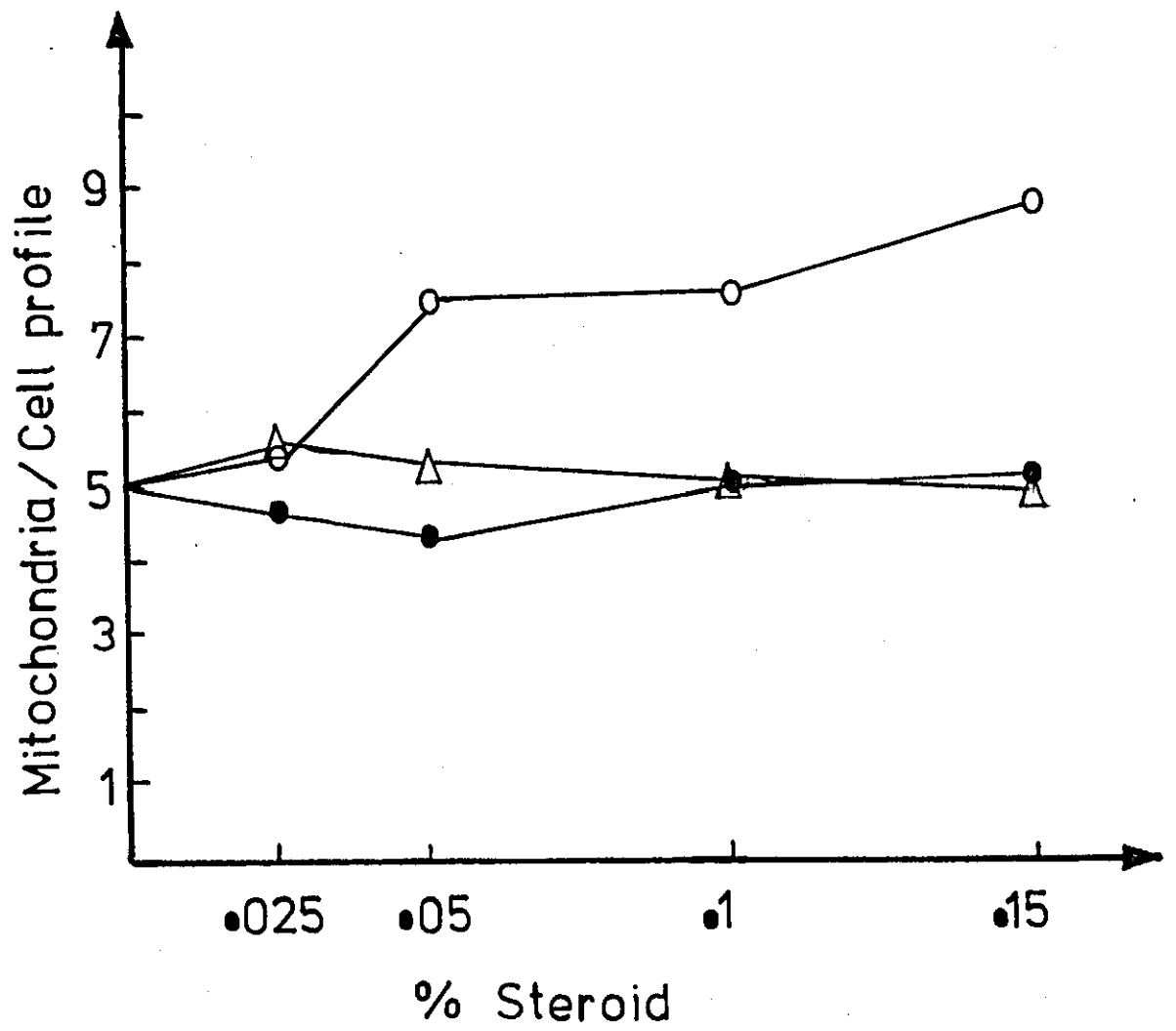
FIG. 13 : Oxygen uptake by a suspension of intact
cells of C. albicans. Gas phase air.
Temperature $30^{\circ}C$. CO_2 was absorbed.
Substrates:□ — □ glucose; ●-● 0.15%
cholic acid salt; X-X 0.1% cholic acid
salt + glucose; Δ - Δ 0.15% cholic acid
salt + glucose and 0-0 0.2% cholic acid ·
salt + glucose.
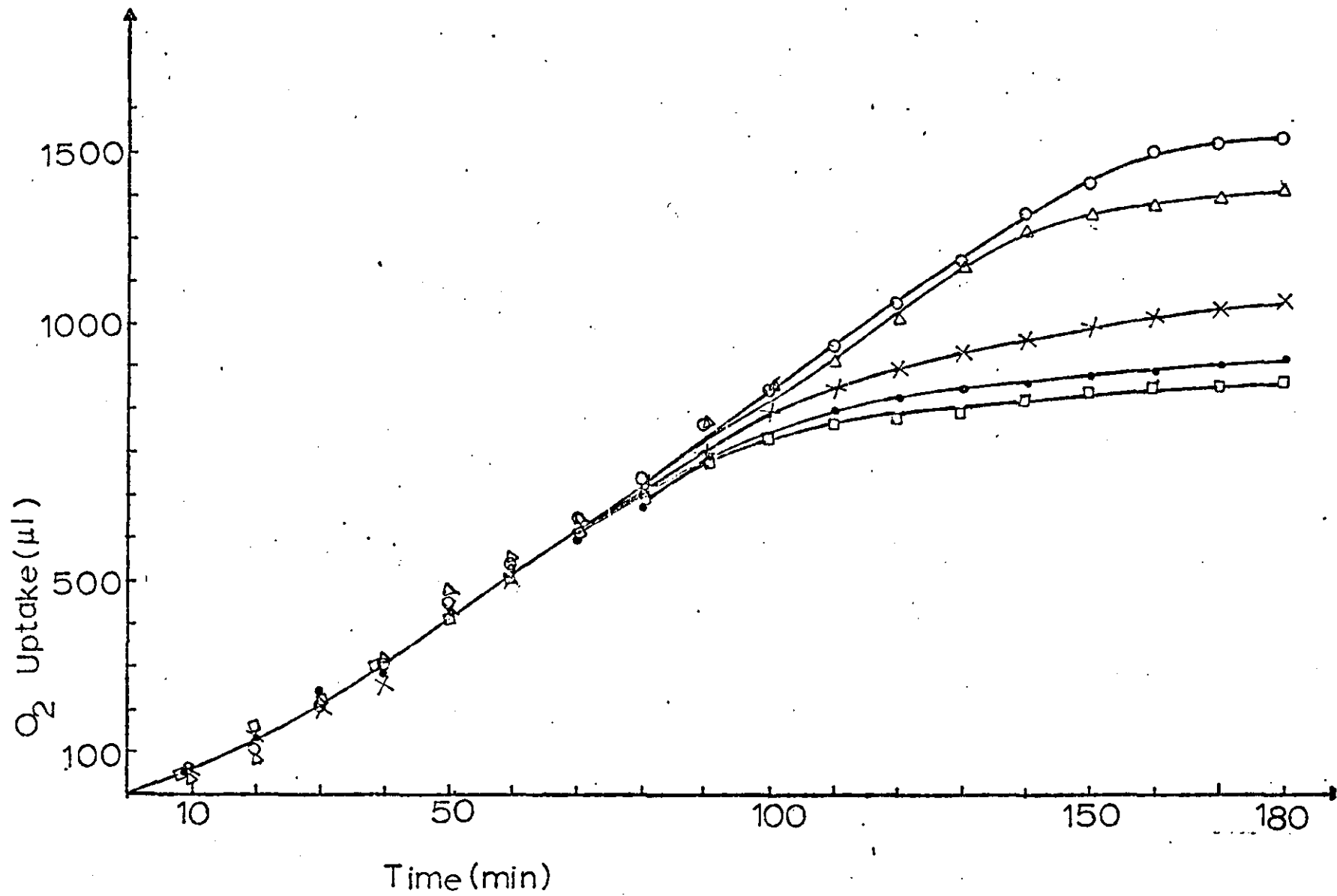
FIG. 14 : Oxygen uptake by suspension of intact

cells of C. albicans. Gas phase air.

Temperature $30^{o}C$. CO_2 was absorbed.

Subtrate: ●-● glucose X-X 0.1% cholic

acid salt + glucose, O-O 0.1% decadron

+ glucose; and Δ - Δ 0.1% alphadolone

acetate + glucose.
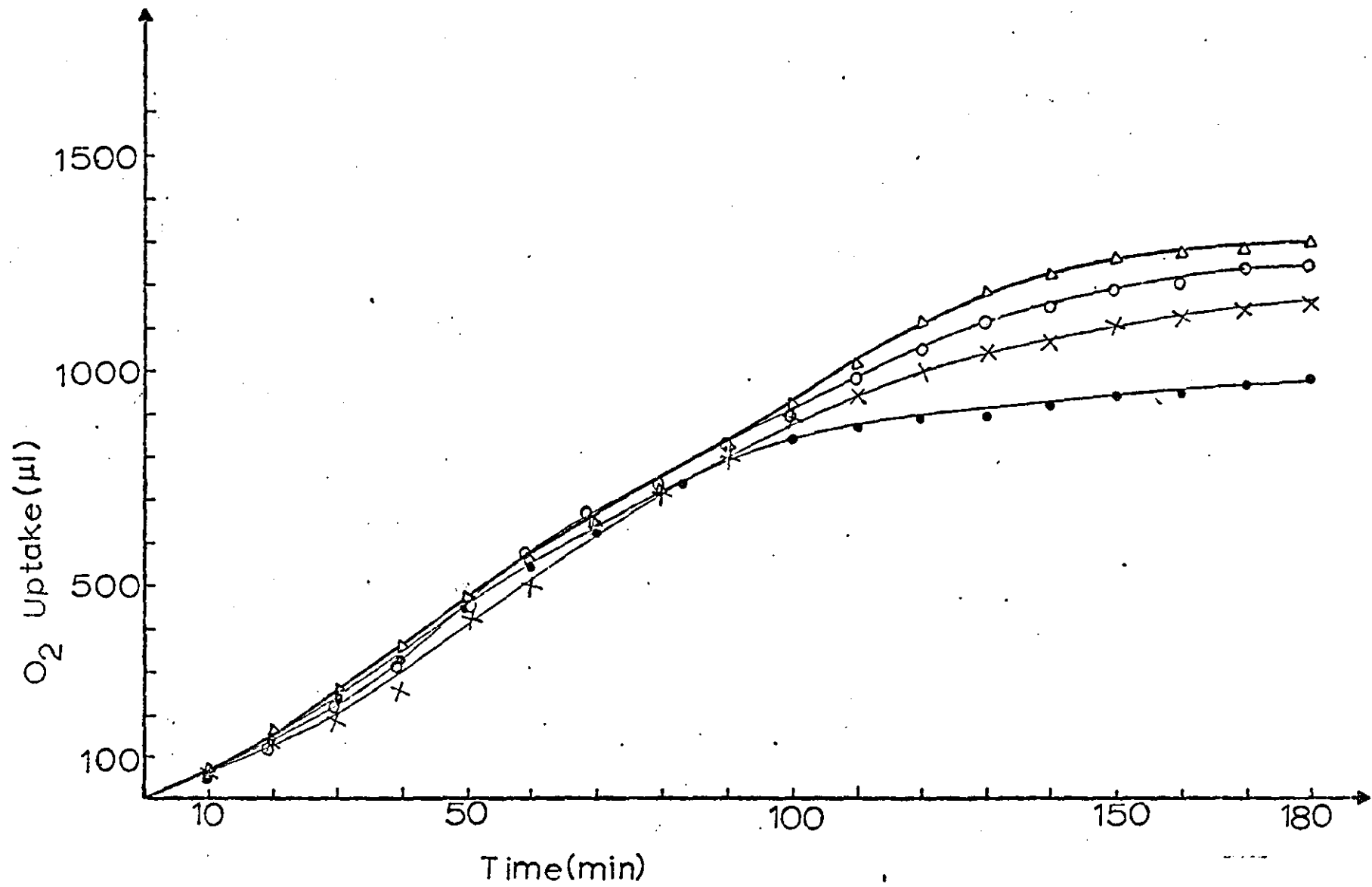
FIG. 15 :   Effect of steroids on oxygen uptake

Gas phase air. Temperature 30°C.
Test organism $\underline{C}$. $\underline{albicans}$. Medium
Neurospora medium $\pm$ 0.15% cholic acid
salt.

<u>KEY</u>

□——□   Subtrate: Glucose+ 0.15% cholic acid salt.
Inoculum grown overnight in the presence
of .15% cholic acid salt.

X - X   Subtrate: Glucose 0.15% cholic acid salt.
Inoculum: grown overnight in the absence
of .15% cholic acid salt.

Δ - Δ   Subtrate: Glucose.
Inoculum: grown overnight in the presence
of .15% cholic acid salt.

O —— O   Subtrate: Glucose.
Inoculum: grown overnight in the absence
of .15% cholic acid salt.
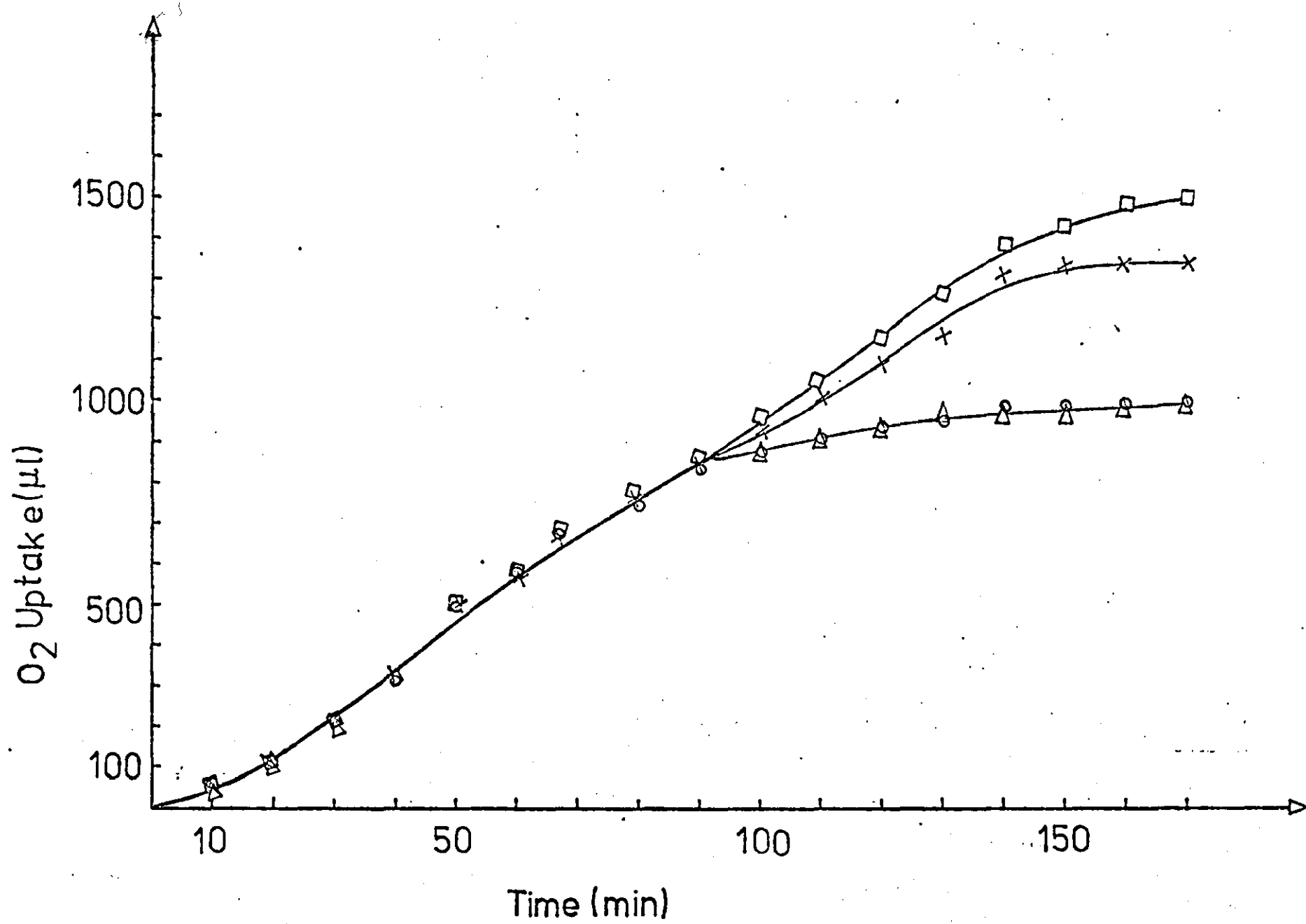
CHAPTER SEVEN

