STUDIES ON THE BIOLOGY OF PATHOGENIC FUNGI

by

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C O N T E N T S

Introduction

Fungi pathogenic to man and other animals represent a small fraction of the total number of organisms known to cause disease. In many cases the effects of fungal pathogens are debilitating rather than lethal, and the relatively rare occurrence of a mycosis in an advanced state has resulted in the exclusion of pathogenic fungi from normal medical studies. Nevertheless, that fungi are responsible for at least a significant proportion of disease is indicated by the statistics quoted by Emmons (1) and Salvin (2). In the latter paper it is shown that of 92,933 deaths due to infectious and parasitic diseases in the United States of America in 1945, 284 were due to mycoses. This represents 0.3% of the total number of deaths, and although low in comparison with the number caused by bacteria and viruses, is approximately equal to the number of deaths attributed to scarlet fever, measles, or the typhuslike diseases caused by the rickettsia: it is more than the total of all deaths caused by rabies, smallpox, relapsing fever, leprosy, brucellosis, paratyphoid fever, plague, cholera, and anthrax. Both Emmons and Salvin however have pointed out that effective measures of control of some of the abovementioned diseases are known and practised; in contrast, the control of mycoses is rarely practised and in several cases not known. The interpretation of these figures is further complicated by the possible failure to attribute deaths to fungal agents, since there exists a range of fungal association with man and other animals

from /

from the active parasite to the harmless commensal. In many cases it is impossible to assess the pathogenic status of a fungus isolated from morbid material, and the association between fungus and animal has to be evaluated by indirect or circumstantial evidence. Many pathogenic fungi have been found in sources external to their host, and it seems likely that association with a host animal is in some instances incidental, the fungus having been acquired by chance from the environment. In other instances, the association may be an adaptive one. The study of soil as a source of human pathogenic fungi has shown that many of these species are capable of existing as saprophytes, suggesting that their occurrence in animal tissue is the result of a biological adaptation, the organisms remaining comparatively unspecialised. Among the animal pathogens, the dermatophytes alone are highly specialised, being adapted efficiently and successfully to a parasitic existence on the keratinised portions of the host. Ajello (3) lists 21 species of pathogenic or potentially pathogenic fungi isolated from soil, including Histoplasma capsulatum Darling. and Cryptococcus neoformans (Sanfelice) Vuillemin., either of which can cause a systemic and fatal mycosis. It is of interest to note that all the common systemic mycoses are considered to occur exogenously, that is, outwith the host animal, whilst other path-:ogens such as Candida albicans (Robin) Berkhout. exhibiting a variety of clinical manifestations appear to be endogenous in origin /

origin, rarely, if ever occurring outside the host.

The fungal organisms associated with humans and other animals represent a small percentage of the group as a whole. Conant et al (4) refer to approximately 70 species of fungi associated with 24 mycoses of the human. Ainsworth (5) lists 88 species of fungi representing 36 genera which have been recorded as pathogenic for man and higher animals in Britain, but this figure includes organisms responsible for poisoning and others where the pathogenic status is uncertain. 65 species are included in a list of the more common fungi recorded as pathogenic to man and animals in Great Britain in a memorandum published by the Medical Research Council (7). Of these, only 43 are considered to be indigenous pathogens. In the most recent edition of "An Introduction to Medical Mycology" Lewis et al (6), it is of interest to note that of 27 chapters dealing with specific mycoses, no fewer than 17 are devoted to the dermatophytes or other representatives of the Fungi Imperfecti which are associated with superficial infections; one chapter deals with the various symptoms caused by infection with the imperfect yeast Candida, whilst 7 of the remaining chapters describe the "deep" mycoses, most of which are also caused by members of the Fungi Imperfecti. Several groups of fungi contain pathogenic members, but as has already been indicated the majority of species belong to the Fungi Imperfecti : amongst these, the most common genera are the dermatophytes Trichophyton, /

Trichophyton, Microsporum, and Epidermophyton, and several other genera also in the Moniliales; the non-ascosporogenous yeasts are also included in the imperfect fungi, and these organisms are capable of producing a wide variety of clinical symptoms, ~~although the diseases they cause are seldom fatal~~. Phycomycetes and Ascomycetes are less commonly represented, and Basidiomycetes are not apparently represented at all, if their toxic or allergic effects are discounted. A single report of a Basidiomycete possibly causing onychomycosis has been described by Kligman (8), the agent being Schizophyllum commune Fries., a member of the gill-forming Agaricales.

Medical mycology has been the subject of several reviews (1, 9, 10, 11, 12, 13, 14) and comprehensive accounts of the scope and significance of the subject can be obtained by reference to them. The limits of present-day knowledge and the current lines of research, the organisms associated with mycoses and descriptions of the diseases they cause; details of the symptoms, prognosis, and treatment; the morphology and physiology of the pathogens can be obtained by reference to the text books (4, 6, 15, 16, 17, 21), and symposia volumes (18, 19, 20). Detailed and invaluable abstracts are presented in the Review of Medical and Veterinary Mycology. (22).

Interest in medical mycology has increased enormously in the last 15 years and a constant and increasing production of papers from medical and related sciences has increased the know-:ledge of such aspects as clinical appearances and characteristics,

pathology /

pathology, epidemiology, treatment, identification, biology, chemistry, biochemistry, immunology, and to a certain extent, the morphology and mycology. The approach to the subject has been primarily from the medical side, but valuable contributions have been made by a number of mycologists, particularly in America. In this country medical mycology has not yet been developed in proportion to its recognition and advance in America, and the number of research departments carrying out investigations on this subject is small. Nevertheless it seems reasonable to suggest that the problems associated with medical mycology are not less significant in Britain than elsewhere, and that there is both a need and an opportunity for an elucidation of some of the numerous difficulties associated with these interesting organisms.

In 1953 an experimental mycoses unit was formed in the University of Edinburgh Department of Botany under the supervision of Dr. Walter Blyth, and sponsored in part by a grant from the Advisory Council for Medical Research. The work carried out in this unit has involved a study of one of the fundamental problems, viz. the influence of antibiotic substances on the course and nature of certain mycoses in vivo, with particular reference to the pathology of the host and the morphology of the fungus. This work has largely been carried out with pathogenic members of the genus Candida, with particular reference to Candida albicans and C. tropicalis (Cast) Berkhout. The results of some of these experiments /

experiments are at present in the Press.

The writer joined the mycoses unit in January 1956 and became interested in several aspects of the yeast pathogens. Since it was intended to establish the means of providing an advisory service for the hospitals and clinics in the Edinburgh district, it was decided to commence studies on the incidence of various species of fungi associated with a variety of human sources. In this manner it was intended to build up a culture collection of representative species, and to design a routine method for their isolation and identification. It was therefore decided to implement these intentions by carrying out a survey of the fungi occurring in a series of random samples from a variety of human sources, the material being obtained from hospitals in the Edinburgh district. This survey was limited in that the incidence of dermatophytes was not investigated, emphasis in the course of the work being laid on the distribution of yeasts. A second survey was initiated, the purpose of which was to examine a number of extra-human sources to determine which of these fungi isolated in the investigation of human sources are also found outwith the host.

In the second section of the present work, several of the yeast isolates obtained in the course of the survey were examined in relation to their early development <u>in vivo</u>, and an attempt made to distinguish the characteristics of their growth within the host. It was also hoped to demonstrate differences in methods and rates of growth between pathogenic species of the genus <u>Candida</u>.

Nature and Extent of the Survey

The object of the survey was to determine the species of fungi associated with various human sources, and to note their relative distributions amongst the sources examined. No attempt was made to correlate the presence of an organism with any clinical symptom or disorder, or to study the species present in relation to past or present treatment with therapeutic agents. The emphasis of the survey was on yeast-like organisms and filamentous forms were not selected for special study. The patients from whom samples were obtained for mycological examination were chosen at random, and although all the patients examined were suffering from some disorder which necessitated their being retained in hospital, either for treatment or observation, not all of the samples were from pathological material. Many of the samples which were examined were removed from patients for routine bacteriological examination, and a proportion of these samples were shown by hospital laboratory personnel to be free from pathogenic bacteria. Thus the patients and sources examined, and consequently the survey itself are considered to represent a cross-section of the hospital population.

A number of surveys of the distribution of fungi from human sources have been made in the past, and these will be considered /

considered in due course. Almost all of these are concerned with organisms isolated from a specific source or a specific disorder; for example Kurung (23) has described the isolation of pathogenic fungi from sputum, Pearson (24) has reported the occurrence of yeast-like fungi from the vagina, whilst other sources examined include neoplastic tissue (25), faeces (26), nails (27), teeth root-canals (28), human bile (29), urine (30), skin (31), and blood (32). Probably the source most frequently examined is sputum, and a number of papers (33, 34, 35, 36, 37, 38) deal with the fungi isolated from patients who are healthy or suffering from pulmonary tuberculosis. Particular interest in the organisms present in these sources has originated from the belief that the widespread use of broad-spectrum antibiotics has resulted in an increase in the significance of the yeast flora. It is suggested that the removal of bacteria (including the harmless commensals) by the antibiotics also removes the element of competition, and as a result the yeasts, which are not affected by the antibiotic, are able to multiply to such an extent that they may actually become pathogenic. In exceptional cases a yeast may bring about a systemic infection, and one such instance encountered in the course of the present work is described below in a separate section.

The present survey differs from those mentioned above in its more comprehensive nature, and represents the first survey of this kind to be undertaken in Scotland. The area covered by the investigation is the Edinburgh district, and samples from the following /

following hospitals were examined :

1. Astley Ainslie Hospital, Edinburgh
2. Bruntsfield Hospital (Edinburgh Hospital for Women and Children) Edinburgh
3. Chalmers Hospital, Edinburgh
4. Deaconess Hospital, Edinburgh
5. Edenhall Hospital, Edinburgh
6. Elsie Inglis Maternity Hospital, Edinburgh
7. Liberton Hospital, Edinburgh
8. Longmore Hospital, Edinburgh
9. Military Hospital, Glencourse, Penicuik
10. Princess Margaret Rose Hospital, Edinburgh
11. Rosslynlee Mental Hospital, Roslin
12. Royal Edinburgh Hospital for Mental Disorders, Edinburgh
13. Royal Hospital for Sick Children, Edinburgh
14. Jordanburn Nerve Hospital, Edinburgh.

The survey lasted for a period of 13 months, and covered the period from June, 1957 to August, 1958.

Material and Methods

I Isolation

Specimens for bacteriological examination from the above hospitals are sent as a normal routine to the bacteriological laboratory at the Astley Ainslie Hospital, Edinburgh. This laboratory /

laboratory therefore receives a variety of material from a number of human sources, which is examined by the microscopical observation of films made from fresh samples, and also by culture, for the presence of bacteria. As a result of the willing and invaluable co-operation of the laboratory personnel it was found possible to extend the routine at this laboratory to the extent of inoculating the surface of agar plates provided by the mycoses unit. In this way it was possible to obtain a much larger number of samples than would have been practicable by direct sampling at the hospitals. All samples were treated in the same manner, a small amount of the material being streaked on the surface of the agar by means of a sterile inoculating needle, after preliminary preparation of a smear for microscopic examination. After inoculation, the plates were stored lid downwards at room temperature until transferred to the mycosis unit, whereupon incubation was carried out at 37°C. It is obvious that this method could have led to the isolation of organisms which were incapable of growth at blood temperature, and whose presence in the sample would therefore be incidental. However, many of these "non-thermophils" were shown to be capable of tolerating a temperature of 37°C, and although probably not representing a significant portion of the flora, their presence was duly recorded.

A number of different agar media were used in the course of /

of this survey, including

1) Sabouraud's glucose agar :

peptone ("Oxoid")	.. .. ..	10 g
glucose	.. .. ..	20 g
agar (Davis)	.. .. ..	20 g
water	.. .. ..	1000 ml

The peptone and glucose were dissolved in water, titrated with hydrochloric acid to pH 5.6 and sterilised at 15 atmos for 15 minutes.

2) Yeastrel agar :

yeast extract (Brewers' Foods Supply Co.)	.. .. ..	7 g
peptone ("Oxoid")	.. .. ..	3 g
agar (Davis)	.. .. ..	20 g
water	.. .. ..	1000 ml

No pH adjustment made. Sterilised at 15 atmos for 15 minutes.

3) Malt agar :

malt extract ("Arlannis")	..	25 g
agar (Davis)	.. .. ..	25 g
water	.. .. ..	1000 ml

No pH adjustment made. Sterilised at 15 atmos for 15 minutes.

4) Acid malt agar :

As for malt agar, but the pH is adjusted with hydrochloric acid to 4.5 before autoclaving.

To reduce the number of bacteria present in the sample, penicillin (20 units per ml.) and streptomycin (40 units per ml.) were incorporated in the medium. These were added aseptically

to /

to the medium just before it was distributed into the Petri dishes. The antibiotics (sodium penicillin G, and dihydrostreptomycin sulphate) were obtained from the Distillers Company (Biochemicals) Ltd. Preliminary trials were carried out to determine the most suitable medium, and although the acid media (without antibiotics) were moderately free from bacteria, the most suitable medium, and the one which was finally adopted, was 2.5% malt agar incorporating antibiotics. No antifungal antibiotics were employed as it was considered that their use was unnecessary. At no time in the course of the survey was a yeast isolate lost by overgrowth with a mould.

The prepared agar containing the antibiotics was poured in approximately 12 ml. portions into sterile Petri dishes, and placed in a clean Petri dish carrier for transportation to the Astley Ainslie laboratory. The carriers were rinsed with 5% lysol before use to minimise the possibility of contamination.

The number of samples received weekly varied, but over the period of the survey averaged about 18. In the course of the 13 months, 1,004 samples were examined.

On receipt from the hospital, the cultures were examined for the presence of yeasts and other organisms, and incubated thereafter at 37°C. The surface of the agar was examined daily, and when any growth on the streaks was seen, a small portion of the colony was removed with the point of a flamed inoculating needle, transferred /

transferred to a malt agar slant and incubated at 25°C. The code number of the sample was recorded, together with the date of inoculation onto the plate, the hospital, source, and the name of the patient from whom it was obtained. A brief note on the appearance and number of colonies developing was also made. In a number of cases more than one organism was isolated from a single plate, whilst the process of identification occasionally revealed that what were originally considered to be different yeasts were in fact different colonies of the same species. The number of colonies developing on the plate can be considered significant only on the assumption that the inoculation of the plate is made within a short time of the sample being obtained from the patient. It is known that the number of yeast cells in a sample can increase rapidly after it has been withdrawn from the patient, and if plated out after a delay, can give an entirely erroneous impression of the actual number of cells present in the original material. For this reason no particular significance was attached to the recorded number of colonies. The plates were retained at 37°C for a minimum of 7 days and then discarded. As a means of reducing the possibility of any one colony on the plate having developed from airborne spores, and thus indicating a false association between organism and source, agar plates were period-:ically exposed to the atmosphere. This was carried out at the bacteriology laboratory at the Astley Ainslie Hospital, and at the /

the University Department of Botany, where the identifications were carried out. Species of Penicillium, Mucor, Aspergillus, Cladosporium, and Botrytis were often present as young colonies at room temperature, but their growth was reduced considerably by the incubation at 37°C. Any filamentous fungi developing at the higher temperature were isolated on malt agar and retained for identification. In most cases, these colonies were identified as Aspergillus fumigatus Fresenius., a ubiquitous species which was present in the atmosphere of both laboratories tested. As a result, no attempt was made to associate the mould with the original sample.

When a yeast had been subcultured successfully, it was "purified" in the manner described below, transferred to a malt agar slant contained in a 1 oz. screwtop bottle, and identified.

II Identification

The identification of yeast-like organisms, unlike other fungi, calls for special methods; for classification is based not only on morphology but on a combination of morphological and physiological characters. Special attention must be paid to the methods used in determining the characteristics of any particular yeast, since the result of any one test is largely dependent on the technique involved. As a result, although the criteria used in the identification of yeast isolates were those described by Lodder and Van Rij (39), the methods used in their assessment are described /

described below.

The yeast culture obtained by transfer from the original plate was incubated at 25°C for 48 hours. It was then "purified" by streaking out on a malt agar plate and incubating for at least 3 days. After the colonies have begun to develop, a single isolated colony was removed and transferred to a fresh malt agar slant in a 1 oz. screwtop bottle. This was the stock culture, and after incubation at 25°C for 48 hours, was used as the source of inoculum for the tests described below.

1. Growth in malt extract. This was carried out in 100 ml Erlenmeyer flasks to which had been added 30 ml of malt extract (25 g Arlannis brand malt extract in 1000 ml distilled water). This method was not altogether satisfactory, and an alternative synthetic medium was devised. This medium, the composition of which is indicated below, gave more consistent results than the malt extract. Such features as ring and pellicle formations were more readily assessed on this medium, and it was adopted for routine use in the process of identification. The formula is as follows :

ammonium sulphate	5 g
potassium dihydrogen phosphate ..	1 g
magnesium sulphate (hydrated) ..	0.5 g
glucose	15 g
distilled water	1000 ml

The medium was distributed in 30 ml quantities into the flasks which were then stoppered with non-absorbent cotton wool and sterilised /

sterilised at 15 atmos. for 15 minutes.

Cells from the stock culture were transferred to the flasks, and after a vigorous shaking to ensure even distribution of the inoculum the flasks were transferred to a constant temper-:ature room at 25°C. After 3 days they were examined and the type of growth noted. The presence of a ring at the interface between medium and flask, surface growth such as islets or a pellicle, and the quantity of growth in the bottom of the flask were recorded. The flask was then shaken to distribute the cells evenly, and a small quantity withdrawn by means of a sterile pipette. This was transferred to a microscope slide and camera lucida drawings of the cells were made. By this means, an accurate record of the size and shape of the cells, and the method of budding, was obtained. All drawings were made at a magnification of 3,000, and cell sizes were determined by reference to the drawings. The number of cells drawn varied between 20 and 50.

The flasks were also examined for the presence of ring, islets or pellicle after one month at 25°C.

2. Appearance of streak cultures. Malt agar slants in test tubes stoppered with cotton wool were inoculated in the form of a streak from the stock culture. They were then incubated at 17°C for one month in a constant temperature room. The appearance of the streak was then recorded. The isolate was then preserved by addition of sterile /

sterile paraffin oil, using the technique described by Buell and Weston (40).

3. Production of pseudomycelium. This was tested on maizemeal agar prepared as follows : 42 g of yellow maizemeal was heated in 500 ml of water at 60°C for one hour. After filtering through paper 500 ml of water, 20 g of Davis agar was added and the volume made up to 1000 ml. After melting the agar in a steamer, the medium was distributed in 250 ml quantities into conical flasks, and sterilised at 15 atmos. for 20 minutes.

Petri dishes containing approximately 10 ml of this medium were inoculated from the stock culture. Using a short inoculating needle and sufficient pressure to force the point containing the inoculum just below the surface of the agar, three streaks 4 to 5 mm apart were made of each isolate. A sterile coverslip was placed over part of each inoculum. Each plate contained four isolates inoculated in this manner. Incubation was at 25°C, and after 4, 5, or 6 days the microscopic appearance of the streaks was noted, including the presence or absence of pseudomycelium, its appearance when present, and the presence of chlamydospores.

4. Ascospore production. All isolates were examined for the production of ascospores. This was carried out by the use of carrot plugs, gypsum blocks, and /

and malt agar. Test tubes containing sterile portions of carrot, or sterile gypsum blocks in Petri dishes were inoculated from the stock culture. These were incubated at 25°C and examined for ascospores at intervals of 1, 2, and 4 weeks. Occasionally, old malt agar cultures (3 months or more) were examined for the presence of ascospores. In one instance an isolate was tested for the production of ascospores in sterile water.