DISCUSSION

The effect of steroids supplementation on growth

The effect on steroids on microorganisms has been widely studied and the results obtained have been diverse and contradictory. Hasany, Basu and Kazdan (313) used decadron and studied its effect on the growth of C. albicans and A. niger using dry weight as a measure of cell growth. They found that both microorganisms showed a progressive increase of their growth rates as the concentration of the steroid in the growth medium was increased. Under similar experimental conditions, A. niger showed a higher growth rate than C. albicans. Stimulation of growth was found in Phytophthera infestans (314) in the presence of cholesterol, Mycobacteria species when ergosterol was used (315), or low concentrations ($10^{-7} - 10^{-9}$M) of a variety of androgenic, oestrogenic and adrenal hormones in the case of streptomycin-bleached Euglena sp. (316)

Candida albicans grew well in the presence of cholic acid, decadron and alphadolone acetate as judged by an increase in dry weight or optical density of the cultures. The increase in cell numbers in the presence of the added steroids was seen as an increase in viable numbers.

However, stimulation of growth in the presence of exogenous sterols does not always occur because Neurospora crassa is specifically inhibited by deoxycorticostercone (317). This compound also inhibits the growth of many Gram-positive and Gram-negative bacteria as well as moulds (N. crassa, A. niger, Penicillium lilacinum and P. puberulum) and yeasts (Torulopsis utilis and S. cerevisiae) (318)

In a study by Jefferson et. al (52), several natural steroids and diethylstilbesterol inhibited the growth of Torula utilis. Mule and Garufi (319) showed that cortisone (10^{-4}M) inhibited the growth and caused cytoplasmic changes in Salmonella typhosa. Recently, Kushwaha (320) showed that prednisolone (5, 10, 50 ppm) inhibited mycelial growth of a number of keratinophilic fungi. Other workers showed that steroids exhibited neither stimulation nor inhibition. Cicchini, and Cao-Pinna (321) reported that testosterone had no effect on Salmonella and Proteus species. Both testosterone (321) and cortisone (322) had no effect on E. coli.

Steroids affected not only the growth rates of fungi but also those of cells in tissue cultures. Crossfeld and Regan (323) reported inhibition of growth of chick embryo fibroblasts by hydrocortisone at concentrations of 400-500 μg/ml. Rasche and Uliner (324) reported that the effect of glucocorticoids on the cell growth of L-cells and epithelial cells is biphasic, i.e. growth inhibitory at low concentrations. Similar results were obtained by Ueki, Fukushima, and Kimato (325) using dexamethasone sodium sulphate. They found that with concentrations of 450-900 μg/ml dexamethasone sodium sulphate, the multiplication of fibroblasts was depressed. On the other hand with 90-230 μg/ml of steroid in the medium, cell multiplication was accelerated.

The response to steroids may be due to the concentration used or to the particular molecule. Concentration has been shown to be important (326) as low concentration of oestradiol stimulated growth of E. coli. whilst higher levels were inhibitory.

The effects observed when cholic acid, decadron or alphadolone acetate were used showed an increase in stimulation up to 0.15% and no inhibitory effects were seen with C. albicans

The molecular structure necessary for the stimulation action of steroids was reported by a number of workers. Cailleau (327) was among the first to relatesterol structural configuration and growth factor activity for a group of microorganisms. He stressed the importance of side chain at C-17 and the 3-β-hydroxyl group. Suppression of the hydroxyl group at position 3 or the degradation or complete elimination of the side chain inactivated the molecule. Van Wagtendonk (328) showed in a study of the molecular structural requirements for activity in Paramecium aurelia that the addition of a side chain at position 24 coupled with a 3-ᴄhydroxyl group was required for enhancement of growth. Degradation of the side chain leading to bile acids or replacement of the side chain by a ketone or hydroxyl group inactivates the molecule.

Buetow and Levedahl (316) suggested different functional groups for the stimulatory action of steroids in Euglena system. This included either a ketone or hydroxyl groups at Carbon 3 or 17 or both and an overall planar configuration. Jefferson et al (52) in a study of A. niger concluded that the fungus has a metabolic requirement for a steroid with a hydroxyl group at C-3, a double bond in the 5-6 position and a side chain similar to that in ergosterol or cholesterol. Elliott, et al (54) have examined many sterols and steroids and concluded that active compounds must possess a 3-β-hydroxyl with methyl groups at C-10 and C-13 and a side chain containing 8-10 carbons at C-17.

Recently, Langcake (314) testing cholesterol and a number of other 4-demethyl sterols, suggested that the presence of a free hydroxyl groups at C-3 appears to be necessary for growth promoting activity. Thus, cholestane, cholesterone and cholesteryl stearate were inactive.

In this study, it was observed that alphadolone acetate enhanced the growth of C. albicans to a greater extent than decadron. The least enhancement was obtained by cholic acid salt. The three steroids have molecular structural features that should enable them to enhance the growth of microorganisms. Alphadolone acetate contains a 3-β - hydroxy group, methyl groups at C-10 and C-13 and a side chain at C-17. Cholic acid salt contains a 3-β-hydroxyl group, methyl groups at C-10 and C-13 and a side chain at C-17. Both of these steroids meet the criteria of Elliott et al (54) Cailleau (327) and Langcake (314) (see above) for structural features required for growth enhancement. The results obtained with Decadron agree with the criteria of Buetow and Levedahl (316) that possession of a keto group at C-3 and hydroxyl group at C-17 gives growth stimulation.

The presence of a keto group at C-11 and C-20 of alphadolone acetate may account for its maximal enhancement. Also the possession of hydroxyl group at C-11, methyl group at C-16, 9-fluorination by decadron may explain why this compound enhances growth better than cholic acid salt. The structural difference of steroids may be another reason for the diversity of the response of microorganisms to steroids (see above).

The use of sterols and steroids as carbon substrates by micro-organisms was first observed by Sohngren (329). He showed that microorganisms could use a sterol as a carbon source for growth.

In this study, it was found that C. albicans utilised acetate, cholic acid salt and glucose as a carbon source. This is in agree-ment with the findings of Santer, Ajl and Turner (330). They showed that Pseudomonas sp. grew well with acetate or glucose or progesterone, testosterone, cholesterol or related steroids as carbon sources. However, Schatz, Savard and Printner (331) showed that whilst Nocardia spp. used cholesterol testosterone and progesterone as a carbon source, estradiol, estriol and ergosterol were found to be inhibitory. The fungus, in the present work, utilised cholic acid salt better than glucose but not as well as acetate (Fig. 11). This suggests that the fungus used cholic acid salt as an alternative carbon source and was able to utilise the steroid more efficiently than glucose. This agrees with Hasany et al (313) who suggested that fungi grown in media containing deca-dron were not entirely dependent on the presence of dextrose in the media. They suggested the steroid as an alternative carbon source.

It is not known, during this study, whether the fungus utilises the breakdown products of steroids, or incorporates the steroid intact. This would require more work and the use of radio labelling techniques. However, Tak (332) suggested that three Mycobacteria species were able to decompose cholesterol when it was the sole carbon source. Turfitt (333) showed that Proactinomyces spp. were able to breakdown choles-terol in fertile soils, whilst many other bacteria, moulds and actino-mycetes were inactive.

He suggested that this bacterium oxidised hydroxy steroids to keto steroids, through ring fission and removal of the side chain (334). Furthermore, Schatz, Savard and Printner (331) showed that compounds underwent further degradation so that they were utilised by the microorganisms. Santer and Ajl (335) showed that when Pseudomonas sp. were grown in the presence of labelled testosterone, the label appeared in protein, nucleic acid and some tricarboxylic acids as well as in carbon dioxide.

Effect of exogenous steroids on morphology

The effect of exogenous steroids on the morphology of micro-organisms has received little attention and most of the work reported deals with the effects of steroids on the morphology of mammalian cells. Herbert, Cisneros and Rennels (336) in a study of the morphological changes in prolactin cells of male rats reported that after testosterone propionate administration, cells became more ovoid and the golgi complex enlarged displaying dilated cisternae and many immature secretory granules. In addition, the diameter of the secretory granules was greater in the animals given the steroid. Laishes and Williams (337) observed that dexamethasone supplementation ($1 \mu M$) of the culture medium maintained the polygonal epithelial morphology of adult rat hepatocytes and increased longevity.

In this study when steroids were added to the growth medium it was shown that the yeast cells were of two different types. Scanning electron microscopy showed that the basic difference between the two types lies in surface smoothness and size. One type of cells was large and more elongated with a smooth surface, the others were smaller and rougher.

Transmission electron microscopy showed the difference in the internal structure of these two types. Large cells with smooth envelopes containing distinct intracytoplasmatic organelles, and small cells with convoluted envelopes and obscure internal structures.

The alterations in the surface of rough cells are most probably due to a change in cell permeability, which is in agreement with ultra-structural observations that showed electron thin areas. The electron thin areas are probably due to decrease in density, such as decrease in density can occur only through loss of material through the cytoplasmic membrane. The effect on the plasma membrane is either a permeability or interference with the energy - dependent membrane - transfer mechanism.

Rough cells examined by S.E.M. were found by T.E.M. to have damaged internal structures. However, results from T.E.M. showed that some smooth surfaced cells were in fact damaged internally. Thus it is likely that many of the apparently healthy smooth cells seen under S.E.M. were in fact non-viable and smoothness is therefore not a criterion for viability. This agrees with the findings of De Nollin and Borgers (338). They found that C.albicans cells with a normal looking smooth surface as seen by S.E.M., were found inside to be completely necrotic upon T.E.M. examination.

Examination by Light Microscopy showed that the mycelial forms of C. albicans exhibited shorter swollen hyphae when grown in the presence of steroids.

The presence of these shorter hyphae was confirmed by S.E.M., also hyphae with constrictions were observed.

In addition S.E.M. showed the presence of collapsed hyphae. This may be due to a loss of cytoplasmic contents as a result of steroid effect, or may be partly due to the method of preparation (vacuum damage).

In this study it was shown that as the concentration of steroid increased the percentage of mycelial to yeast form decreased. This transformation may occur in one of two ways (i) the formation of budding, club-shaped structures at the tips of the hyphae or on the lateral hyphal branches and (ii) the formation of chains of oidia and subsequent fragmentation of the chains into their constituent yeast-like elements (339). The transformation which took place in the present work, most probably follows the second scheme, where constriction of the hyphae (see above) was followed by fragmentation into yeast like forms.

The effect of steroids on the transformation of mycelial to yeast form may have an implication on the pathogenicity of C. albicans. As mentioned in Chapter Four, the pathogenicity of C. albicans may be dependent on the form of fungus takes in vivo. In this case the transformation into the yeast form, in the presence of steroids may enhance the pathogenicity of the fungus in vivo. It should be remembered that the relationship of pathogenicity and form is not a simple one, and that other factors have to be considered (Chapter Four).

The results obtained from the effect of steroids on morphology seem to contradict their effect on growth. As reported earlier steroids enhanced the growth rate of C. albicans. The morphology shows that some of the cells are dying due to cytoplasmic leakage and internal disorganisation. This would imply that the growth rate of cells grown in the presence of steroids should be slower due to the loss of certain cells.

This contradiction could be explained on the basis of adaptation. Adaptation is a fundamental property by means of which microbial cells react to adverse environmental conditions. Salkin (340) reported adaptation of numerous strains of filamentous fungi to cycloheximide. The extent of this adaptation varied in part with the species and strain of the microorganism. Capek and associates (341) reported that adaptation of C. albicans to 2-methyl-thio-5-(3-iodo-propargyloxy) pyrimidine. They demonstrated the presence of an enzyme system catalysing the splitting-off of the iodopropargyloxy chain of the antimycotic; giving rise to antimycotically inactive 2-methyl thio-5-hydroxy pyrimidine. Adapted cells are capable of undergoing this transformation, unadapted cells, however, are incapable of doing so.

It is possible that steroids are toxic to C. albicans and interfere with the dynamics of cellular homeostasis and induce in the cell system reactions which to some extent abolish their toxic effect. Cells that adapt to the steroids start growing and multiply rapidly (as noticed with the involvement of cells in cell division at high steroid concentrations) and more than compensate for the loss of the unadapted cells.

Effect of exogenous steroids on the sterol content of C. albicans

The effect of steroid supplementation on the lipid content of microorganisms was studied by Bulman (342) who found little change in their lipid composition after steroid supplementation. He suggested that the membranes of E. coli and S.aureus had therefore remained unchanged.

In this study it was shown that C. albicans grown in the presence of exogenous steroids showed an increase in the total lipid content compared to a decrease in sterol content. The variation of lipid composition, due to steroid supplementation, was not studied. However, Bulman (342) showed that in the case of C. albicans and S.cerevisiae the action of alphaxolone (0 to 600μg/ml) resulted in an inhibition of the synthesis of the ninhydrin-positive lipids. Alphaxolone was the only compound, out of a number of steroids tested, which could modify the synthesis of the lipids. However, some of the sterols (5-androstan-3β -ol, 5β - cholestan -3α-ol and 5-cholesten-3α-ol) did modify the response of C. albicans to the thiol-active compound bronopol and phenyl-mercuric nitrate.

A prerequisite for the study of the change in sterol content by steroid supplementation, is the ability to identify the different sterol fractions in C. albicans. This was done by a combination of t.l.c. g.l.c. and various spectrophotometric techniques (IR, U.V. NMR and mass spectrophotometry). It was found that a combination of these techniques was necessary to identify most of the sterol fractions. The availability of standards was also important.