Observations were made under oil immersion (Cooke 1.8 mm objective), the cells being stained by the Schaeffer-Fulton modification of the Wirtz method (44) or by mounting in a drop of dilute (0.05%) methylene blue solution. When present, the shape of the ascospores, and the number present in the asci was recorded. An attempt was also made to determine whether or not ascus formation was preceded by conjugation.

5. Fermentation. Originally, sugar fermentations were carried out in ¼ oz. screwtop bottles, using the technique described by Martin et al (41). This method was later discarded in favour of the one described below, which was found to give more consistent results. All isolates were tested in the following manner. Cells from the stock culture were transferred to approximately 1 ml of peptone water. This was prepared by the addition of 100 ml of water to 1 g of peptone ("Oxoid") and 0.5 g of sodium chloride. Sterilisation was carried out at 15 atmos. for 15 minutes. The yeast suspension was allowed /

allowed to stand overnight. After shaking thoroughly, single drops of the suspension were added by means of a sterile glass pipette into the fermentation tubes. These contained a nutrient broth incorporating an indicator, an inverted Durham tube, and a sugar. The fermentation reaction of each isolate was tested against five different sugars, viz. glucose, galactose, sucrose, maltose, and lactose. The inoculated tubes were incubated at 25°C and readings made every day for a period of 15 days. In some cases the period of observation was extended to 20 days, but normally, a positive fermentation reaction was noted between 1 to 10 days. Production of acid, acid and gas, or a negative result were recorded. Where fermentation occurred, the time in days from inoculation and the strength of the fermentation (strong or weak) were noted. Fermentations were considered positive when both acid and gas were produced, and negative when there is no reaction or if acid only is produced.

The fermentation broth has the following formula :

peptone ("Oxoid")	10 g
sodium chloride	5 g
distilled water	900 ml

0.04 g of bromo-thymol blue were dissolved in 100 ml of distilled water. (The indicator goes into solution more readily if the water is made alkaline by the addition of a drop of 1N sodium hydroxide). When the indicator had dissolved, the solution was added to the fermentation broth and the pH adjusted electro-:metrically to exactly 7.2. The broth was distributed in 5 ml quantities /

quantities into test tubes (125 mm x 12 mm) containing an inverted Durham tube (26 mm x 6 mm). The tubes were stoppered with non-absorbent cotton wool and sterilised at 15 atmos for 15 minutes. Different colours of cotton wool were used to indicate the different sugars used in the test. Sugars were prepared as 20% solutions, steam sterilised for 3 days, and 0.5 ml portions added aseptically by sterile glass pipettes to the fermentation tubes. This gave a final sugar concentration of 2%. The tubes, now containing the sugar were steam sterilised for 30 minutes as a precaution against accidental contamination, and on cooling were ready for inoculation. This method was adopted as both the sugar solution and the fermen-:tation broth could be retained for long periods without breakdown or change in pH. The sugars were added to the fermentation tubes just before use. During fermentation, production of acid was revealed by the change in colour from blue to yellow, whilst any gas production was shown by formation of a bubble in the inverted Durham tube.

Occasionally, the fermentation of inulin was tested, and this was prepared as above, the concentration of the inulin also being 2%.

Quantitative fermentation of the trisaccharide raffinose is of considerable taxonomic importance, and following the procedure adopted by Lodder and Van Rij, all sugars which fermented sucrose were also tested against raffinose. The method used was that described /

described by Wickerham (42). The strain of Saccharomyces carlsbergensis Hansen. used for the test was obtained from Dr. Wickerham (Strain Y-379).

On one occasion it was necessary to determine whether or not an isolate fermented melibiose. An adaptation of Wickerham's technique was used for this purpose. A 4% solution of raffinose was inoculated with a species known to ferment only the fructose portion of the raffinose molecule. The yeast used for this purpose was Saccharomyces fragilis Jörgensen., isolate H7981, which was obtained from the stool of a patient suffering from an intestinal complaint. When gas production had ceased only the melibiose portion of the raffinose molecule remained. The solution was then inoculated with the isolate under consideration. As no further gas was evolved, it was concluded that the organism did not ferment melibiose.

6. Sugar assimilation. The ability of each isolate to assimilate glucose, galactose, sucrose, maltose, and lactose was tested by the auxanographic method. The procedure adopted by the writer differed only in detail from the method described for this test by Lodder and Van Rij. Thus, the drop of yeast suspension was added to the melted and cooled agar before pouring into the Petri dishes. Vitamins are provided by the addition of a drop of sterile yeast extract which was also added to the agar just before pouring.

It /

It was found necessary to use two Petri dishes for each isolate, as attempts to assess plates on which all five sugars had been placed were seldom satisfactory, owing to the overlapping of the zones of enhanced growth. Glucose, galactose, and lactose were tested on one plate, sucrose and maltose on the other.

Cells from the stock culture were transferred into a test tube containing approximately 1 ml of sterile water. After mixing, a drop of this suspension was placed in each of two tubes containing about 15 ml of the washed agar which had been melted and cooled to 45°C. After thorough mixing, the agar was poured into a Petri dish, and after setting, small quantities of the sugars to be tested were placed on the surface. Readings were made after incubation at 25°C for 48 hours, although in some cases it was found possible to extend this time. The appearance of a positive reaction is shown in Plate I Figure 1. Slow-growing yeasts and isolates which had given doubtful results were tested by growth in a liquid medium as described by Wickerham and Burton (43).

7. Assimilation of potassium nitrate. The ability of a yeast to assimilate nitrate nitrogen is an important taxonomic character, and may even have generic significance. All isolates were examined by means of the auxanographic method for their ability to assimilate potassium nitrate. The method was similar to that described for

sugar assimilations except that a carbon source (2% glucose) was incorporated in the medium. The melted and cooled agar was inoculated with a drop of the same yeast suspension used for the sugar assimilation test. After pouring and allowing to set, the agar surface was inoculated with small quantities of peptone and potassium nitrate. It was found that the quantity of nitrate used was significant as it tends to diffuse rapidly over the surface and if too much was added, the zone of enhanced growth (if present) was too large, spreading over the greater part of the plate.

The plates were incubated lid downwards at 25°C and the results noted after 1 or occasionally 2 days. A positive reaction is shown in Plate I Figure 2.

8. Ethanol as sole carbon source. This test was carried out for all isolates.

A basic medium containing the following materials was distributed in 5 ml portions in test tubes.

ammonium sulphate	1 g
potassium dihydrogen sulphate ..	1 g
magnesium sulphate (hydrated) ..	0.5 g
distilled water	1000 ml

After sterilisation at 15 A for 15 minutes, a drop of sterile yeast extract and ethanol to the concentration of 3% was added. The tubes were then inoculated with a drop of the same yeast suspension used in the sugar fermentation tests. After inoculation, the tubes were incubated at 25°C and the results noted after 7 days and 3 weeks. Presence of a sediment, ring, or pellicle /

pellicle were noted.

9. Splitting of arbutin. Each isolate was examined for its ability to split the glucoside arbutin, using the method described by Lodder and Van Rij. The medium containing the arbutin and the ferric chloride was poured into a Petri dish. Two isolates were inoculated onto each plate. Readings were made after 6, 10, and 14 days.

10. Reaction in litmus milk. This test was employed only in the case of species of the genus Candida. Since the identification of this genus is dependent on the results of ascospore production and pseudomycelial development tests, this test was carried out after all other tests had been completed. The method used was that described by Lodder and Van Rij.

11. Production of "starch". The production of starch-like compounds is a characteristic of the genus Cryptococcus. All non-ascosporogenous, non-fermenting isolates which did not form pseudomycelium were tested by the method described by Lodder and Van Rij.

12. Production of carotenoid pigments. The red or pink colouration characteristic of the genera Rhodotorula and Sporobolomyces was always conspicuous in any of the media on which the isolate was growing. The presence of a red or pink colour of an isolate was recorded. /

recorded.

13. Production of ballistospores. All red yeasts were examined for the prod-:uction of ballistospores. In the process of "purification" which was carried out for all isolates, the Petri dishes were incubated lid downwards. Ballistospores were readily detected by the appear-:ance on the lid of a mirror image of the developing colonies on the inoculated agar surface.

14. Thermophilic nature. The ability of the isolate to grow at 37°C was determined by inoculating the surface of Petri dishes containing malt agar and incubating at 37°C. Four isolates were streaked onto each plate. Results were noted after 1, 2, and 4 days.

Replications of most of the tests were carried out before final identification of any isolate was made. With certain exceptions, the number of replications of each test were as follows :

1. Liquid medium : twice. The second test was carried out by placing drops of yeast suspension in Petri dishes containing malt extract.

2. Streak culture : once. In a number of cases where the result of the original test did not appear to be typical of the species, a second trial was carried out. If this differed markedly from the original a check on /

on the purity of the isolate was made by streaking out again on malt agar. A third test was then made from the new culture.

3. Formation of pseudomycelium. If pseudomycelium was absent, the test was repeated twice more. If pseudomycelium was present a second test was made to confirm its appearance, method of branching, and method of production of blastospores. An exception was made in the case of chlamydospore production, when no further test was made.

4. Ascospore production : once. Occasionally, an isolate was re-tested to confirm the presence of ascospores.

5. Fermentation. The number of fermentation trials for each isolate varied. At least two fermentations were carried out, but this number was increased to 3, 4, or 5, if reproducible results were not obtained.

6. Sugar assimilation. Normally 3 trials were carried out, but if doubtful results were obtained, this number was increased to 4, 5 or more. In such cases, the isolate was tested in liquid media as described above. One exception was made in the case of those isolates producing chlamydospores in maizemeal agar. If these isolates also fermented glucose, galactose, and maltose, and the result of the first sugar assimilation trial indicated that glucose, galactose, sucrose, and maltose were assimilated, the organism was identified as Candida albicans, and no further sugar assimilation tests /

tests were considered necessary.

7. Nitrate assimilation. Normally twice, but for species of Cryptococcus, this was repeated a third time.

8. Ethanol as sole carbon source : twice.

9. Splitting of arbutin : twice.

10. Reaction in litmus milk : once. Occasionally, this was repeated.

11. Production of starch : if positive once. If negative, the test was repeated.

The results of the tests were recorded on cyclostyled sheets which were designed for the purpose. One of these sheets is shown below.

On the first part of the sheet are recorded :

1) The identity of the isolate

2) Its source, and the date of isolation

3) The code number of the isolate. (This number is the one given to the sample when received at the Astley Ainslie Hospital).

4) Growth in malt extract at 3 days and 1 month. As indicated above, malt extract was later replaced by a synthetic medium.

5) Malt agar - 1 month. Streak character noted.

6) Maizemeal agar. Presence or absence of pseudomycelium noted. If present, the type is recorded, or a brief description given. A freehand drawing is also made when /

when the type of pseudomycelium cannot be referred to any one of the forms recognised by Lodder and Van Rij.

7) Camera lucida drawings are made of a number of cells, showing the variation in size and shape. Cell measurements are made directly from these drawings. A brief description is also given of the cell shape, whether the cells are single, in pairs, clusters, or chains. The method of budding is also noted. Details of ascospore production are also noted here, including ascospore size and shape, and the number per ascus.

On the reverse side are noted :

8) Fermentation of glucose (D), galactose (G), sucrose (S), maltose (M), lactose (L), and raffinose (R).

9) C/M refers to the presence of the highly diagnostic chlamydospores (C) or the production of pseudomycelium (M). If neither is present, a -ve is recorded.

10) EtOH refers to the growth with ethanol as the sole source of carbon. Recorded as + or - . If +, the presence of a ring, or a pellicle is noted.

11) Splitting of arbutin (Arb)+ or - .

12) Growth in litmus milk (milk); reaction noted.

13) Assimilation of glucose, galactose, sucrose, maltose and lactose noted + or - .

14) Assimilation of peptone (Pep) and potassium nitrate (NO_3) noted + or - .

15) Production of ascospores (A'spore) noted + or - in

Car - carrot plugs
Pot - potato discs
VJM - vegetable juice medium
Gyp - gypsum blocks

note: potato discs and a vegetable juice medium were used originally, but were discarded in favour of the carrot plugs and the gypsum blocks.

16) Some details of the case history of the patient were recorded.

An /

An example of the method of recording the characteristics is shown in Text Figures 1 and 2.

Text Figure 1

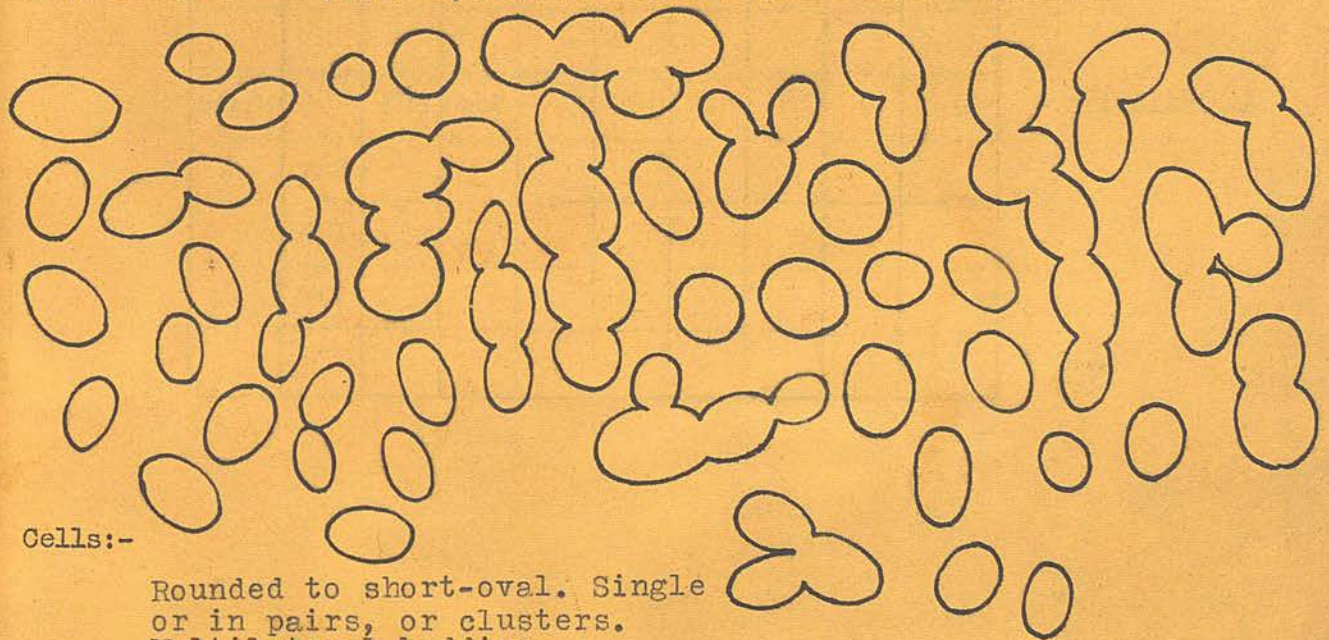
Torulopsis glabrata — Throat swab 19.9.57. — J 3444

Malt extract 3 days:- Sediment only

Malt extract 1 month:- Sediment only : no surface growth

Malt agar 1 month:- Soft, smooth, shiny, spreading, grey. Margins slightly irregular. Mycelial growth absent

Maizemeal agar:- Pseudomycelium absent. 2nd trial - absent



Cells:-

Rounded to short-oval. Single or in pairs, or clusters. Multilateral budding

Text /

Text Figure 2

Fermentation:-

D	G	S	M	L	R	C/M	EtOH	Arb.	Milk
AG 6	-15	-15	-15	-15		-	-	-	
AG 5	-17	-17	-17	-17		-			
AG 3	-18	-18	-18	-18					

Assimilation:-

D	G	S	M	L		Pep	NO3		A'spor
+	-	-	-	-		+	-	Car.	-
+	-	-	-	-		+	-	Pot.	-
+	-	-	-	-				VJM	
								Gyp	-

Male 53 Years. Post appendicectomy chest infection.
Pneumonia ?

Culture : Pneumococcus ++
Film : Epithelial cells +

Results of the Survey

The species of yeasts encountered in the course of the survey are listed below. The numbers of yeasts and other organisms, and the sources from which they were isolated are indicated in Table 1. The distribution of the species of yeasts in relation to the sources from which they were obtained are shown in Table 2.

List of species

ASCOMYCETES (Family SACCHAROMYCETACEAE)

Hansenula anomola (Hansen) H. et P. Sydow

Pichia membranaefaciens (Hansen)

Saccharomyces cerevisiae (Hansen)

Saccharomyces carlsbergensis (Hansen)

Saccharomyces fragilis (Jörg)

FUNGI IMPERFECTI (Family CRYPTOCOCCACEAE)

Trichosporon cutaneum (de Beurm., Gougerot et Vaucher) Ota

Candida albicans (Robin) Berkhout

Candida tropicalis (Cast.) Berkhout

Candida parapsilosis (Ashf.) Langeron et Talice

Candida zeylanoides (Cast.) Langeron et Guerra

Candida krusei (Cast.) Berkhout

Candida robusta Diddens et Lodder

Candida guilliermondii (Cast.) Langeron et Guerra

Torulopsis glabrata (Anderson) Lodder et De Vries

Torulopsis /

Torulopsis famata (Harrison) Lodder et Van Rij

Torulopsis candida (Saito) Lodder

Cryptococcus laurentii (Kufferath) Skinner

Cryptococcus albidus (Saito) Skinner

Cryptococcus diffluens (Zach.) Lodder et Van Rij

Rhodotorula mucilaginosa (Jörg.) Harrison

Table 1. Sources examined with the number of fungi recorded

Source	No. of samples examined	No. of yeasts isolated	% of total	No. of filamentous fungi	% of total
urine	182	18	9.9	8	4.4
sputum	174	120	71.3	9	5.2
throat	103	35	33.0	3	2.9
stool	79	43	53.2	5	6.3
vagina	150	13	8.7	4	2.7
wounds etc.	203	16	7.9	6	3.0
others	113	3	2.7	6	4.9
Totals	1004	248	24.9	41	4.1

Table 2. Species of yeast recorded, with number of isolates obtained for each of 8 sources examined

Species	Sources								
	urine	sputum	throat	stool	vagina	wounds &c	other	Total	% of Total
Hansenula anomola	-	2	-	-	-	-	-	2	0.8
Pichia membranaefaciens	-	2	-	-	-	-	-	2	0.8
Saccharomyces cerevisiae	-	4	-	-	-	-	-	4	1.6
S. carlsbergensis	-	1	-	-	-	-	-	1	0.4
S. fragilis	1	-	1	1	-	-	-	3	1.2
Trichosporon cutaneum	-	-	-	1	-	-	-	1	0.4
Candida albicans	3	70	28	27	7	6	-	141	56.4
C. tropicalis	-	7	2	2	-	1	-	12	4.8
C. parapsilosis	-	1	1	2	-	3	-	7	2.8
C. zeylanoides	1	1	-	2	-	-	-	4	1.6
C. krusei	-	2	-	1	-	-	-	3	1.2
C. robusta	-	1	-	-	-	-	-	1	0.4
C. guilliermondii	-	-	-	-	1	-	-	1	0.4
Torulopsis glabrata	5	15	2	4	3	-	2	31	12.4
T. famata	1	-	-	-	-	-	-	1	0.4
T. candida	-	1	-	-	-	-	-	1	0.4
Cryptococcus laurentii	-	1	-	-	-	-	-	1	0.4
C. albidus	1	1	-	-	-	2	-	4	1.6
C. diffluens	-	-	-	-	-	1	-	1	0.4
Rhodotorula mucilaginosa	4	7	1	2	1	1	-	16	6.4
Total	16	116	35	42	12	14	2	237	95.6
Unidentified isolates	2	4	-	1	1	2	1	11	4.4
Total	18	120	35	43	13	16	3	248	100

Consideration of the various sources examined, with reference to the species isolated are discussed below under separate sections.