In this study it was shown that the yeast form of C. albicans A39 contained less squalene and ergosterol than the mycelial form. However, the yeast form contained slightly higher amounts of lanosterol and 4α-Me zymosterol than the mycelial form. Similarly, the yeast form of C. albicans IMI 45348 contained less squalene. However, there was no significant difference between the two forms in ergosterol content. Unlike C. albicans A39, higher amounts of 4α-Me zymosterol content was present in the mycelial form than in the yeast form. Whereas 4,4' dimethyl-zymosterol was detected in both forms of C. albicans A39, it was present only in the mycelial form of C. albicans IMI 45348.

The relationship between the process of adaptation and sterol content of C. albicans was studied by Capek et al (132). They showed that adaptation to the antimycotic VUF B-9244 was accompanied by a decrease in the sterol content of the cells with a lower ergosterol content.

Results obtained in this study agree with those of Capek et al (132). It was shown here that C. albicans grown in the presence of steroids had a lower ergosterol content. No increase in the theoretically possible steroid breakdown products of ergosterol was detected chromatographically when the fungus was grown in the presence of steroids. This would suggest that the lower ergosterol content is not due to enzymatic degradation of preformed ergosterol, but to inhibition of its synthesis.

This agrees with Capek et al (132). Barton and co-workers (173) showed 4,4' dimethyl zymosterol to be an intermediate in the biosynthesis of ergosterol. In this study, the accumulation of 4,4' dimethyl zymosterol may suggest a possible site of inhibition. Since ergosterol appears to play an important role during adaptation, it would be interesting to resolve the biochemical function of this compound in the the microbial cells.

The accumulation of califerol after growth of C. albicans in the presence of steroids may be due to either one of two possibilities: (i) an alteration in the route of ergosterol precursors leading to calciferol production instead of ergosterol; or (ii) production of calciferol as a product of irradiation of ergosterol brought about by changes in cell boundaries. However, growth of C. albicans in dark, under similar conditions did not alter the effect of steroids on calciferol content. This would rule out the second possibility, and suggest that calciferol accumulation is due, most probably, to a change in the biosynthetic process of sterols.

The appearance of an unidentified sterol fraction may be yet another way of responding to steroid supplementation. This sterol may be incorporated into the cell membrane rendering it adaptable to the exogenous steroids. Attempts to identify this sterol were unsuccessful, it was not detected on t.l.c. However, it was shown that this sterol is not a breakdown product of either calciferol or ergosterol.

The adaptation of the mycelial form of C. albicans manifested
itself with a lowering of the ergosterol content and an increase
in calciferol content. However, unlike the yeast form the "unidentified"
sterol was not detected. This may be due to the differences in the
sterol content and possibly the biosynthetic route of yeast and
mycelial form may differ.

The effect of steroids on the metabolism of C. albicans.

The biochemical responses of microorganisms to added sterols and steroids are almost as diverse as growth responses. These compounds affect the general metabolism of many microorganisms, however, the primary action of a sterol or steroid on any microorganism is not yet known. Buetow and Levedahl (343) reviewed the metabolic responses of microorganisms to sterols and steroids.

In this study it has been shown that all three steroids tested, stimulated oxygen uptake by C. albicans, which agrees with the work of Taylor (344). Using luminous bacteria, he reported a .stimulation of respiration when they were grown on a minimal salt medium containing steroids prepared from adrenal cortex. This stimulatory effect was not observed when a nutrient medium was used. Also concentrations of testosterone (10^{-6} - 10^{-7} M) enhanced both the endogenous and substrate respiration rates of Euglena sp. whether or not the·cells were previously grown in the presence of the steroid (345). Furthermore the stimulatory effect of testosterone was dependent upon the presence of an inorganic nitrogen source, and it was suggested that the effect of testosterone was on the permeability of nitrogen-containing compounds or to some enzyme system involved in the synthesis of proteins from simple nitrogen-containing compounds. However, other workers reported inhibition of respiration by steroids. Meier, and Schuler (346) Lester and Hechter (347) reported that deoxycorticosteroid inhibited the respiration of yeast and Neurospora crassa. Hydrocortisone was reported by Baba, Nagata and Nakajima (348) to inhibit the respiration of Trichomonas vaginalis.

In some cases, there appears to be a concentration effect of the steroids. In this study, the effect of steroids tested did not depend on concentration. However, Baba et al (348) suggested that at low concentration cortisone stimulated the respiration of T. vaginalis and at high concentrations inhibited it. Similar results were found for hydro-cortisone on Salmonella typhosa (349).

In this study, oxygen uptake was highest in the presence of alphadolone acetate followed by decadron and was the least with cholic acid salt. This pattern of metabolic response to steroid supplementation is similar to that for growth (see above). This would suggest a correlation between the two responses.

Since the work of Ephrussi (350) various criteria have been employed for diagnosing respiratory mutation in yeast. They include oxygen consumption, colony size, cytochromes, utilisation of carbon sources (selective culture media) and certain colour differentiation by suitable dyes (351).

The exact nature of this respiratory mutation is not known, however, Nagi and co-workers (351) proposed two possibilities: (i) permanent inactivation or elimination of one or many of the respiratory enzymes and (ii) inhibition of the formation of enzymes by a specific block or disturbance in the cell metabolism. This mutation is affected by a certain factor which resides in the cytoplasm as well as by a nuclear gene mutation.

It was inferred that the "cytoplasmic" factor which controls the formation of a series of respiratory enzymes is autonomous in its replication but is dependent on a dominant nuclear gene in its function.

In this study it was shown that when C. albicans was cultured on a magdala red medium supplemented with cholic acid salt, there was a change in the colour of the colonies from tinted light red in the control to deep red-sheen colonies in the supplemented medium. No change in the colour of the colonies on Magdala red medium supplemented with either alphadolone acetate or decadron was observed. This would suggest that cholic acid salt impaired the respiratory enzymes leading to a phenotypic change, while alphadolone acetate and decadron did not. Bulman (342) showed that when C. albicans and S. cerevisiae were cultured on magdala red medium supplemented with steroids, no change in the colour of the colonies was observed, suggesting that steroid supplementation failed to induce impairment of the respiratory enzymes. His work agrees with the results obtained for alphadolone acetate and decadron.

The difference in response between alphadolone acetate and decadron on one hand, and cholic acid salt on the other would suggest an alternative adaptation mechanism for cholic acid salt.

Hartmand and Liu (352) suggested that mitochondria were present in respiratory mutant cells as well as in normal yeast cells. However, apparent differences were seen between the mitochondria from the two types of yeast cells.

The differences in shape and size of mitochondria whether they are spherical, elongated or threadlike remain unresolved.

In this study, it was shown that the number of mitochondria in C. albicans grown in a medium supplemented with cholic acid salt were higher than those cells grown in an unsupplemented medium. In the literature there is no mention of the difference in the number of mitochondria when cells are grown in steroid containing media.

No apparent difference in the size and shape of mitochondria from the two types was observed. No significant difference was observed in the number of mitochondria of cells grown in the medium supplemented with alphadolone acetate and decadron, or those grown in an unsupplemented medium. This agrees with the results obtained from the Magdala red colour differentiation. However, it is important to draw attention to the fact that electron microscopy of random thin sections of eukaryotic cells such as yeasts and algae gives inadequate visualisation of the three-dimensional arrangements of intra-cellular contents, making it difficult to count the exact number of mitochondria present per cell. The method suggested by Davison and Garland (353) using serial sectioning and model building of mitochondrial structure, to obtain a three-dimensional picture of the mitochondria, is superior.

The relationship between resistance to polyene antibiotics and sterol content of fungi is well established. In view of the change in sterol patterns in the presence of steroids, the change in resistance was also examined.

It was shown in this study that the resistance of C. albicans
to polyene antifungal agents increased in the presence of steroids.
Resistance to amphotericin-B appears to reach the highest level for
C. albicans grown in the presence of alphadolone acetate (16-fold),
with decadron half this level (8-fold) whilst with cholic acid the
resistance was only 4-fold. Resistance to trichomycin reached the
same level for each of alphadolone acetate and decadron (16-fold),
whilst in the presence of cholic acid salt there was only a two fold
increase. Resistance to nystatin was increased to the same level
(2-fold) in the presence of each of the three steroids studied.

The degree of polyene resistance obtained in the present experi-
ments agrees with that obtained by other workers, all used "training"
techniques on C.albicans. Bodanhoff (354) and Boudru (355) reported
increases in nystatin resistance of 2-to-6-fold, while Athar and Winner
(125) obtained rather greater values (30-to-40-fold). Resistance to
amphotericin-B appears to reach higher levels. Lones and Peacock (356)
and Hebeka and Solotorovsky (357) reported increases respectively of
29-to-60-fold, while others reported increases of more than 1000-fold
(358).

In this study the decrease in ergosterol content and development
of resistance to polyene antibiotics of C. albicans suggest that the
development of resistance was a result of the decrease in ergosterol
content. This agrees with the findings of Athar and Winner (125).
However, this conflicts with the results of Hamilton-Miller (359).
He induced sterol deficient C. albicans by mutation and found that
increased ergosterol levels accompanied polyene resistance. Recently,
Russell, Kerridge and Bokor (360) related the sensitivity to polyenes
of A. fumigatus conidia during germination with the membrane sterol
metabolism.

It would appear that the resistance to polyene antibiotic is more complex than relating it to the amount of ergosterol present in the fungal membranes. Other factors need to be considered (Chapter two) such as the presence of wall and the metabolic state of the organism (361).

Recently, the variation in sensitivity to polyenes was attributed to differing fatty acid compositions by Koh, Mariott, Taylor and Gale (362).

. Frenkel (363) in a study of the role of corticosteroids as pre-disposing factors in fungal diseases laid down the criteria for the structural modification of progesterone (as a parent compound) required to enhance acute fungal infections. He suggested that 11-hydroxylation was of primary importance in producing infection-enhancing effects; 17-hydroxylation of secondary importance, and 21-hydroxylation of tertiary importance. This applies only to modification of the basic 21-carbon steroid structure with keto groups in position 3 and 20 and a double bond at 4, i.e. progesterone. The infection-enhancing effects of steroids can be potentiated further by a double bond between 1 and 2, by 6- or 9- fluorination, by 6- methylation, by 16- hydroxylation or methylation, and by certain other modifications (364).

In this study, decadron possesses most of the functional groups required by the criteria of Frenkel for the enhancement of fungal infections.

Decadron possesses hydroxyl groups at positions 11, 17 and 21, a double bond between 1 and 2, 9- fluorination and 16- methylation. The criteria of Frenkel does not apply to alphadolone acetate and cholic acid salt because they are not modifications of progesterone. However, these two steroids possess the functional groups required for enhancing the growth of the fungus, namely, a 3-β-hydroxyl group, methyl groups at C-10 and C-13 and a side chain at C-17. Alphadolone acetate and cholic acid salt affected the morphology of C. albicans in a similar manner to decadron, suggesting that the functional groups they possess are of importance to infection-enhancing effects.

It is important to mention that the enhancement of infections depends not only on steroid structure, but also on biological half-life, especially solubility and rate of degradation (364). Also the route of steroid administration to the host has been shown to be of vital importance (365).

More work is required to establish a structure/activity relationship for alphadolone acetate and cholic acid, and infection-enhancing effects.

The role of steroids as a predisposing factor in the production of fungal infections was attributed to either an effect on the host or the fungus itself. In this study it was shown that the growth of C. albicans in the presence of steroids lead to a response which manifested itself by an adaptation mechanism.

This adaptation mechanism included a number of changes in growth, morphology, sterol content, metabolism and resistance of the fungus. The effect of steroids on the host included direct tissue toxicity, alterations of the gastrointestinal flora and alteration of the immunologic response (Chapter four).

It would appear that steroids have a dual effect in predisposing to fungal infections, affecting both the fungus and the host.

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<u>A P P E N D I X   I</u>

FIG. 1 : U.V. absorption spectrum of sterols

extracted from the yeast form of

C. albicans. The dotted spectrum

is that of standard ergosterol.
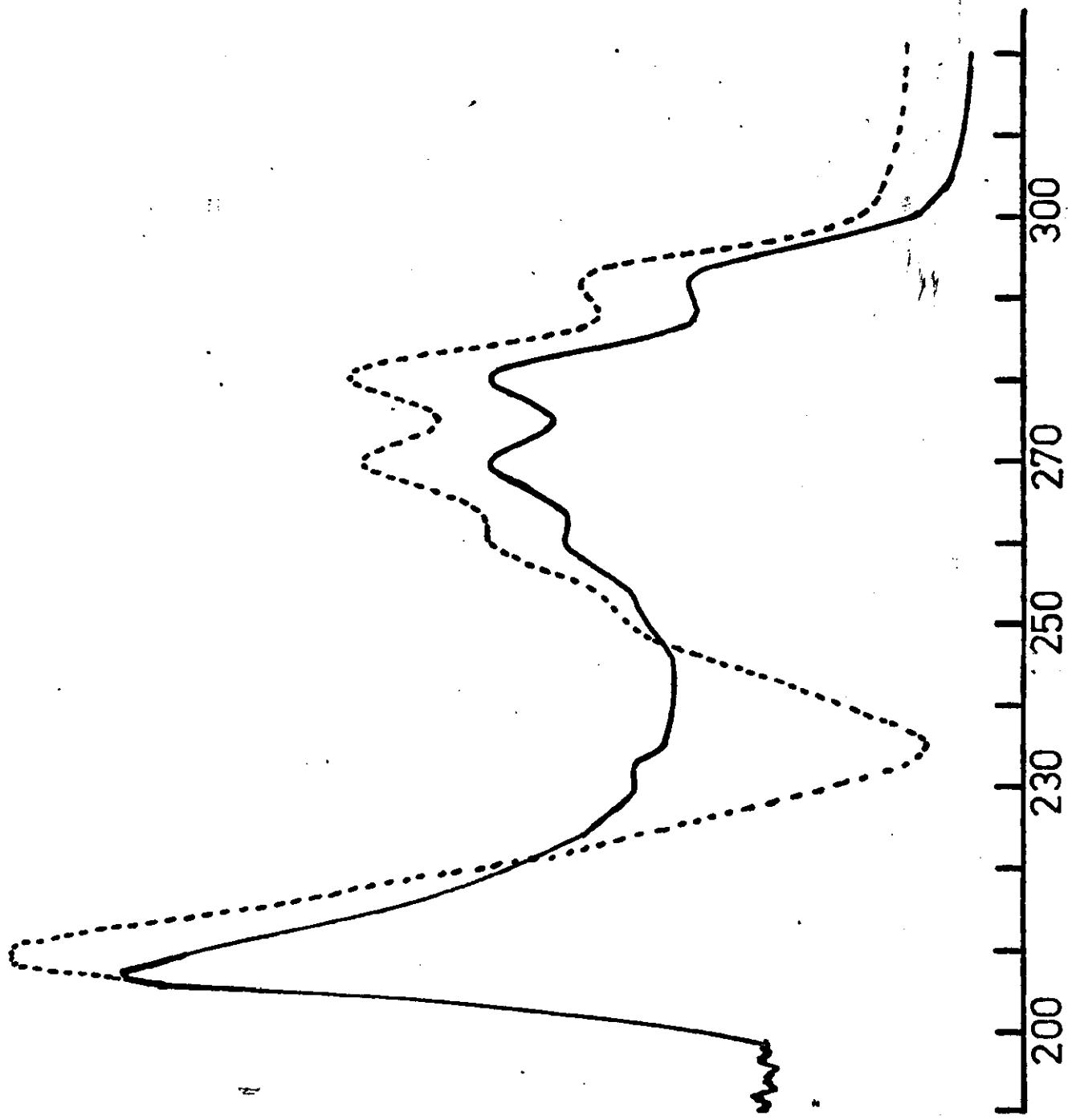
FIG. 2 : I.R. spectrum of ergosterol isolated
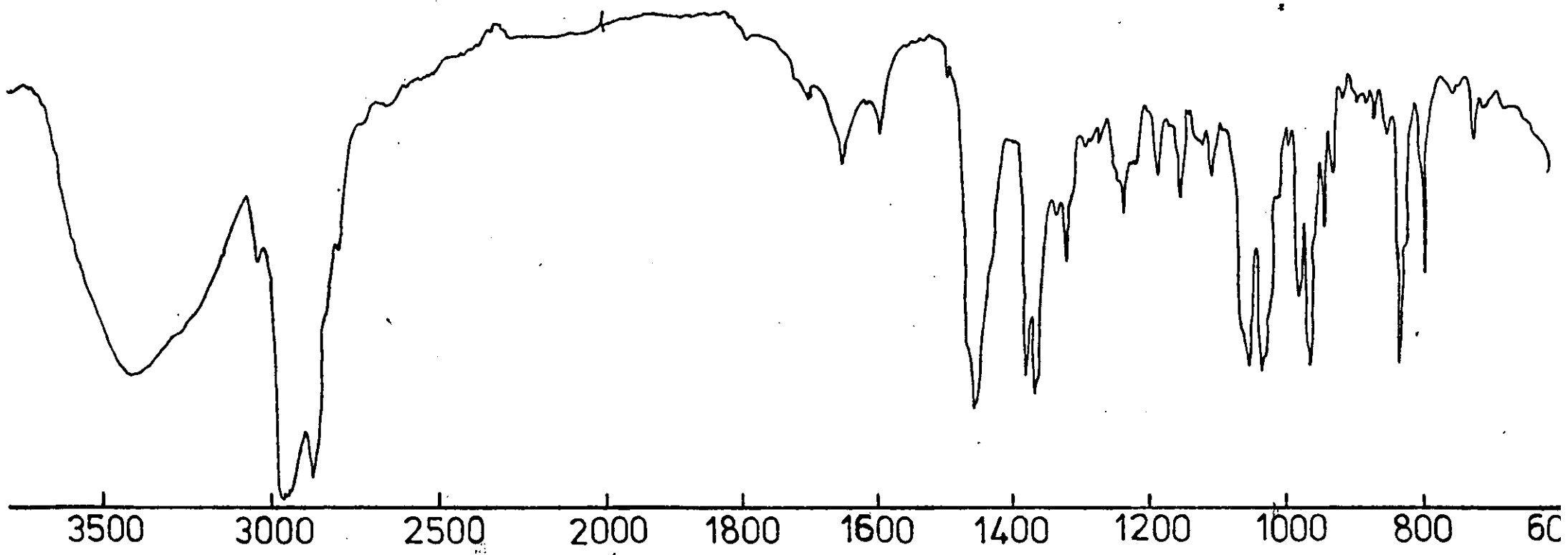
from C. albicans.

FIG. 3 :   N.M.R. spectrum of ergosterol

          isolated from C. albicans.
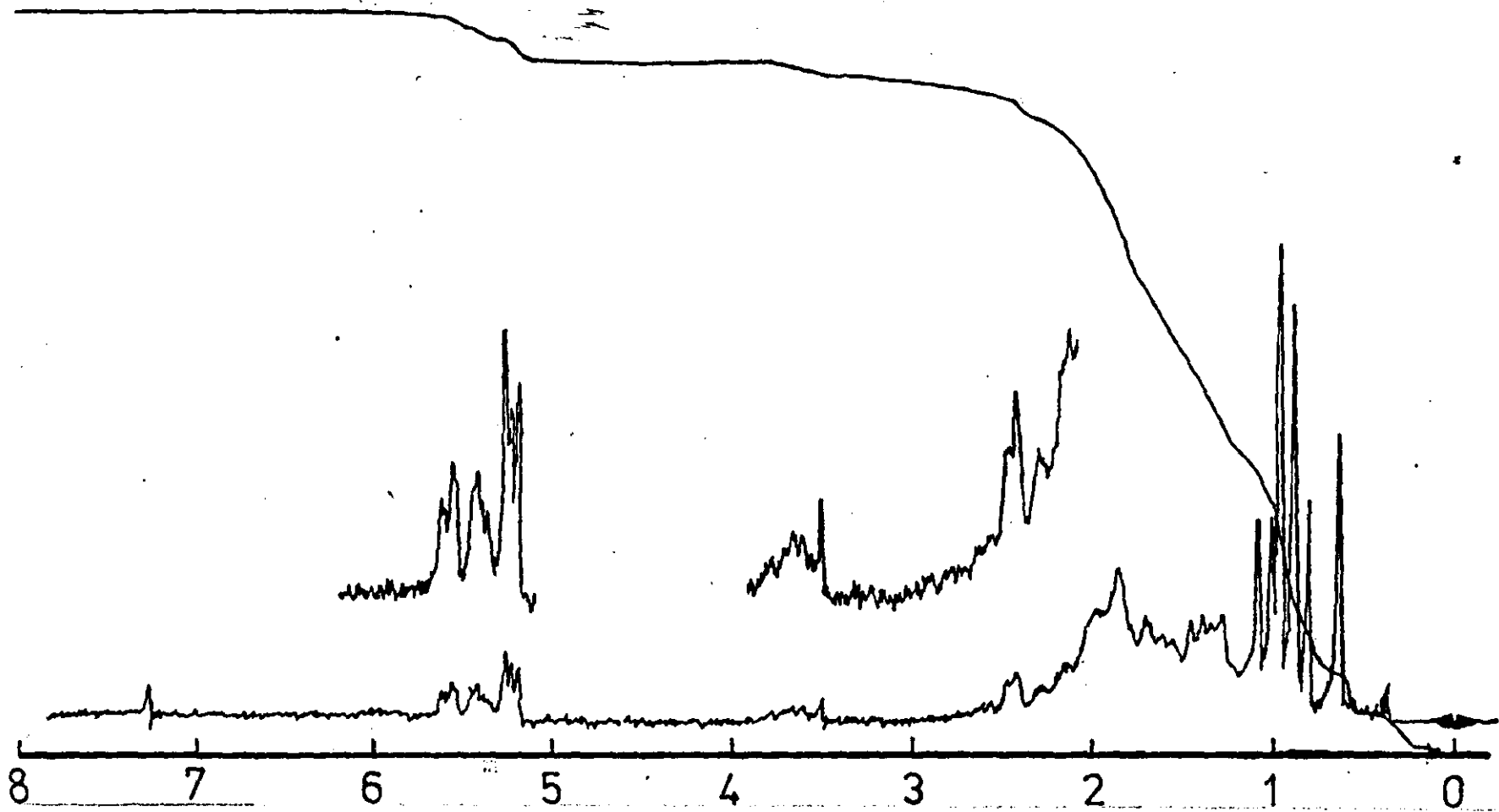
FIG. 4 : U.V. absorption spectrum of calciferol

isolated from using preparative thin

layer chromatography. The dotted spec-

trum is that of standard califerol.
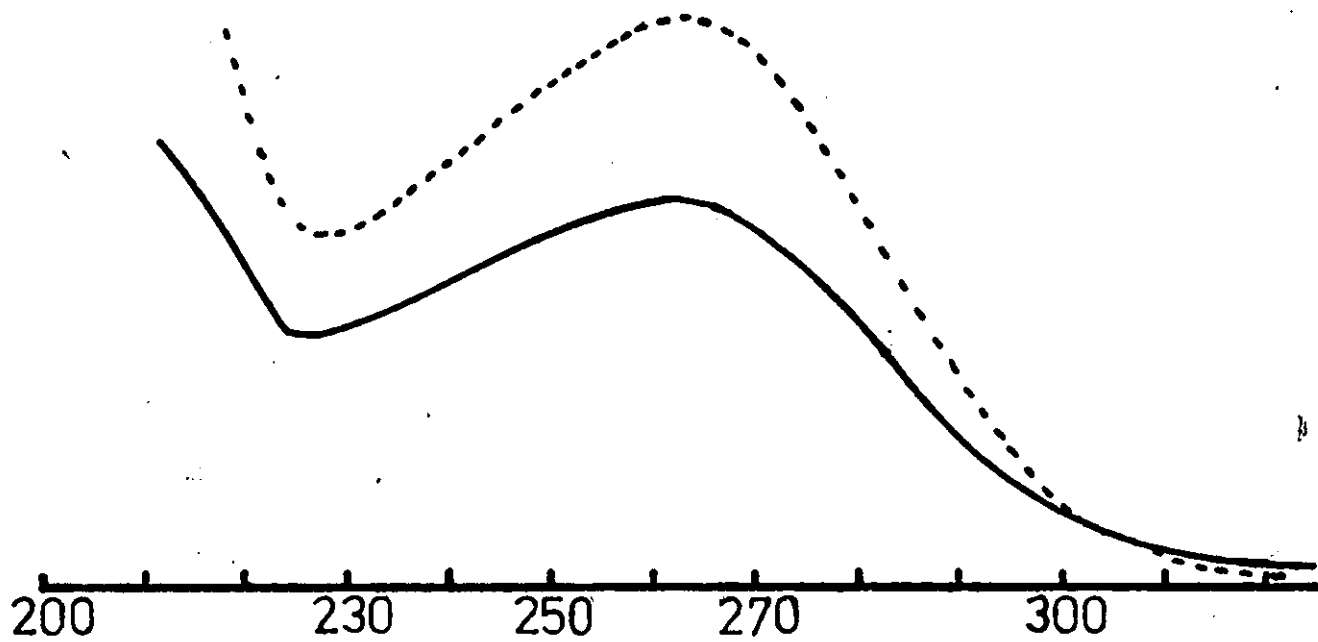
FIG. 5 : I.R. spectrum of calciferol isolated
from C. albicans.

3500　3000　2500　2000　1800　1600　1400　1200　1000　800

FIG. 6 : I.R. spectrum of lanosterol isolated

from C. albicans.

3500 3000 2500 2000 1800 1600 1400 1200 1000 800
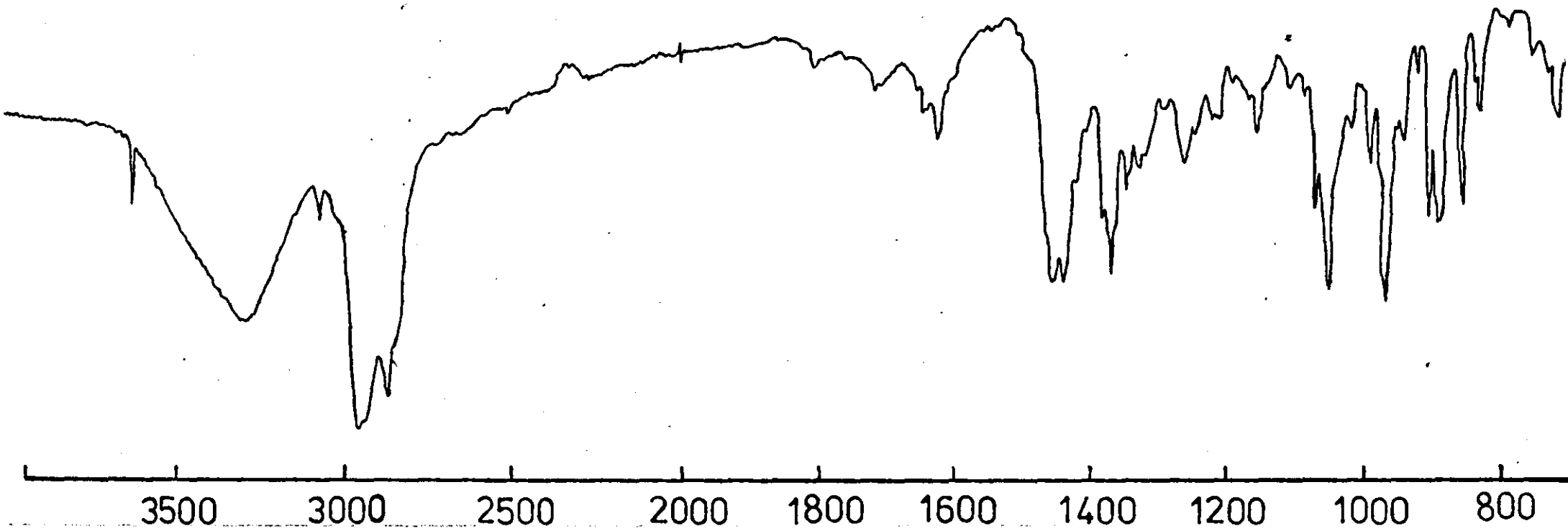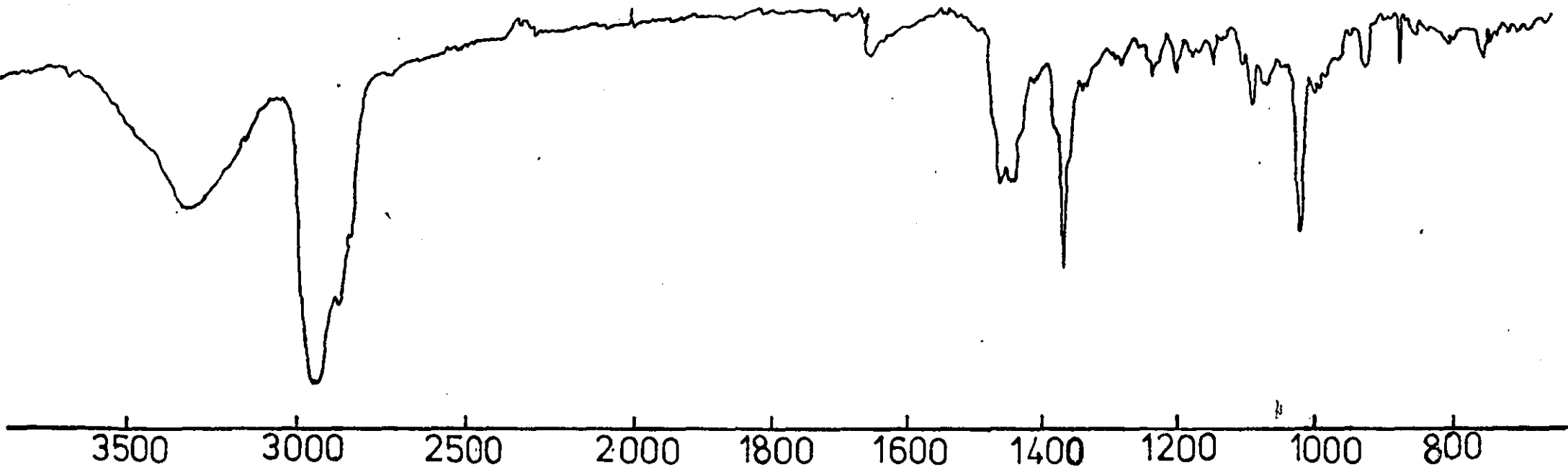
FIG. 7 : N.M.R. spectrum of lanosterol isolated
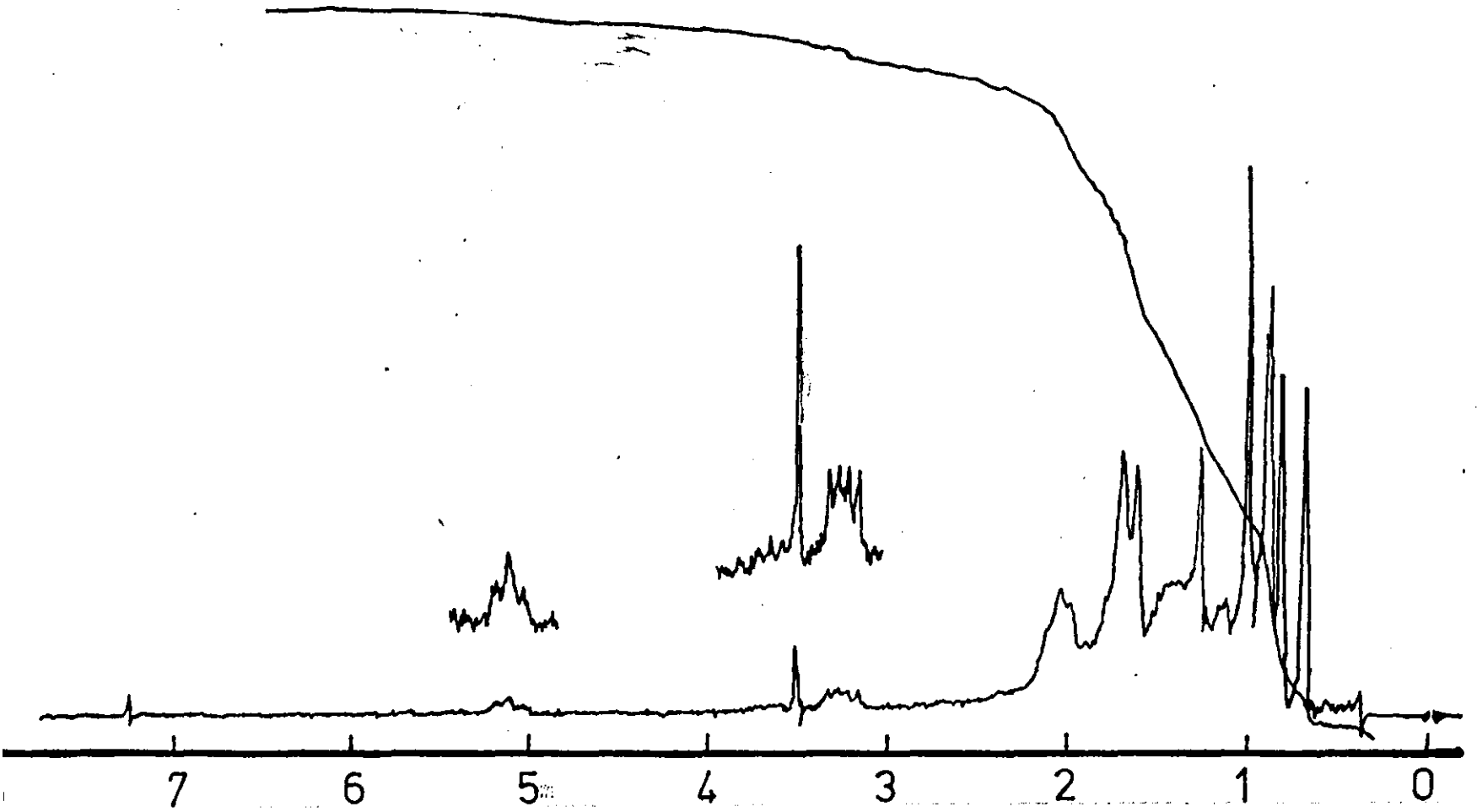
from C. albicans.