1. Urine :

Only 18 isolates of yeast were obtained from 182 samples examined (9.9%). The species obtained, and the number of occasions on which they were isolated are as follows:

Torulopsis glabrata	5
Rhodotorula mucilaginosa ..	4
Candida albicans	3
Candida zeylanoides	1
Torulopsis famata	1
Cryptococcus albidus	1
Saccharomyces fragilis ..	1
Candida sp.	1
Cryptococcus sp.	1

Beck and Löhlein (30) have examined the yeast flora of urine in relation to the administration of penicillin. In 3700 samples of urine from patients not receiving penicillin, only 3 patients harboured yeast-like organisms. Microscopical examin-:ation of the urinary sediment of patients receiving the antibiotic showed the presence of yeast cells in 60% of the samples. No direct comparison can therefore be made with the present survey, where /

where no attempt was made to correlate the presence of organisms with the administration of therapeutic agents.

It is of interest to note that the yeast most commonly isolated from urine was Torulopsis glabrata. Although reports of this yeast are scarce in the literature, Artagaveytia-Allende and Carcia-Zorron (45) have reported its presence in the vaginal tract of the human. Orie (53) has isolated a strain from urine. It is perhaps significant that all 5 isolations of T. glabrata were from female patients, and it may be that in most of the cases, its presence in the urine was incidental. In four of the cases there was no reason to suggest that the organism was not a member of the vaginal flora, and no evidence that this yeast was contained in the urine. In the case of the fifth patient, however, the urine had been removed by means of a catheter. In this instance it is unlikely that the yeast could have originated from the vagina itself since the urine was removed directly from the bladder. Although the possibility remains that the organism could have been acquired by the passage of the tube through the urethral orifice, it is probable that this specimen was isolated from the urine rather than the vagina.

Of the remaining 11 isolates, 8 were from female patients and 3 from male patients. None of these was obtained from catheter samples, hence their presence in the urine of female patients does not exclude the possibility of their being members of /

of the vaginal flora. Two of the 3 isolates of Candida albicans were from male patients. This yeast has been recorded from a variety of sites in the human, and it has been reported as causing infection of the urinary tract (46, 47). Rhodotorula mucilaginosa is also a common member of the flora of a variety of sources, human and otherwise. Like Torulopsis glabrata, it can possibly be regarded as a normal member of the vaginal flora in the human. Three of the 4 cultures obtained in the present survey were obtained from female patients.

All isolates with the exception of Cryptococcus albidus were capable of growth at 37°C.

2. Sputum :

The examination of 174 samples of sputum resulted in the isolation of 120 yeasts (68.9%). The species obtained were:

Candida albicans	70
Torulopsis glabrata	15
Rhodotorula mucilaginosa	7
Candida tropicalis	7
Saccharomyces cerevisiae	3
Candida krusei	2
Pichia membranaefaciens	2
Hansenula anomola	2
Candida parapsilosis	1
Candida zeylanoides	1

Candida /

Candida robusta	1
Torulopsis candida	1
Cryptococcus laurentii	1
Cryptococcus albidus	1
Saccharomyces cerevisiae var. ellipsoideus	1
Saccharomyces carlsbergensis ..	1
Torulopsis sp. (I4837)	1
Endomycopsis sp. (?) (I7094B) ..	1
Cryptococcus sp. (J3104B)	1
Candida sp. (J5069)	1

It has already been noted that several studies on the flora of the mouth have been made (33, 34, 35, 36, 37, 38, 48, 49, 50, 51, 74, 75, 78, 117, 118). The species of yeast recorded and their relative frequencies differ, but a general pattern of distribution is common to them all. Candida albicans is the most common species recorded, followed by a group consisting of C. tropicalis, C. krusei, and C. parapsilosis, and occasionally C. pseudotropicalis (Cast.) Basgal., C. lipolytica (Harrison) Diddens et Lodder, C. macedoniensis (Cast. et Chalmers) Berkhout, C. brumptii Langeron et Guerra, and C. guilliermondii.

The number of yeastlike organisms which have been recorded from sputum is large, and it is generally recognised that many of these are incidental, having been acquired accidentally from the environment. It is well know that yeasts are common on

a /

a wide variety of foodstuffs, and a number have been recorded from the atmosphere (72, 73). The origin of most of the species isolated from sputum in the course of this and other surveys is probably exogenous, that is, from a source outside the host animal. Yeasts occurring in the present survey whose sources are probably exogenous include the ascosporogenous yeasts Saccharomyces, Hansenula, and Pichia, all of which have been isolated from vegetable sources. Some of the isolates of Rhodotorula may also have had exogenous origins, since this yeast has frequently been obtained from air or vegetable sources. Cryptococcus albidus and C. laurentii are both normally saprophytic although the former has been recorded from sputum in Holland (53). Torulopsis candida and Candida robusta are probably not significant members of the flora of the sputum and it is of interest to note that these two isolates, and the two species of Cryptococcus mentioned above, were incapable of growth at 37°C. All the remaining species are those which have been isolated from human sputum.

In common with other studies on the mycological flora of the mouth, Candida albicans is the most common species, representing 57.6% of the total number of yeasts. A feature of unusual interest in the present survey is the exceptionally high incidence of Torulopsis glabrata which was isolated on 15 occasions (12.2% of the total number of yeasts isolated from this source). It has already been noted in the previous section that this organism /

organism was the most common species occurring in samples of urine. Explanations of this unexpectedly high incidence can only be tentative, and are discussed under a separate section. Until the present survey was carried out, T. glabrata was not considered to be a significant member of the yeast flora of the mouth, and the number of occasions on which it has been recorded is small. It has been reported by Artagaveytia-Allende and García-Zorron (45), by Dietrichson (38), and by Black and Fisher (58). It should be noted that the majority of samples received for examination were from patients suffering from a disorder of the respiratory system. In this respect, the material collected for examination should be regarded as morbid rather than healthy. Many, but not all of the patients were suffering from bronchitis or suspected pneumonia, whilst the remaining samples were taken from patients for routine investigation. Included in the latter group were patients whose symptoms were non-specific, and included "tiredness", "pyrexia", "heart-failure", "emphysema", "pulmonary embolism", and others where the presence of a pathogenic organism was not suspected.

3. Throat :

103 plates were examined, from which 34 (33%) yeasts were obtained. The species isolated were:

Candida albicans	28
Candida tropicalis	2
Torulopsis glabrata	2

Candida /

Candida parapsilosis 1

Rhodotorula mucilaginosa 1

Saccharomyces fragilis 1

When the number of isolates for each species is compared with those listed in the previous section, differences are noted in the total number of species recorded. Both Candida albicans and Torulopsis glabrata are less frequently isolated from the throat.

The distinction between throat and sputum samples may be considered an arbitrary one, and there is no reason for supposing that an organism isolated from the throat is confined to this site. On the basis that all the isolates from this source were obtained by direct sampling from the throat by means of swabs, it was felt that the separation was justified. All the species occurring in the throat were also represented in the sputum, and any difference between the sources is quantitative rather than qualitative. Of the 16 species of yeast occurring in the sputum, only 6 were represented in the throat. Their frequency was much lower, occurring in 33% of the total number of samples examined as against 71.3% in the case of sputum. It is clear that the throat harbours a smaller proportion of yeasts than the mouth, and it is suggested that this may be attributed to differences in the nature of the two regions. It seems possible that the greater number of organisms present in the mouth could be explained by supposing /

supposing that many of the yeasts have been acquired from an external source, perhaps by the ingestion of food material. Once inside the mouth they may remain viable for varying lengths of time on particles of the original substrate. It is possible that the mouth retains sufficient particles in crevices between the teeth or beneath dentures. The organisms acquired accidentally may be capable of resisting for a time, the changes in the environment, including a higher temperature, the presence of salivary enzymes, and the competition with normal commensals. Ultimately, however, their inability to survive these conditions would be reflected in a reduction of their numbers. Those organisms which are capable of surviving would be better adapted to their new environment, and might be expected to become estab-:lished as members of the mouth flora. It is suggested that the throat provides a less suitable environment for the growth and propagation of yeasts than the mouth, and that the smaller number of isolates made from throat swabs supports this view.

Patients from whom isolates were made were showing a variety of clinical symptons. "Sore throats", "bronchitis", and "upper respiratory infections" accounted for approximately 43% of the number of symptoms or diagnoses. Other disorders included rheumatic fever, enlarged heart, rheumatism, and pyrexia. In one case, C. albicans was isolated in quantity from white patches in the pharynx: yeast cells were also noted in the film made from the /

the original sample.

The comparatively low number of species isolated from the throat in the course of the present survey is not in accordance with the findings of Reiersøl (54), who obtained yeasts in 114 (45.6%) out of 250 laryngeal swabs. Brygoo (29), in a study of 66 strains of Candida isolated from pharyngeal swabs reported that species of Candida were isolated from more than 30% of the total number (125) examined. Both Brygoo and the writer obtained Candida albicans, C. tropicalis, C. parapsilosis, and C. zeylanoides from throat swabs. Not represented in the present survey was C. robusta, although this species was recorded from sputum. Other species of Candida reported by Brygoo were C. pelliculosa Red., C. guilliermondii, C. macedoniensis, C. krusei, C. catenulata Diddens et Lodder, C. rugosa (Anderson) Diddens et Lodder, and C. heveanensis (Groenewege) Diddens et Lodder (= Cryptococcus laurentii).