FIG.8 : I.R. spectrum of squalene isolated
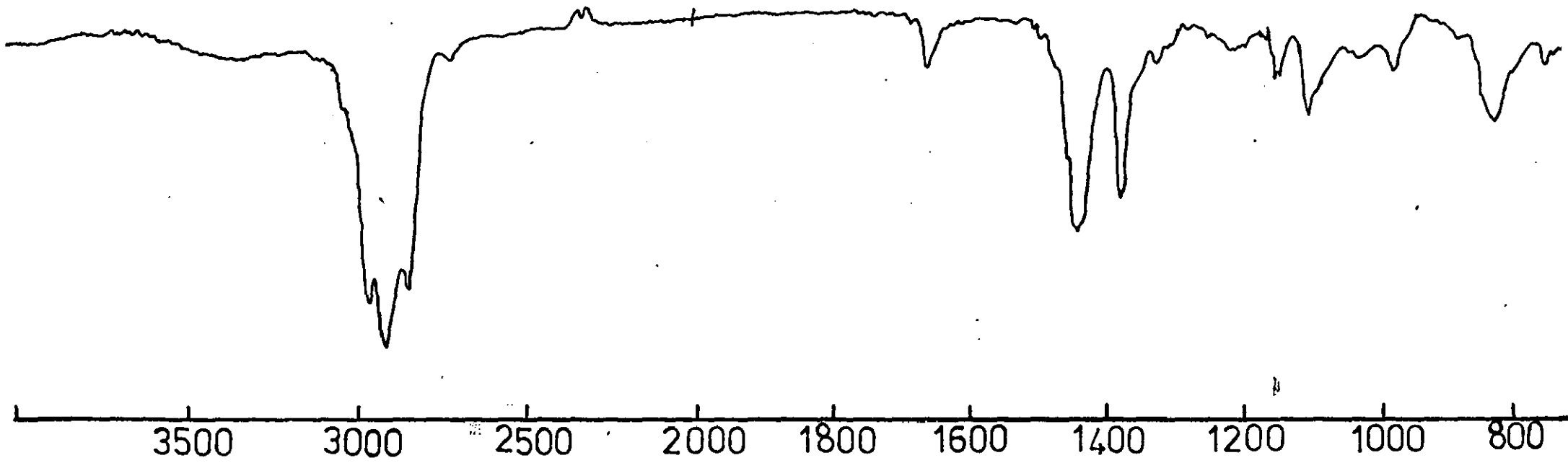
from C. albicans.

FIG. 9 : N.M.R. spectrum of squalene isolated

from C. albicans.
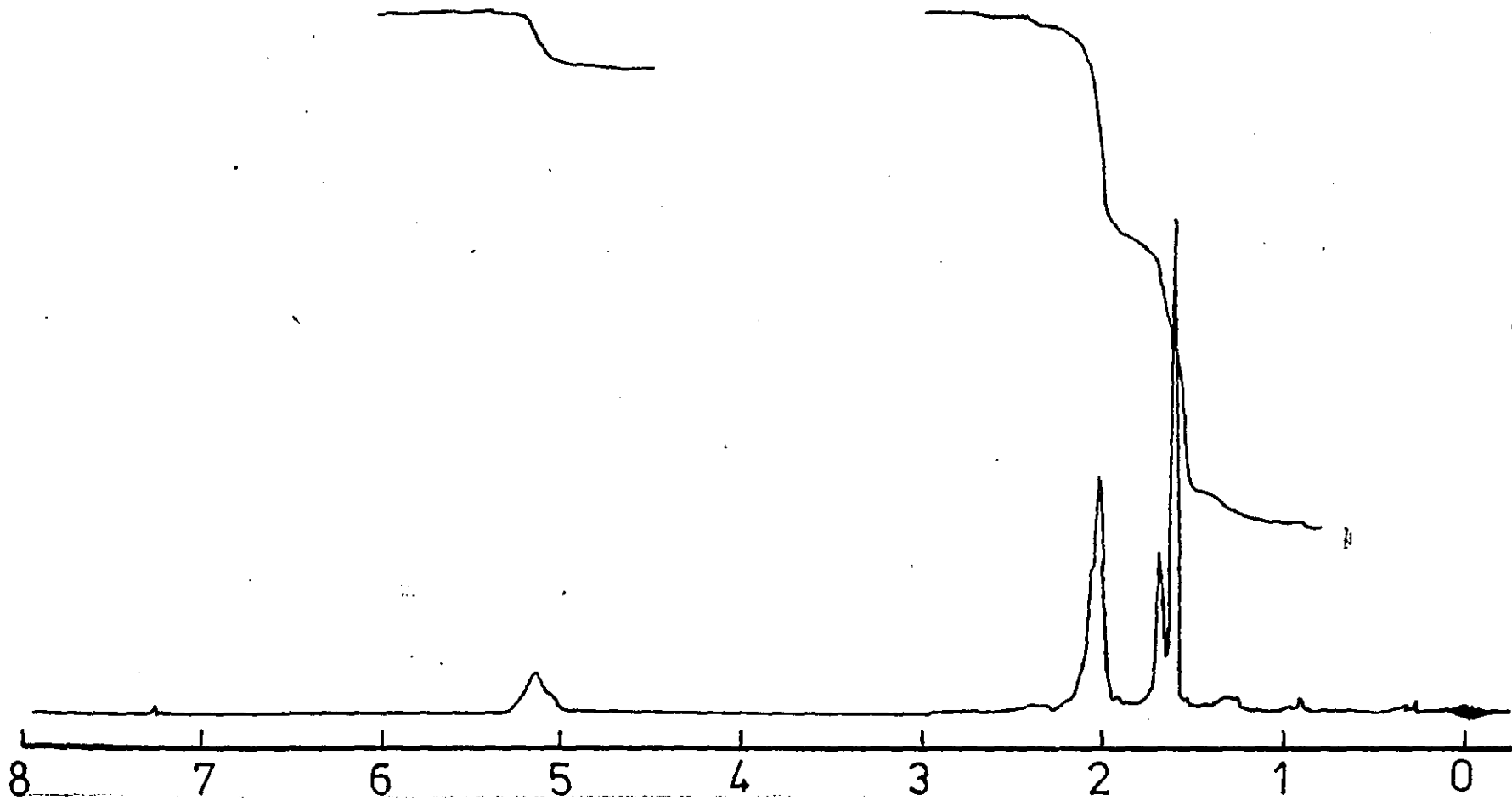
A P P E N D I X   II

KEY TO GAS-LIQUID CHROMATOGRAMS

1   Squalene

2   Breakdown product of ergosterol

3   Breakdown product of ergosterol

U   Unidentified

C   Calciferol

4   Zymosterol

5   Ergosterol

6   24,28 dehydroergosterol

7   4 me zymosterol
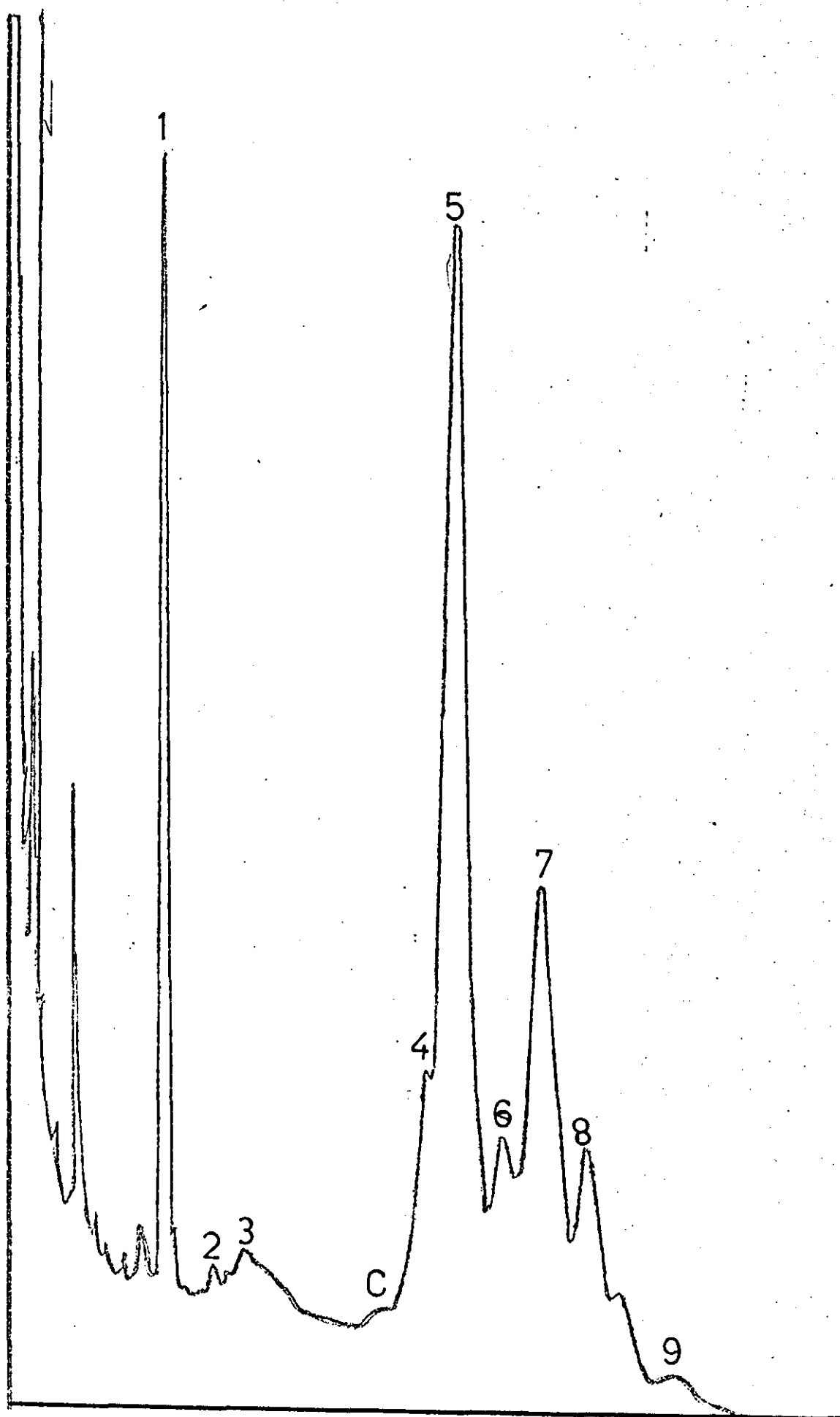
8   Lanosterol

9   4,4', dimethylzymosterol

FIG. 1 : Gas-liquid chromatogram on 3%

JXR   of trimethylsilyl (TMS)

derivatives of total sterols from

the yeast form C. albicans A39

grown in the absence of steroids

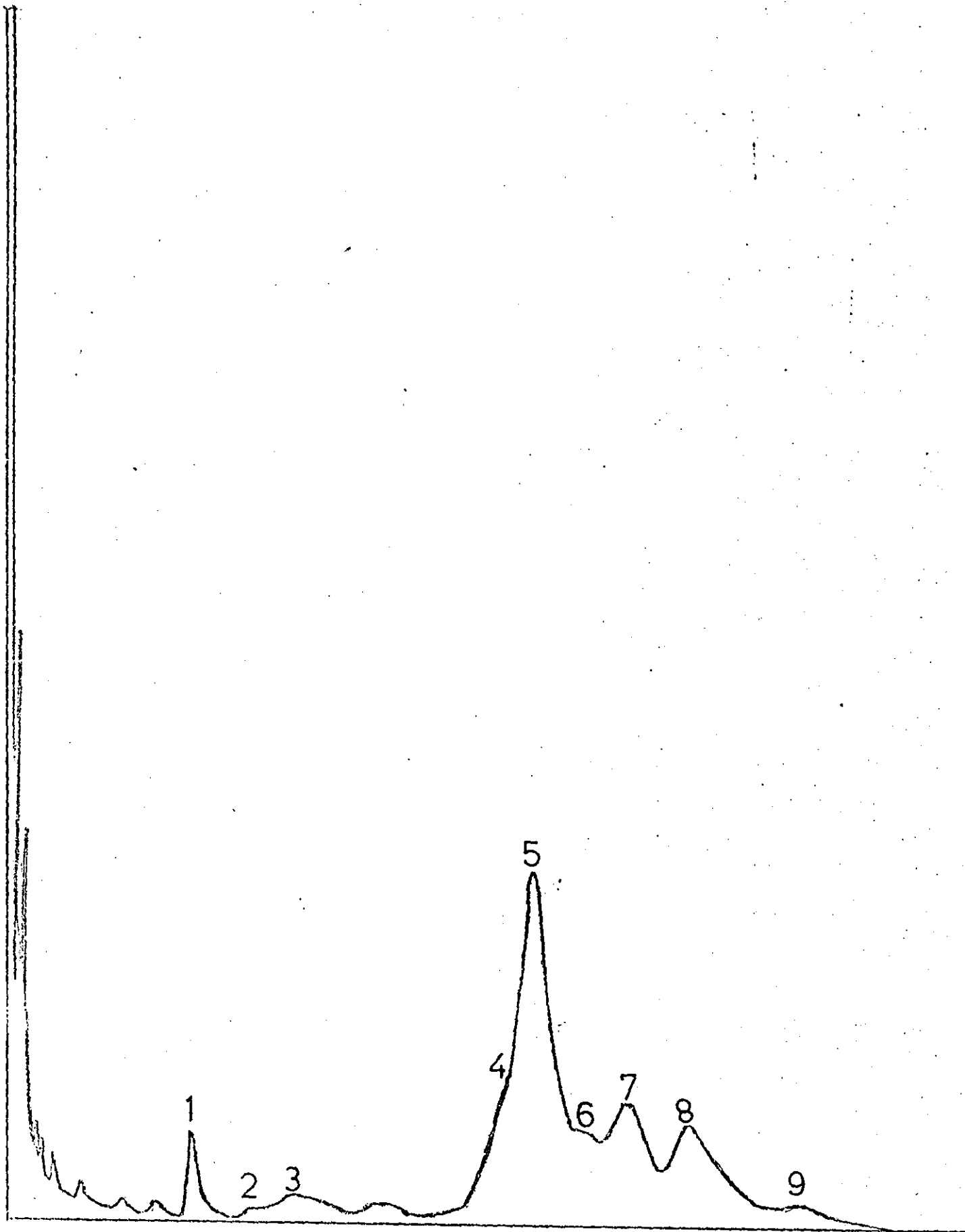(control) Column temperature was

230°.
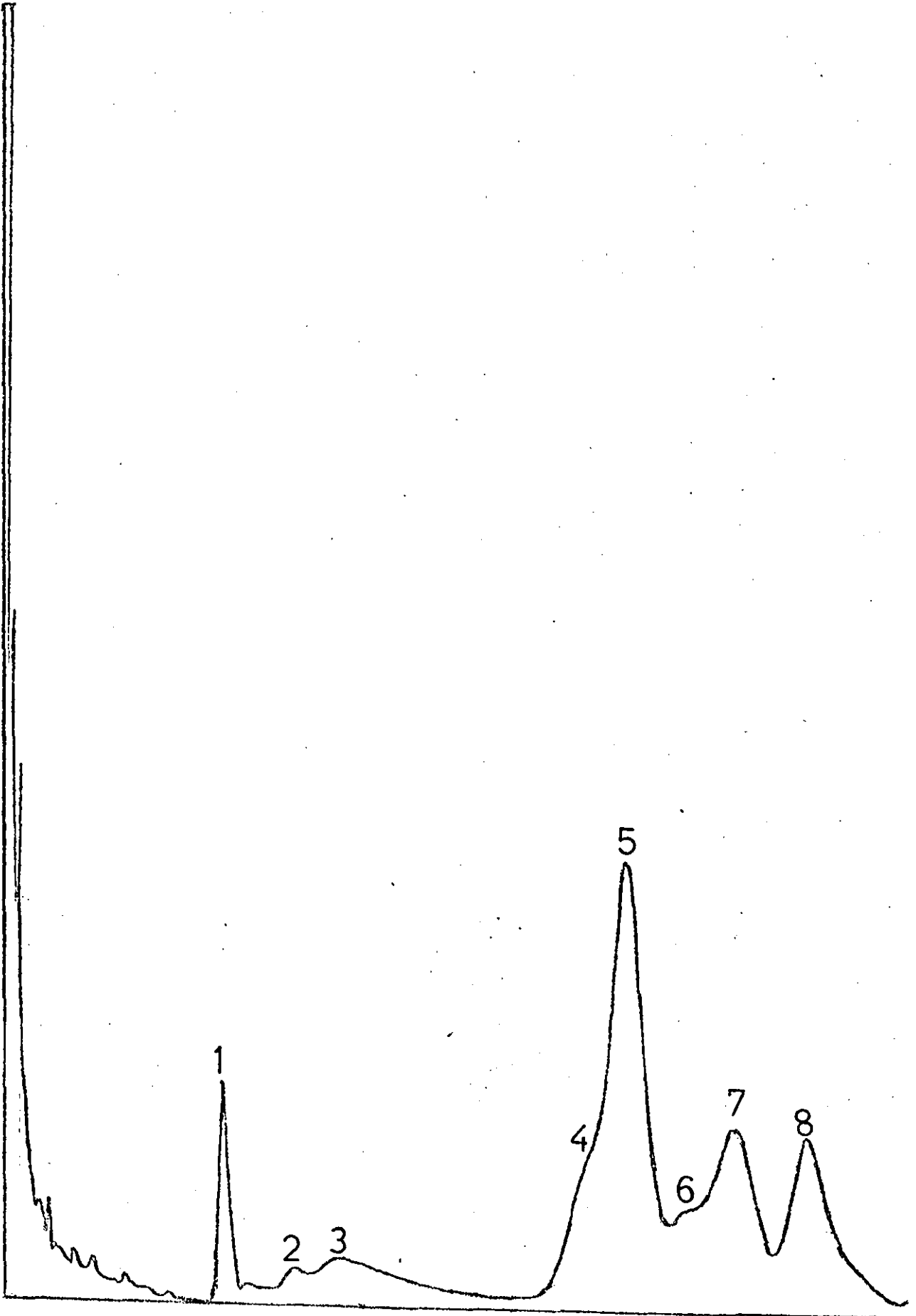
FIG. 2 : Gas-liquid chromatogram on 3%

JXR   of trimethylsilyl (TMS)

derivatives of total sterols

from the yeast form of C. albicans

IMI 45348 grown in the absence of

steroids (control). Column tem-

perature was 230°.
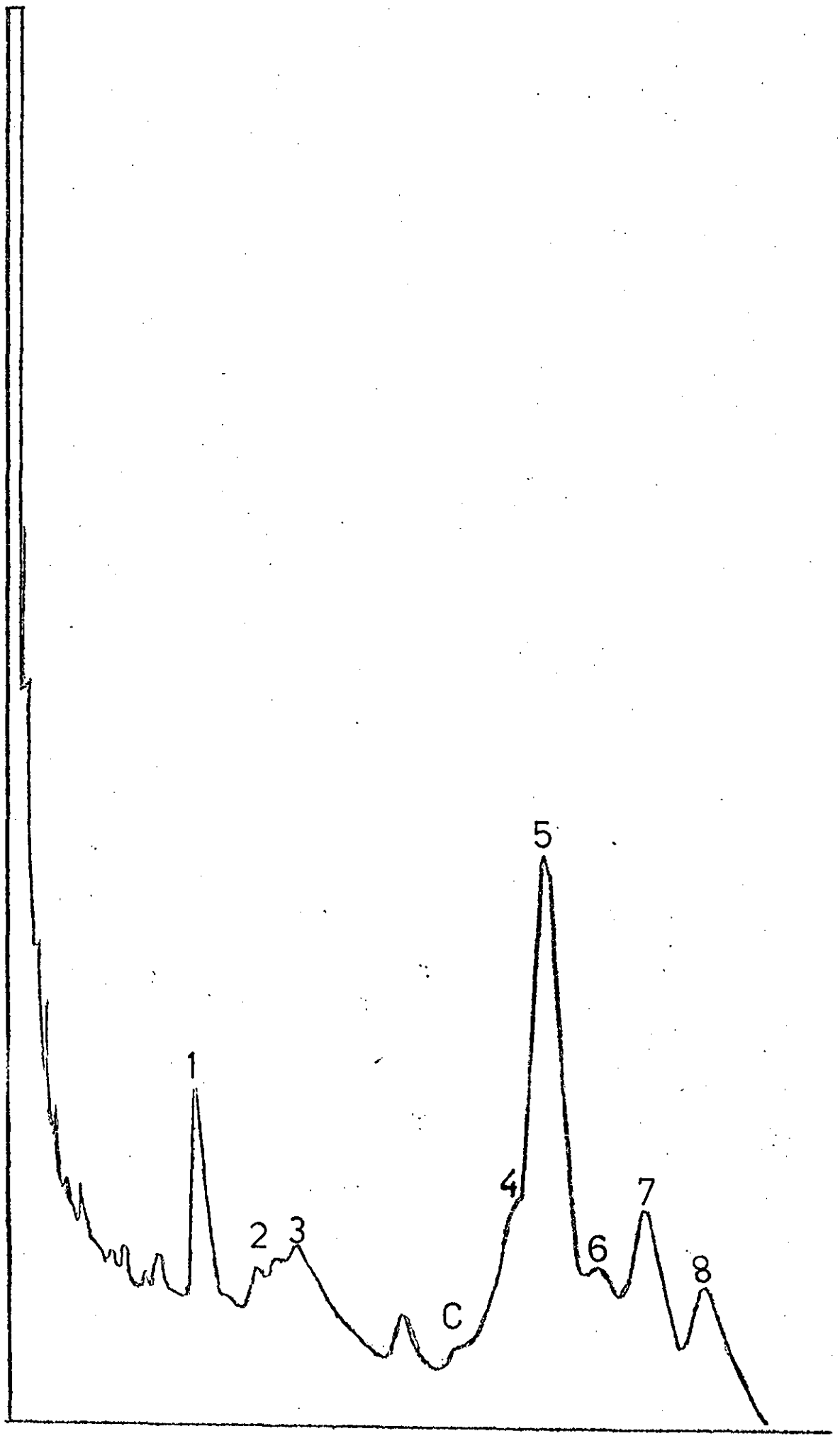
FIG. 3 : Gas-liquid chromatogram on 3%

JXR of trimethylsilyl (TMS)

derivatives of total sterols

from the mycelial form of C.

albicans A 39 grown in the absence

of steroids (control). Column

temperature was 230°.
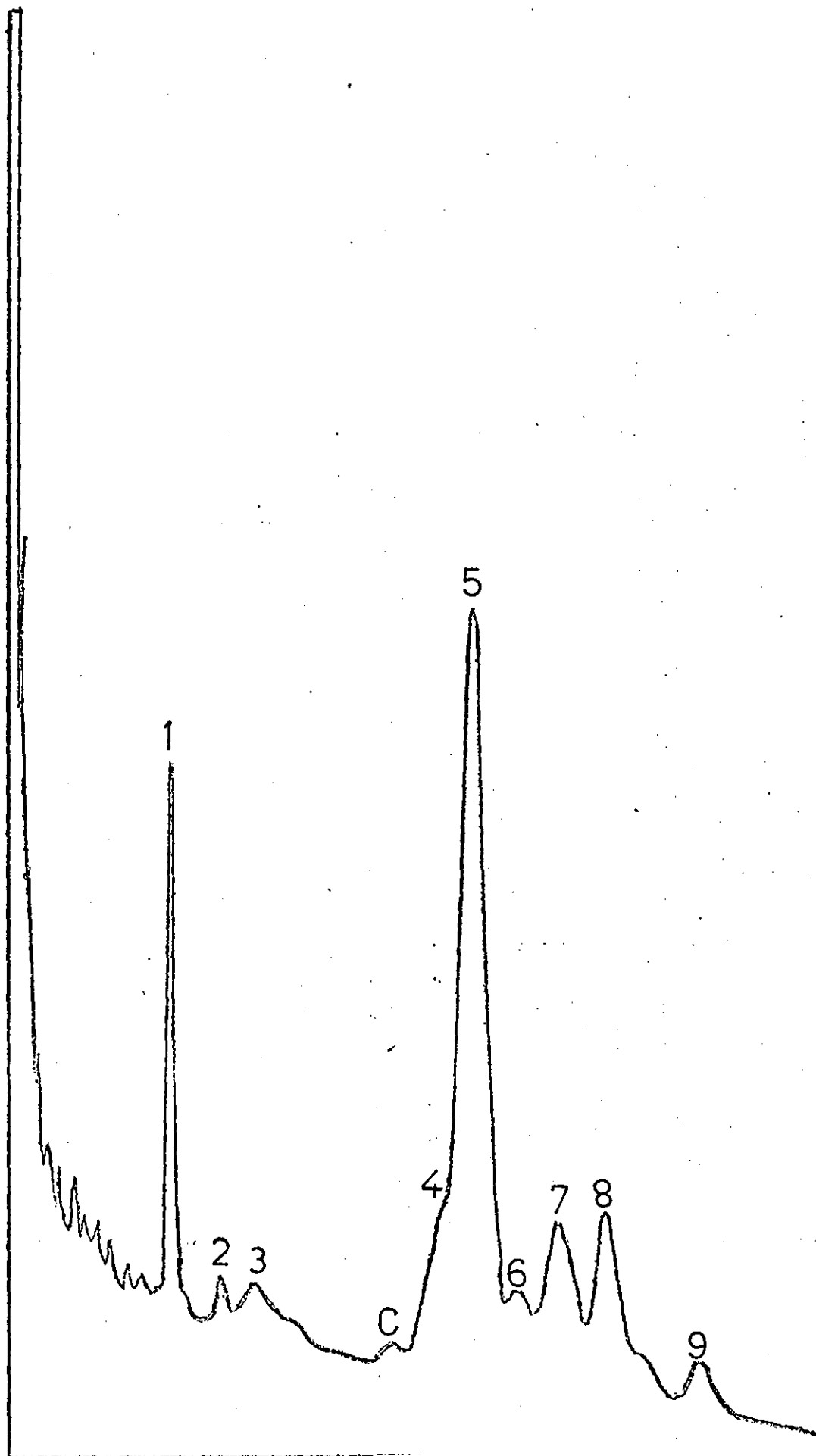
FIG. 4 : Gas-liquid chromatogram on 3%

JXR of trimethylsilyl (TMS)

derivatives of total sterols

from the mycelial form of

C. albicans IMI 45348 grown in

the absence of steroids (control).