All cultures obtained from this source were capable of growth at 37°C.

4. Stool :

79 samples were examined from which 43 (54.4%) cultures of yeasts were obtained. The following species were isolated:

Candida albicans	27
Torulopsis glabrata	4
Candida tropicalis	2
Candida parapsilosis	2
Candida zeylanoides	2
Rhodotorula mucilaginosa	2

Candida /

Candida krusei	1
Trichosporon cutaneum	1
Saccharomyces fragilis	1
Candida sp	1

A number of studies on the yeasts occurring in the human intestinal tract have been made (26, 45, 55, 56, 57, 59, 60, 61). Many of these deal specifically with the incidence of Candida albicans. Negroni (62) has recorded the presence of yeasts in 36 out of 50 specimens of faeces. He reports the species most commonly associated with this source to be Candida krusei, followed by C. parakrusei (= C. parapsilosis), C. albicans, C. zeylanoides, C. tropicalis, and C. chalmersi (= C. parapsilosis). Both Windisch and Staib (55) and Mehnhert (26) have reported a different pattern. The former paper reports the presence of yeasts in 37% of 1,158 samples of human faeces, whilst Behnhert has shown the incidence to be 26%. The figure obtained by the writer was 54.4%. Both papers have reported that C. albicans was the most common species, and this was also noted in the present survey. Candida krusei is frequently associated with the human intestinal tract, but was found on one occasion only in the present work. Candida parapsilosis (55, 62), Trichosporon cutaneum (55), and C. zeylanoides (26, 62) have all been reported from faeces and each of these species was isolated from this source in the course of the present study. Saccharomyces fragilis has also been reported from this site (63) and although members of the ascosporogenous /

ascosporogenous yeasts have frequently been isolated from faeces, they appear to be incidental rather than normal inhabitants. Rhodotorula mucilaginosa has also been reported from faeces and two of the strains studied by Lodder and Van Rij (39) were isolated from this source.

Torulopsis glabrata was again prominent amongst the species isolated in the present survey. This organism was originally isolated from human faeces (64) but it has seldom been reported since then from this source. Parle (65) has isolated 3 strains in the course of his investigation on the yeasts occurring in the alimentary tract of several mammals.

All the organisms isolated from stool samples were capable of growth at 37°C.

5. Vagina : (High vaginal swabs)

Out of 150 specimens examined, 13 (8.7%) yeast isolates were obtained, representing only 4 species. These were:

Candida albicans	7
Torulopsis glabrata	3
Candida guilliermondii	1
Rhodotorula mucilaginosa	1
Candida sp (C. zeylanoides?) ..	1

Studies on the vaginal flora of the human (45, 66, 67, 68, 70) have consistently revealed that the most common member of the yeast flora is C. albicans, which is often associated with a form of vaginitis. Other species isolated from the vagina are /

are C. pseudotropicalis (Cast.) Basgal. C. krusei, C. parapsilosis, C. stellatoidea (Jones et Martin) Langeron et Guerra., C. guilliermondii, and C. tropicalis. Rhodotorula species have been reported by Vaccaro and Ferrada Urzua (66), and Torulopsis glabrata by Artagaveytia-Allende (71).

The specimens received for mycological investigation from the Astley Ainslie laboratory were almost exclusively high vaginal swabs. These represent routine examination of the upper portion of the vagina following parturition. These swabs were all obtained during puerpurium and are therefore not comparable to those made from the vagina at any other time. Following parturition, the environment of the vagina is altered considerably: mechanical and physical changes almost certainly bring about the removal of many of the organisms present in the superficial layers of the vagina. This is reflected by the reduced number of isolates made from this source. No other record of the fungi present in puerperial swabs could be found so that comparisons in this case are not possible. In view of the specialised nature of the source from which the isolates were derived, it is considered that no particular significance can be attributed either to the identity of the organisms or their incidence in the samples.

6. Wounds etc. :

16 isolates (7.9%) were obtained from 203 cultures examined, viz.:

Candida /

Candida albicans	6
Candida parapsilosis	3
Cryptococcus albidus	2
Candida tropicalis	1
Cryptococcus diffluens	1
Rhodotorula mucilaginosa	1
Cryptococcus sp	1
Candida sp (C. krusei ?)	1

Samples in this section were those which had been taken from surgical and other wounds, including surface swabs and pus. Samples were also taken from discharging sinuses, lesions, boils, abscesses, carbuncles, septic stitches and bed sores. More than 95% of the samples were obtained from septic wounds or sores occurring on such varied regions as the heel, ear, breast, finger, leg, scrotum, eye, hand, neck, scalp, and groin. A small number, accounting for less than 5% of the total number of samples examined were from internal abscesses or ulcers, but no yeasts were isolated from these samples.

The superficial position of the majority of the wounds or sores together with the presence of dead tissue suggested that from this material a number of saprophytic forms might be isolated. It is interesting to note that of the species isolated from these sources only C. albicans, C. parapsilosis, and C. tropicalis were capable of growth at 37°C. The Candida sp. tentatively identified as /

as C. krusei showed reduced growth at this temperature, whilst none of the species of Cryptococcus were able to grow on cultures maintained at blood temperature.

Yeasts have occasionally been reported from pus (97, 116) and pathological conditions of the human skin (76). Both C. albicans and C. parapsilosis have been reported from cutaneous lesions (77). Drouhet (102) considers that the isolation of C. albicans from cutaneous lesions is of pathological significance, since it is the only species of Candida not found in the saprophytic state on the skin, but only on mucous membranes. If this is so, it is possible that the isolates of this yeast in the present survey may be implicated in the sepsis. In this respect it probably differs from the other species isolated from this source, where there is little evidence to suggest that these fungi are actively associated with inflammation. In none of the cases were yeast cells recorded in the smear, and in only one instance were "Candida" colonies recorded at the bacteriological laboratory.

From the methods used and the results obtained it was clear that the presence of yeasts in these sources was exceptional. Over 92% of the plates examined were negative for yeasts although bacteriological examination by hospital personnel showed that bacteria were invariably present. It is of interest to note that the number of isolates of C. parapsilosis occurring in pus accounted for 50% of the total number recorded in the course of the complete survey. /

survey.

7. Other sources :

Only 3 isolates of yeast were made in the remaining 113 samples (2.7%) examined. Two of these were referable to Torulopsis glabrata. In one case, the fungus was isolated from "sticky eyes" of a male infant, 9 days old. The other isolate was made from a rheumatic nodule in a 71 year old female patient. The third (unidentified) species is tentatively referred to the genus Rhodotorula, and was isolated from a submaxillary gland. It was not capable of growth at 37°C and as only a single colony developed on 2 plates inoculated with the original material, its presence is regarded as accidental.

The sources examined in this heterogenous group consisted primarily of routine swabs from ear, nose, and eye. Also examined were swabs from infected nail beds (paronychia), skin scrapings, olecranon bursitis, and appendicitis.

8. Fungi isolated from the atmosphere :

In an attempt to establish which of the yeasts isolated in the course of the present survey were also present in the atmosphere, Petri dishes containing malt agar and incorporating antibiotics were exposed periodically to the air. This was carried out at the Bacteriology laboratory, Astley Ainslie Hospital, and at the Mycology laboratory, Royal Botanic Garden. Each plate

was /

was duplicated and the presence of mould and yeasts was recorded after 24 to 48 hours incubation. One of the plates was incubated at 37°C, the other at 25°C. No yeasts were isolated from the plates incubated at the higher temperature. It has already been mentioned that Aspergillus fumigatus was frequently observed, both in the atmospheric plates and in those plates used in the course of the survey of human sources. No quantitative estimation was made either of this species or of the yeasts which were isolated. Only Aspergillus fumigatus among the filamentous fungi was recorded. Identification of the yeasts was achieved by means of the tests described above (pp. 14-27). Of the organisms studied, the most common yeasts were members of the genus Cryptococcus. The atmosphere at the Astley Ainslie Hospital was shown to harbour Cryptococcus laurentii, Pullularia pullulans (deBary) Berkhout., and Sporobolomyces roseus Kluyver et Van Niel. Also present were Candida guilliermondii and an unidentified species of Cryptococcus. In the Mycology laboratory at the Royal Botanic Garden, where the identifications were carried out, Cryptococcus albidus, Pullularia pullulans and Rhodotorula mucilaginosa were isolated.

Of the species isolated from human sources, Cryptococcus albidus, C. laurentii, Candida guilliermondii, and Rhodotorula mucilaginosa were also recorded from the atmosphere. The possibility therefore exists that their presence in the plates inoculated from human sources was due to aerial contamination.

When /

When only one or two colonies developed on the inoculated agar surface this possibility could not be discounted. It is suggested that the paucity of colonies appearing on the inoculated surface gives an indication of the probability of an exogenous origin. Thus the greater the number of colonies appearing, the more likely it is that they have been present in the sample.

Di Menna (72) and Connell and Skinner (73) have carried out analyses of the yeasts isolated from air, and have recorded the presence of all of the above species with the exception of Candida guilliermondii. This yeast, however, is relatively common in sources outwith the animal body, and its presence has been reported in the aerial flora by Saito (79).

Discussion

The results of the survey show that only 4 species can be regarded as relatively common. Candida albicans accounted for 56% of the total number of yeasts isolated. Second in incidence was Torulopsis glabrata (12.4%), followed by Rhodotorula mucilaginosa (6.0%) and Candida tropicalis (4.8%)

The original discovery of C. albicans was made by Robin (80), who was the first to describe its association with the disorder of the throat known as "thrush". Since then it has been isolated repeatedly from a variety of animal sources, human and otherwise : it is undoubtedly pathogenic although it is also frequently found in association with apparently healthy individuals. Many studies have been made of the varied and numerous associations of this fungus with the animal body. The number of occasions that this species has been isolated from sources other than the animal body is small, and only 5 such records have been made (81, 82, 83, 84, 85). There seems little doubt that its presence in soil or vegetation is exceptional. It seems likely that its occurrence in soil can be attributed to the previous presence of animals in the vicinity i.e. derived from faeces, but it is interesting to note that Di Menna (82) demonstrated the ability of the fungus to exist in garden soil for at least 9 months. The number of qualitative studies of the yeast flora of soil and other sources is small,/

small, and it is possible that further investigations would provide additional records of the occurrence of C. albicans and other pathogenic yeasts.