Column temperature was 230°.

FIG. 5 : Gas-liquid chromatogram on 3%

JXR of trimethylsilyl (TMS)

derivatives of total sterols from

the yeast form of C. albicans A39

grown in the presence of .05%

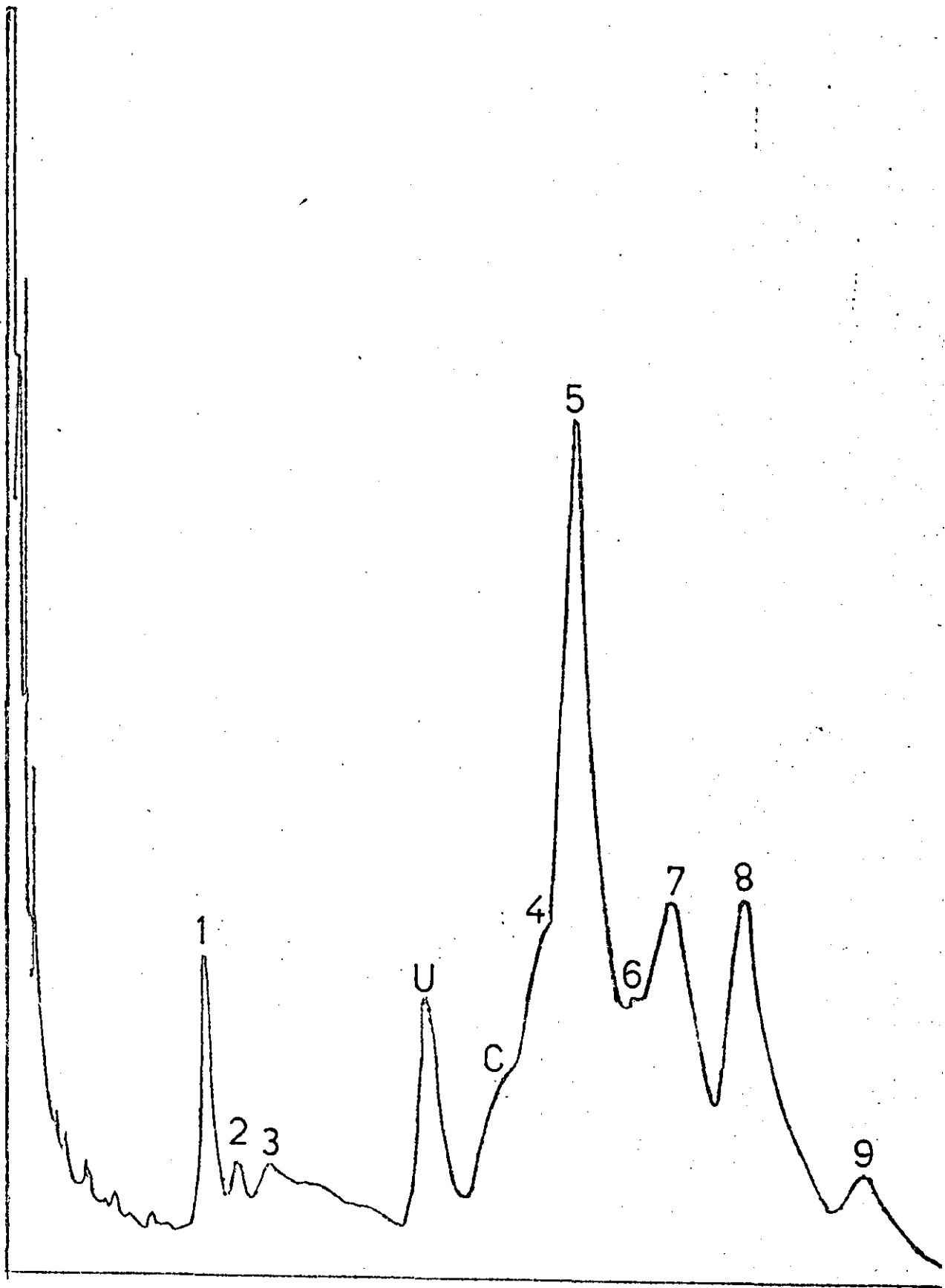cholic acid salt (experimental).
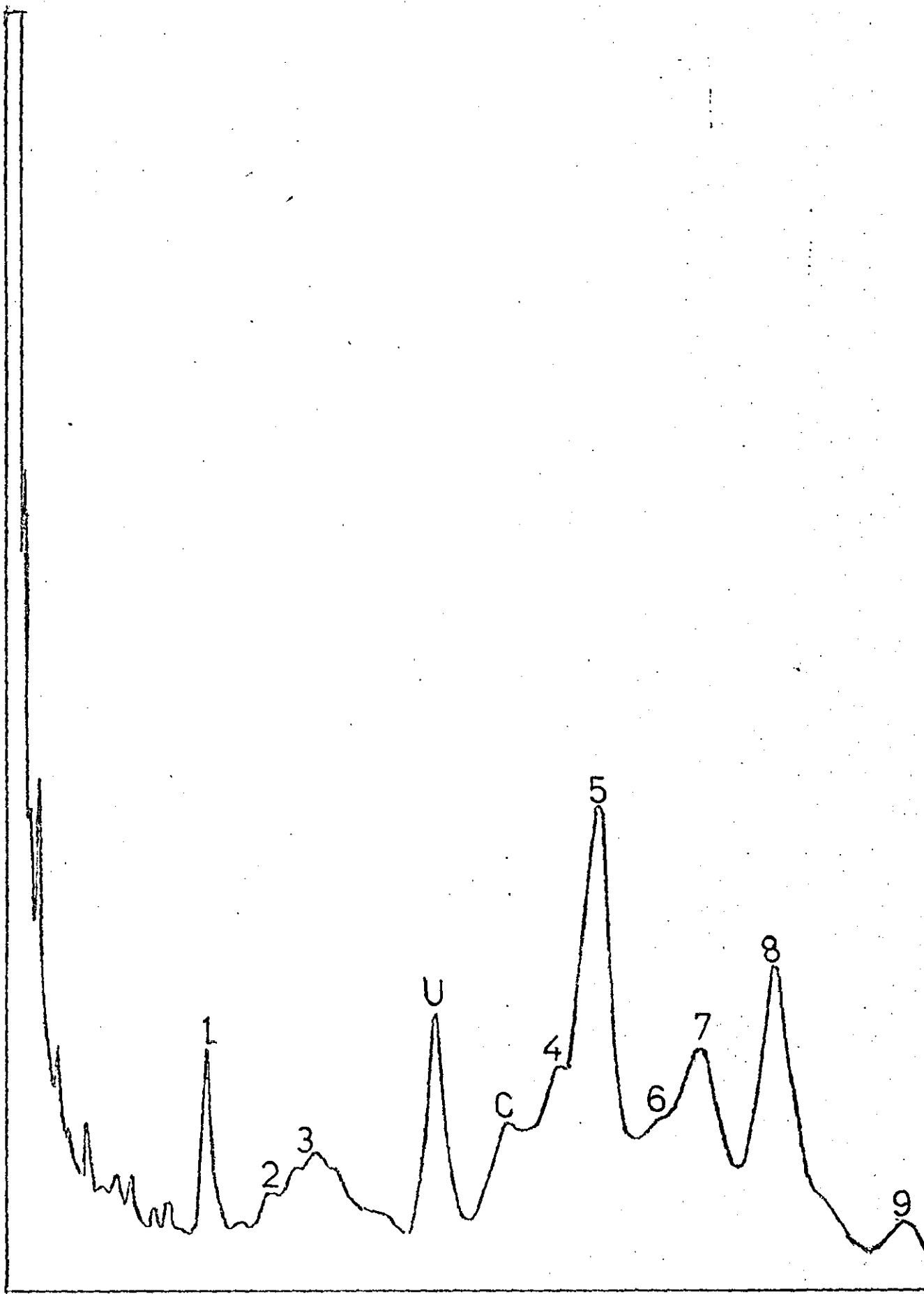
Column temperature was 230°.

FIG. 6 : Gas-liquid chromatogram on 3%

JXR of trimethylsilyl (TMS)

derivatives of total sterols from

the yeast form of C. albicans A39

grown in the presence of .1% cholic

acid salt (experimental). Column
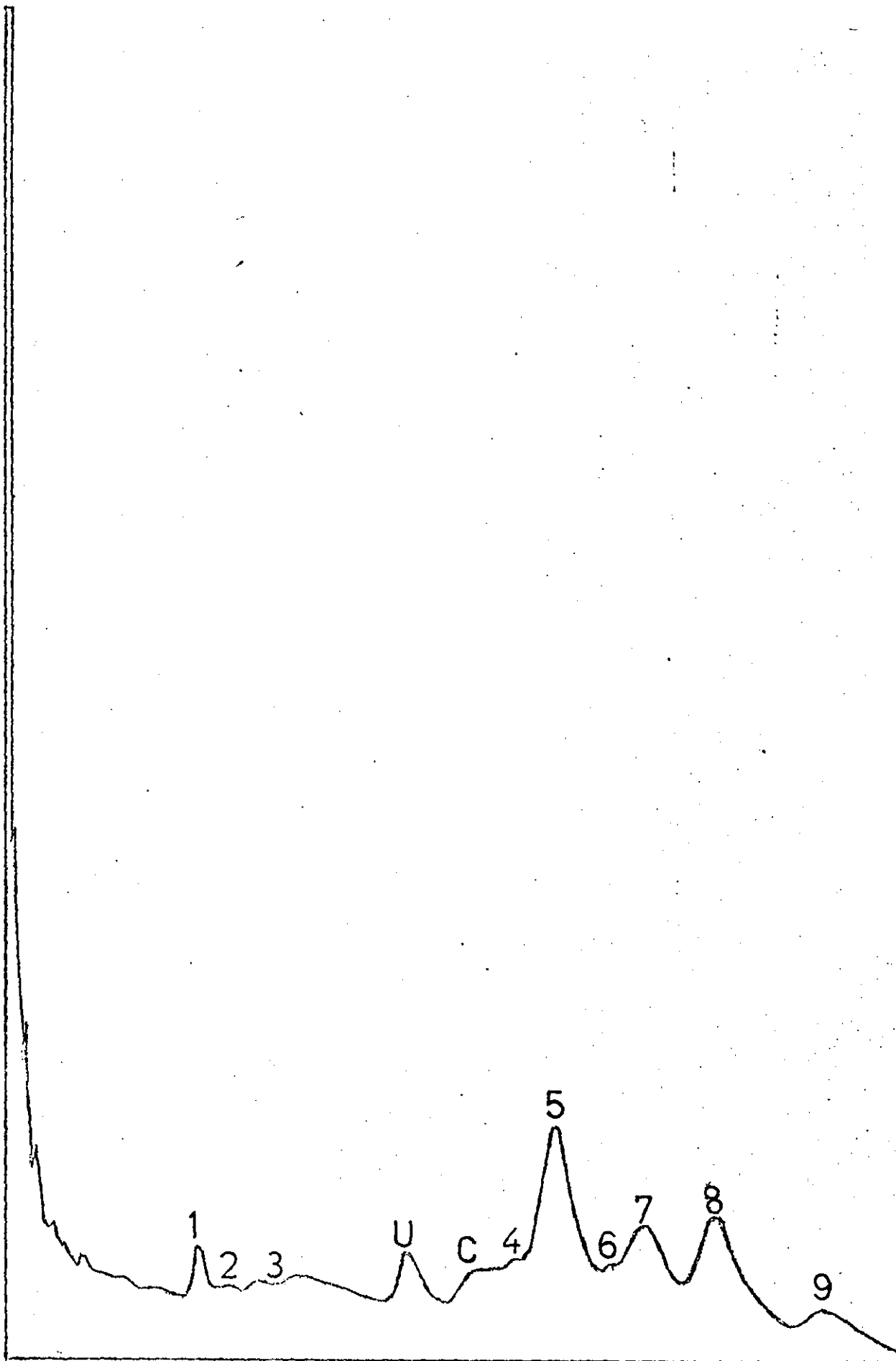
temperature was 230°.

FIG. 7 : Gas-liquid chromatogram on 3%

JXR of trimethylsilyl (TMS)

derivatives of total sterols from

the yeast form of C. albicans A39

grown in the presence of .15%

cholic acid salt (experimental).

Column temperature was 230°.

FIG. 8 : Gas-liquid chromatogram on 3%

        JXR   of trimethylsilyl (TMS)

        derivatives of total sterols

        from the yeast form of C. albicans

        A39 grown in the presence of .2%

        cholic acid salt (experimental).

        Column temperature was 230°.

FIG. 9 : Gas-liquid chromatogram on 3%

JXR   of trimethylsilyl (TMS)

derivatives of total sterols from

the yeast form of C. albicans A39

grown in the presence of .05%

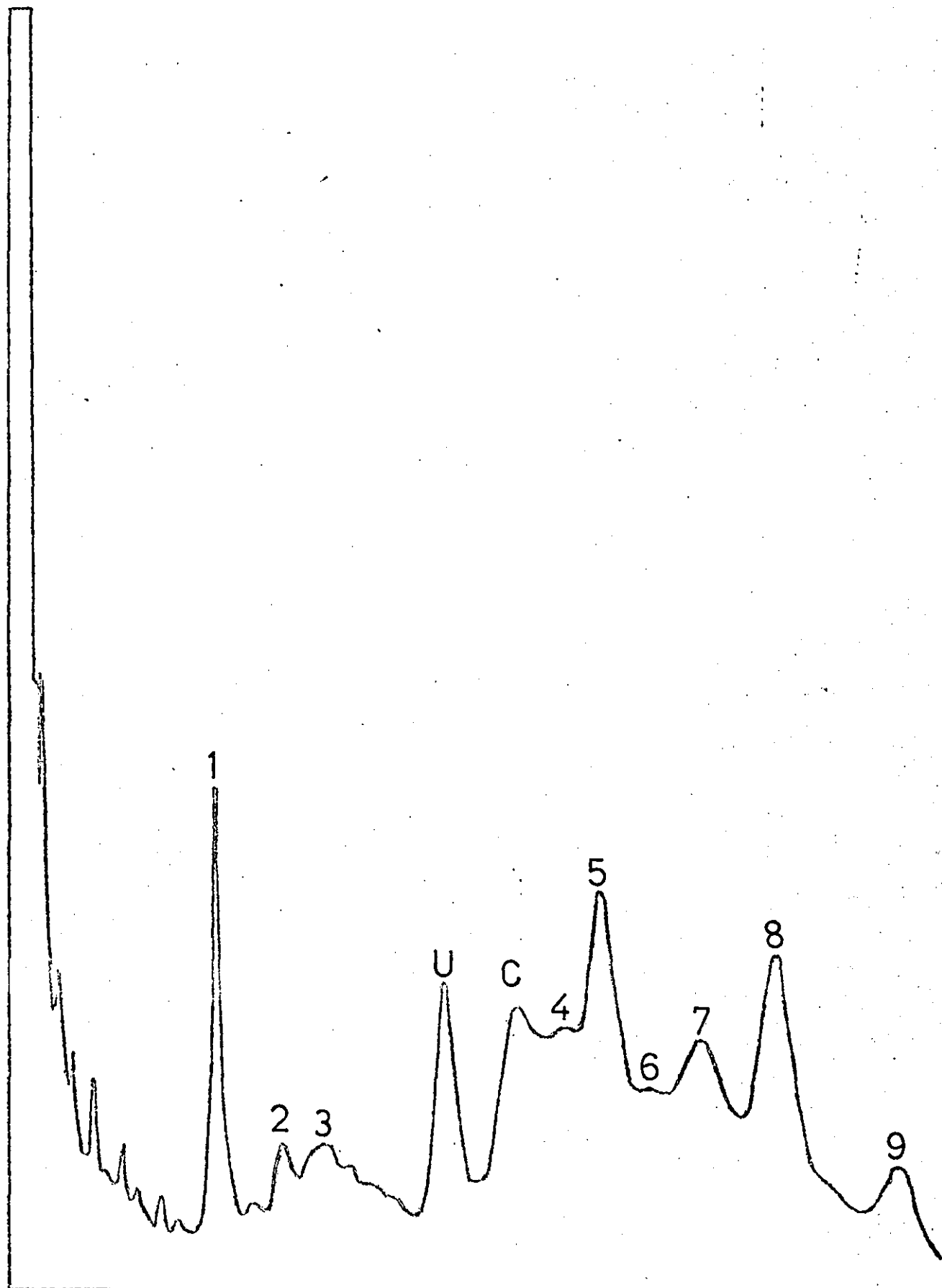Decadron (experimental) Column

.temperature was 230°.