In the present survey, C. albicans was isolated from stools, urine, sputum, throat, swabs, vaginal swabs, and from wound discharges. It was the yeast most frequently isolated from all sources, with the exception of urine, where Torulopsis glabrata was the most common form. The comparatively high incidence of this species is considered to confirm the reports of its widespread distribution .

Torulopsis glabrata, since its initial recovery from human faeces (64), has been isolated subsequently from a number of human sources (38, 53, 58, 71, 86, 87, 88). Reports of its occurrence were originally exclusively from human sources, but later studies revealed its association with other animals. It has been isolated by Phaff et al. (89) from the Shrimp, and by Parle (65) from the mammallian alimentary tract. Recca and Mrak (90) have isolated a strain from concentrated orange juice, and a single report of its isolation from garden soil in Holland has been made (91). Nevertheless, on the basis that the organism is normally obtained only from the animal body, it is considered that the yeast is endogenous in origin. In this respect it is comparable to C. albicans where spread of the fungus is from one individual to another rather than from an external source.

The /

The pathogenic status of this yeast is uncertain, and the results of inoculation tests on animals have been inconsistent. Black and Fisher (58) using a strain of T. glabrata isolated from a patient suffering from broncho-pneumonia, inoculated the yeast intraperitoneally into rats. The rats were sacrificed after 5 to 10 days, and the yeast recovered from some of the organs. Lopez-Fernandez (92) has found it pathogenic to laboratory animals. Lodder and De Vries (87) obtained evidence of pathogenicity of this species towards rats. Later experiments carried out by Lodder and De Minger (93) showed that no pathological changes could be observed following intraperitoneal and subcutaneous inoculation into mice and rats : it was considered that the strain of yeast used in the inoculation was not pathogenic. From these reports, it appears that the species is a marginal pathogen in laboratory animals, the degree of pathogenicity probably depending on the strain of yeast employed, and the susceptibility of the individual host animal.

Wickerham (94) has suggested that this yeast is apparently increasing in incidence, and has reported its association with 2 cases of septicaemia. He suggests that the increase in incidence may be the result of the widespread use of broad-spectrum anti-:biotics. This would be directly comparable to the increase in the number of infections caused by C. albicans following antibiotic therapy. The number of yeasts made in the course of the present survey /

survey was high, 31 isolates being obtained. It seems possible that this is the first record of the occurrence of this species as a significant member of the yeast flora of the particular sources examined. The organism was isolated most frequently from sputum, and was also recorded from urine, throat swabs, stools, high vaginal swabs, an eye swab, and a swab from a rheumatic nodule. The failure of atmospheric plates to reveal its presence, and the absence of any reports of this fungus being isolated from air, suggest that the possibility of aerial contamination of the plates can be discounted. The production of colonies from inoculated plates is therefore considered to be an indication of their presence in the original sample. In this respect, the number of isolations of Rhodotorula mucilaginosa must be viewed rather more critically, since it has been shown that this species is capable of aerial dispersal. This yeast (R. mucilaginosa) was isolated repeatedly from the atmosphere of the Mycology laboratory, Royal Botanic Garden, although it was never detected in the atmosphere of the Bacteriology laboratory, Astley Ainslie hospital.

Rhodotorula mucilaginosa has been isolated from a variety of sources. In the strains studied by Lodder and Van Rij (39) original isolation had been made from water, beer, sputum, faeces, air, human hair, diseased nails, bile, and a wound secretion. Clark et al (95) have reported its presence on fresh apples, and Ainsworth and Austwick (96) have recorded its presence in a number of /

of pathological specimens obtained from domestic animals. In common with C. albicans and T. glabrata, the incidence of R. mucilaginosa was greatest in the sputum : it was also found in urine, throat swabs, and stools.

Candida tropicalis has been isolated from a variety of disorders since it was originally described by Castellani (103). Unlike C. albicans and T. glabrata it has been isolated from a number of vegetable sources, although in common with them, it is normally regarded as a typical member of the mycological flora of certain animal sources. In the human it has been found associated with disorders of the lungs (98), intestine (57), mouth (99), nails (99, 100), skin (99), toes (116), and pulmonary tuberculosis (34). It has also been found as the cause of a fatal septicaemia in an Edinburgh hospital. Identification of this organism was carried out in the mycoses unit, and a detailed description is noted below in a separate section. Although this yeast has been isolated from several human sources, it is generally confined to the respiratory tract, the intestine, or the skin. No isolates were made in the present survey from urine or vaginal swabs, although records have been made of the occurrence of this species in both of these sources by Drouhet (101). The incidence of this species recorded by the writer is low, and only 12 isolates were obtained from the 1,004 samples examined. In all of the sources from which it was obtained (sputum, throat swab, stool, and wound) it accounted /

accounted for between 5 - 6% of the total number of yeasts recorded from that particular source.

Unidentified isolates :

Of the isolates obtained in the course of the survey 11 could not be ascribed with certainty to any particular species. An account of each of these species with comments on its taxonomic affinities and tentative identification is given below.

Unless otherwise stated, the organisms were capable of growth at 37°C.

1. H 7681 Isolated from a high vaginal swab 3.7.57

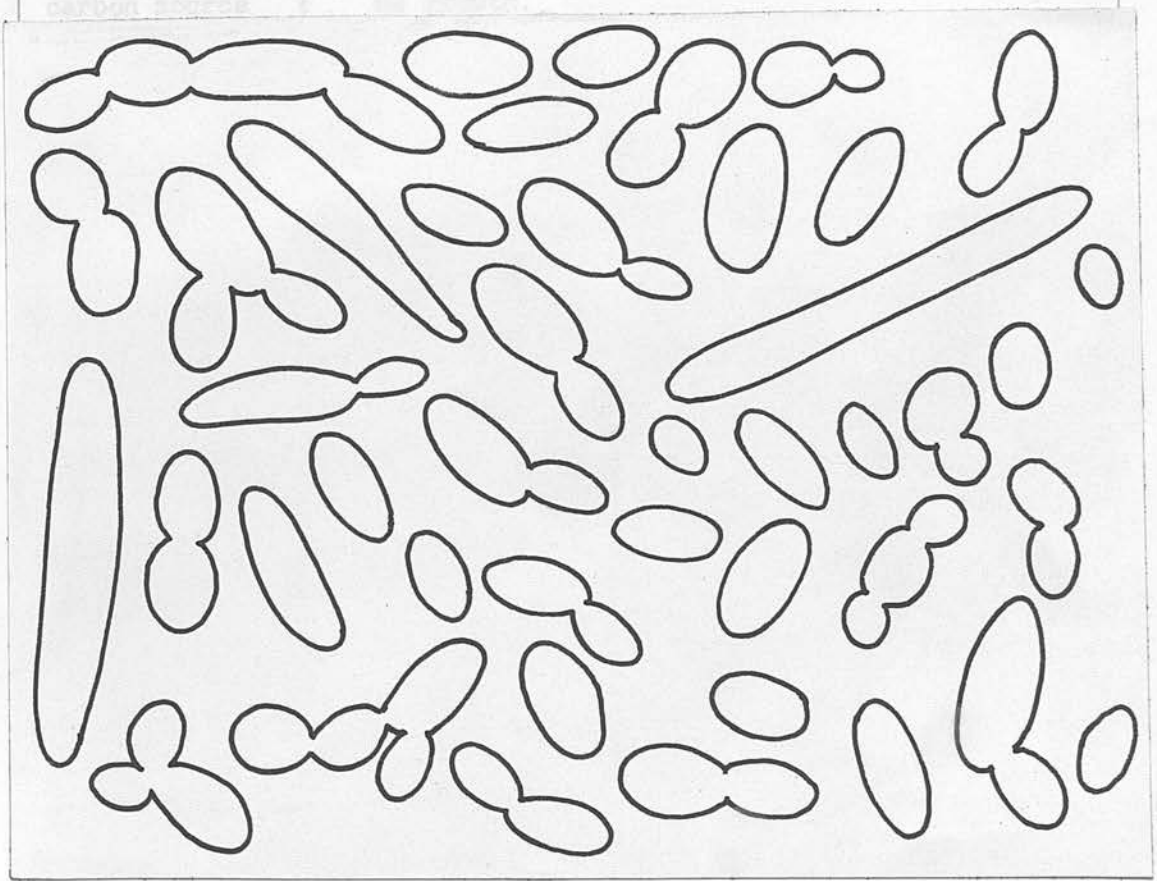
Liquid culture : 3 days - slight trace of a ring; no other surface growth.
1 month - discontinuous ring, no islets or pellicle.

Malt agar, 1 month : streak soft, smooth, grey, glistening. Margin smooth, surrounded by a narrow fringe of pseudomycelium. Submerged growth absent.

Pseudomycelium : well developed, much branched, blastospores in small verticils. Pseudomycelial cells often curved, giving strands a sinuous appearance.

Cells : /

Cells : (Text Figure 3) X 3200



Oval to long-oval, some cylindrical. Single, in pairs, or in small clusters. (2-5)μ X (3-12)μ. Elongate cells may be up to 20μ in length.

Fermentation : a very weak fermentation of glucose was recorded in 4 out of 5 trials, and only after a minimum period of 15 days.

Sugar assimilation : only glucose assimilated.

Nitrate assimilation : absent.

Ethanol /

Ethanol as sole carbon source : no growth.