FIG. 10 : Gas-liquid chromatogram on 3%

JXR of trimethylsilyl (TMS)

derivatives of total sterols

from the mycelial form of C.

albicans A39 grown in the presence

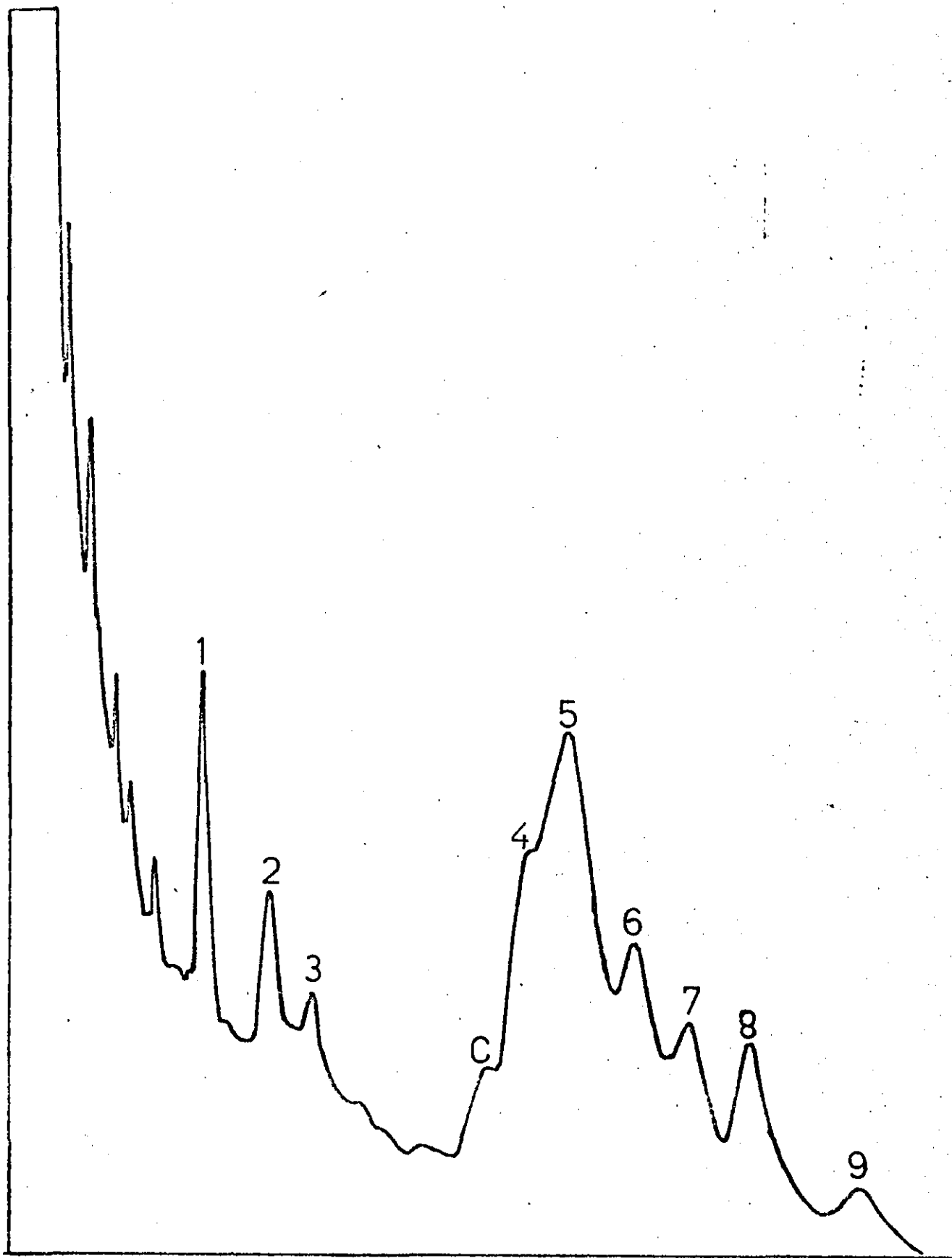of .15% cholic acid salt (experi-

mental). Column temperature was

230°.

FIG. 11 : Gas-liquid chromatogram on 3%

JXR   of trimethylsilyl (TMS)

derivatives of total sterols

from the mycelial form of C.

albicans A 39 grown in the

presence of .05% Decadron

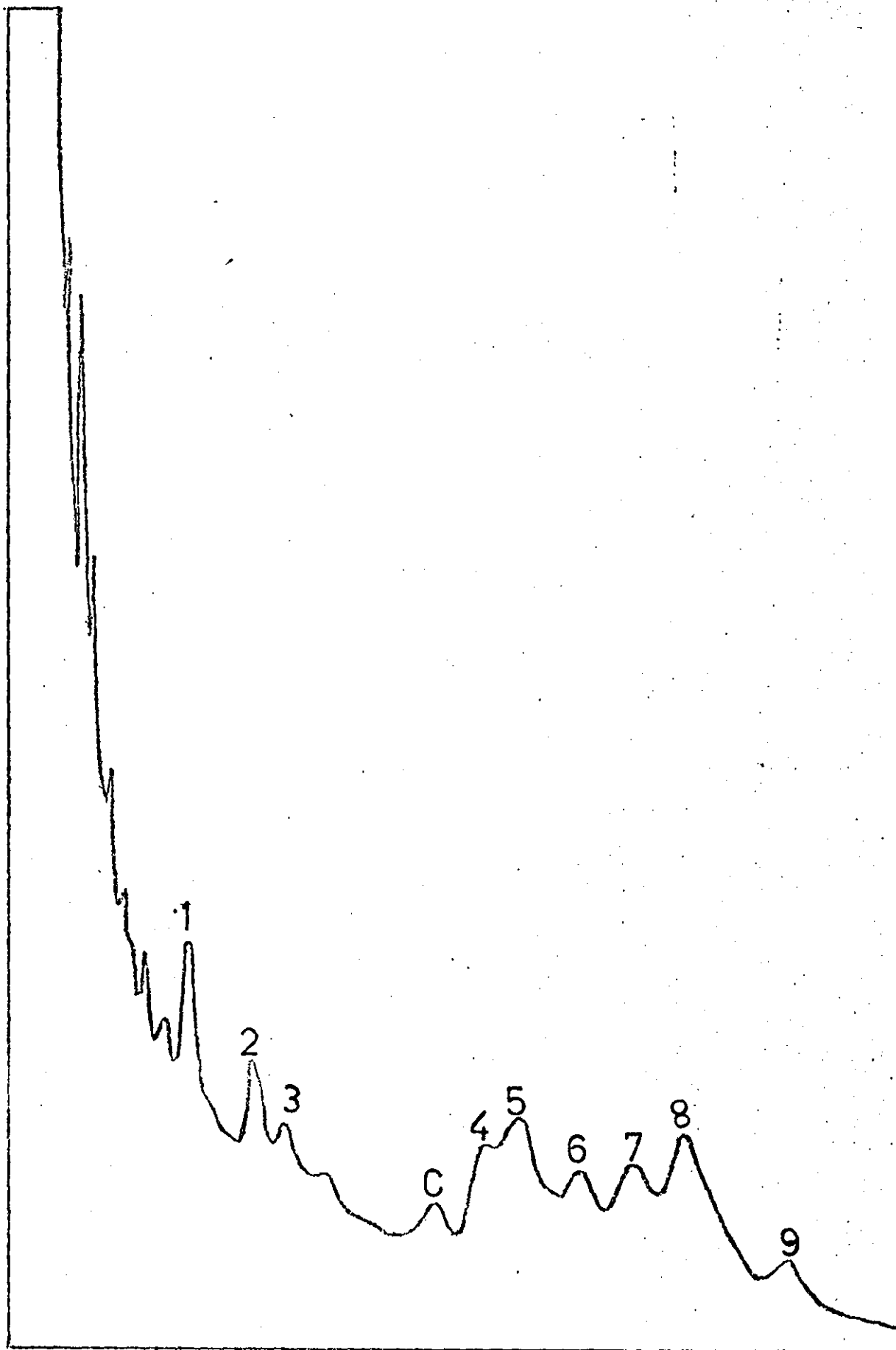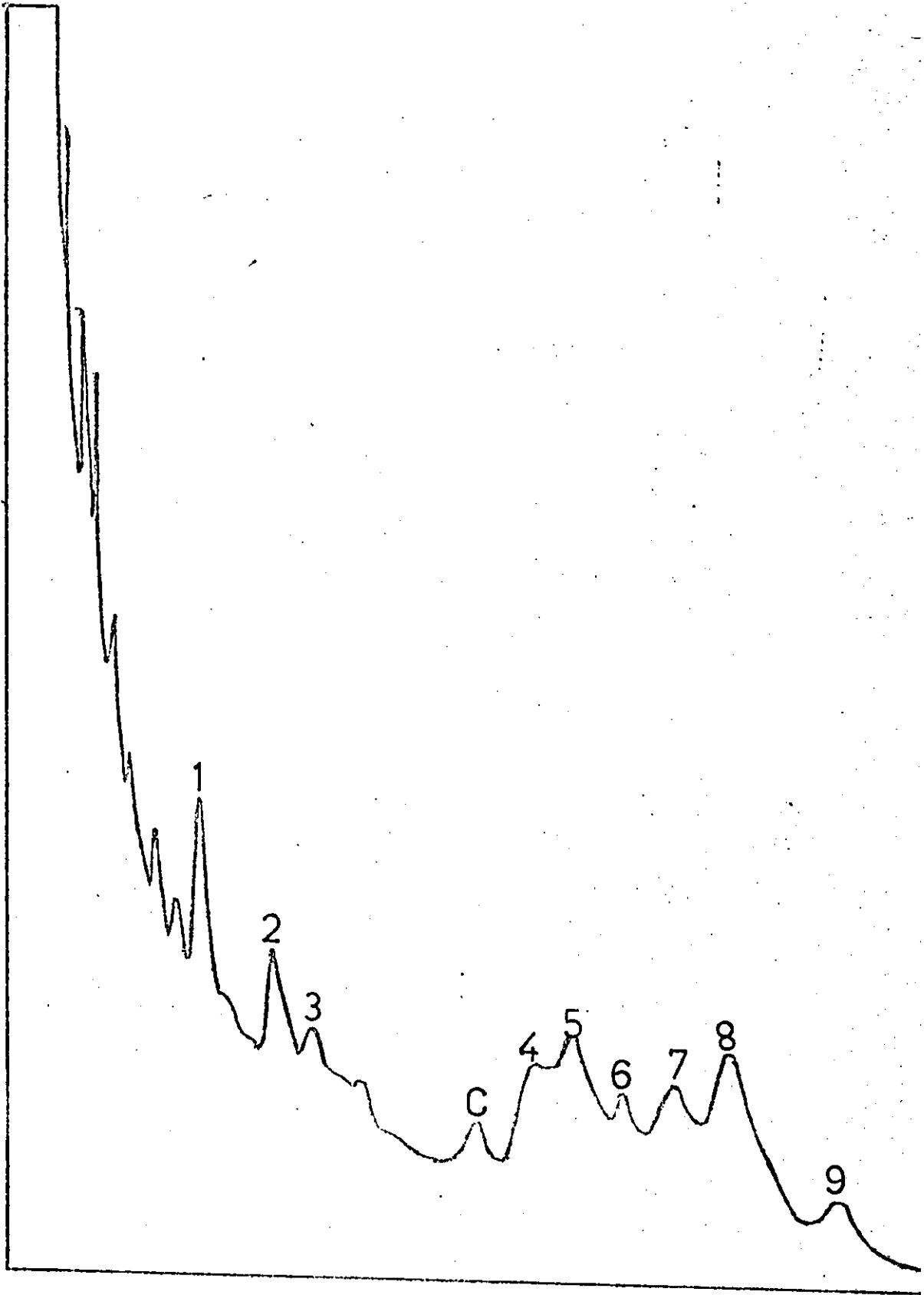(experimental). Column Tempera-

ture was 230°.

FIG. 12 : Gas-liquid chromatogram on 3%

JXR of trimethylsilyl (TMS)

derivatives of total sterols

from the mycelial form of C.

albicans A39 grown in the presence

of .05% Alphadolone acetate (experi-

mental). Column temperature was

230°.

APPENDIX III

Statistical Treatment of Data

1. The Mean

$$\bar{x} = \Sigma \, x/_n$$

$\bar{x}$ signifies the mean.

x signifies each of the values observed.

n signifies number of terms.

2. Standard deviation

$$SD = \frac{\Sigma(\text{data value} - \text{average value})^2}{\text{number of terms} -1}$$

3. Confidence Limits

$$\text{Confidence Limits} = AV \pm \frac{t}{\sqrt{n}} \cdot SD$$

AV = Average value

Value of t may be obtained from statistical tables.

n = number of terms.

4. Least Squares

$$y = mX + C \quad \ldots\ldots\ldots \text{ equation of a straight line.}$$

$$m = \frac{\Sigma(x_i - \bar{x})(y_i - \bar{y})}{\Sigma(x_i - \bar{x})^2}$$

m = The slope.

x_i and y_i are individual x and y data points.

$\bar{x}$ and $\bar{y}$ are the average values for x and y.

$$c = \frac{\Sigma y_i \Sigma(x_i - \bar{x})^2 - \Sigma x_i \Sigma(x_i - \bar{x})(y_i - \bar{y})}{n \Sigma(x_i - \bar{x})^2}$$

c = intercept

5. Chi-squared statistical test

$$X^2 = \frac{1}{t} \Sigma(v_i - t)^2$$

X^2 = Chi-square value.

t = is the theoretical frequency.

v_i = observed frequency.

The probability values associated with X^2 values calculated can be obtained from statistical tables.