Growth in litmus milk : no change noted.

Splitting of arbutin: absent.

Ascospores : absent.

The absence of ascospores and the production of pseudomycelium indicate that this isolate is a member of the genus Candida. The species which it most closely resembles are C. mycoderma (Reess) Lodder et Van Rij and C. zeylanoides. These two species differ essentially in the production of a pellicle, which is quickly produced by the former species and is absent in the latter. The ability to ferment glucose, however, is not characteristic of C. zeylanoides. Nevertheless, the writer is of the opinion that the absence of a pellicle, the size and shape of the cells, the appearance of pseudomycelium, and of the streak, are quite typical of this species. The isolate is therefore referred to Candida zeylanoides, differing from the other strains of this species in its weak fermentation of glucose.

2. I 4837 Isolated from sputum 24.8.57

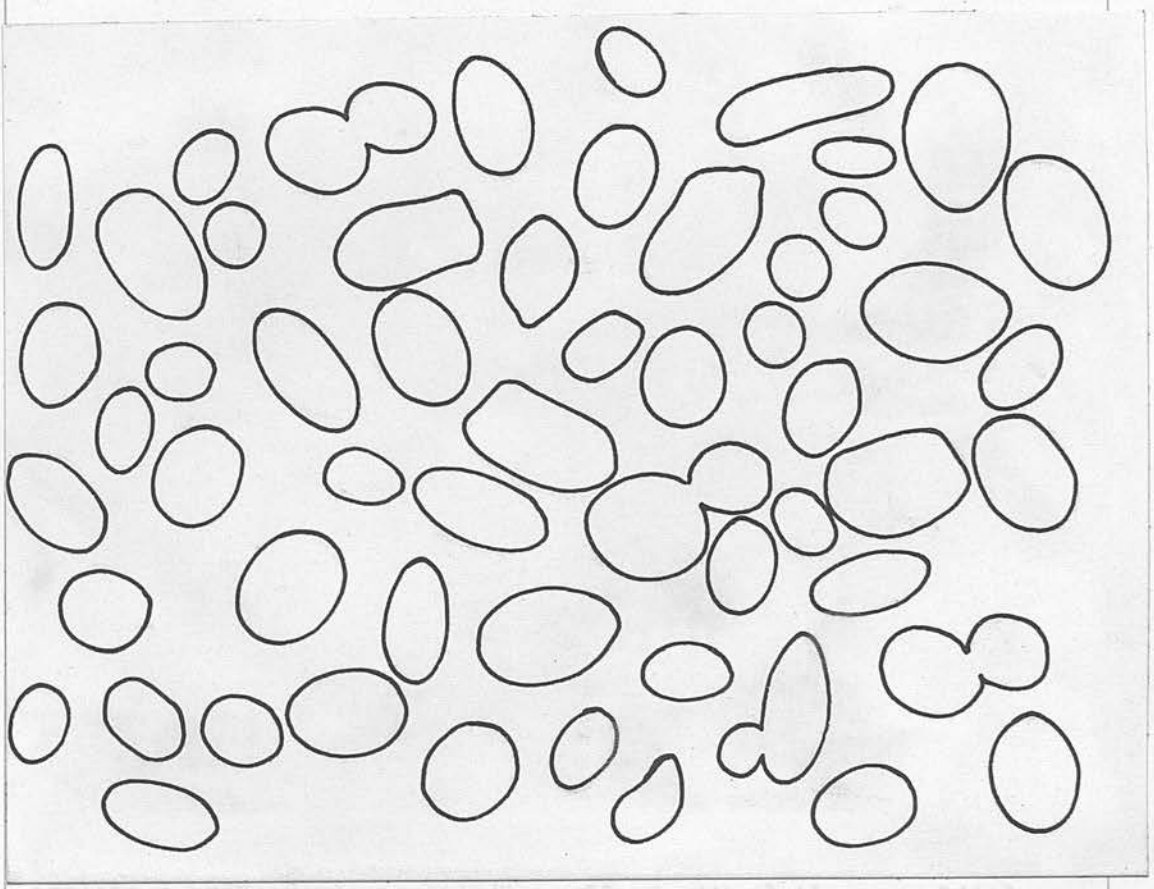
Liquid culture : sediment only, no surface growth.

Malt agar, 1 month : streak soft, smooth, spreading, thin, glistening, grey-white. Margins smooth. Pseudomycelial growth absent.

Pseudomycelium : /

Pseudomycelium : absent. X 3200

Cells : (Text Figure 4)



short-oval, occasionally oblong, or long oval. Single or in pairs. Multilateral budding. (2-4)μ X (3-8)μ.

Fermentation : glucose, galactose, sucrose, maltose, and raffinose +$\frac{1}{3}$ fermented.

Sugar assimilation : absent.

Nitrate assimilation : absent.

Ethanol as sole carbon source : no growth.

Splitting /

Splitting of arbutin : weakly positive.

Ascospores : absent.

This organism was incapable of growth at 37°C.

The absence of ascospores and pseudomycelium, the presence of oval cells, and a fermentive ability would suggest that this isolate is a representative of the genus Torulopsis. No species of Torulopsis, however, has been described which has the fermentation and assimilation patterns of the isolate under discussion. The possibility exists that this species is a strain of Candida which does not produce pseudomycelium, and a comparison was therefore made with species of Candida which showed similar fermentation and assimilation reactions. The species which most closely resembles the present isolate is Candida robusta, which is characterised amongst other features, by a poor development of pseudomycelium. Several similarities between this species and isolate I 4837 are seen, but the cells in the latter are little more than a half of the size reported for C. robusta. The inability of the isolate under discussion to produce pseudomycelium appears to be consistent, and no trace of its development was noted in the course of 5 separate trials. It is therefore considered that this isolate should be referred to the genus Torulopsis.

3. I 5460 Isolated from urine 23.9.57

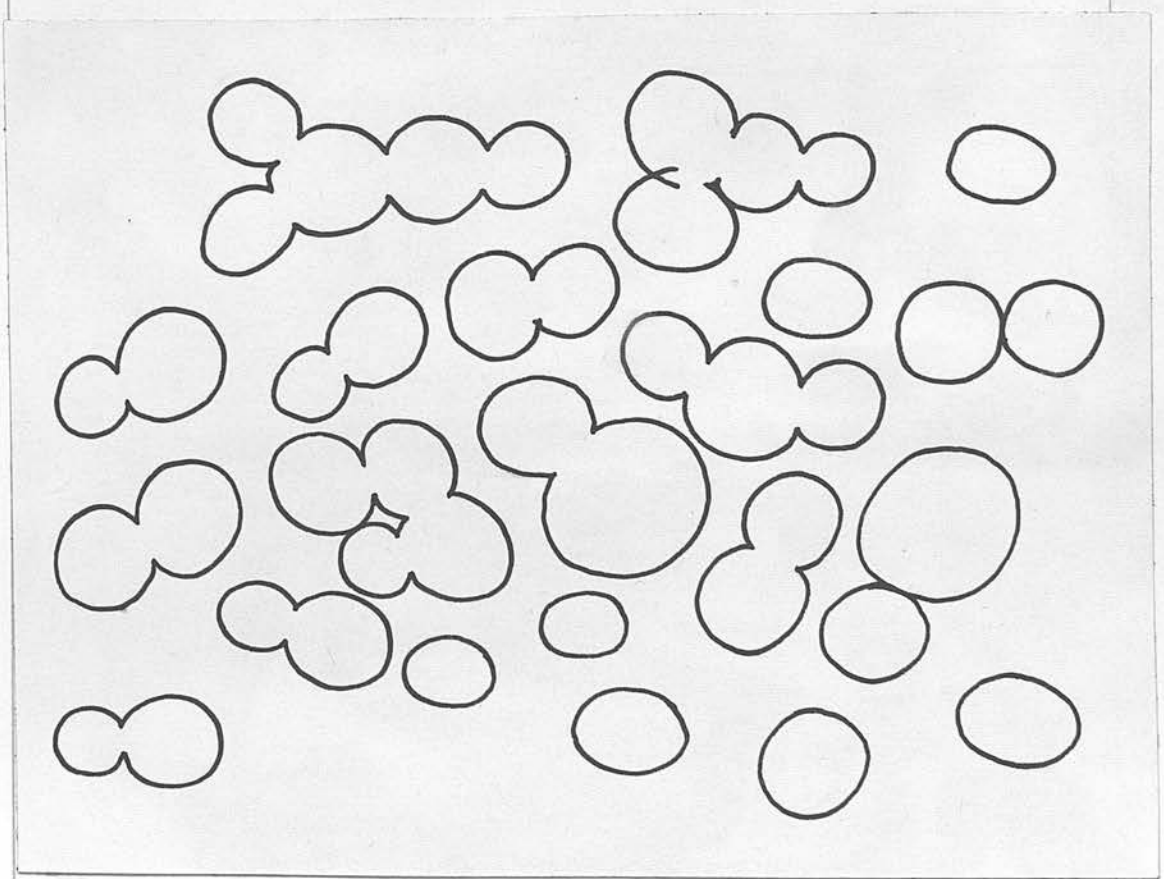
Liquid culture : sediment only, no surface growth.

Malt /

Malt agar, 1 month : streak soft, smooth, white, rather flat, margin slightly irregular, more or less glistening. Submerged growth present.

Pseudomycelium : abundant, branched, forming a conspicuous fringe to the inoculated streak. Blast-:ospores few, arranged in small verticils. Giant cells present.

Cells : (Text Figure 5) X 3200



rounded, seldom single, usually occurring in irregularly branced clusters (3-5.5)μ X (4-6.5)μ /

(4-6.5)μ. Multilateral budding.

Fermentation : glucose and galactose (weakly) are fermented.

Sugar assimilation : glucose, galactose, sucrose, and maltose are assimilated

Nitrate assimilation : absent.

Ethanol as sole carbon source : no growth.

Growth in litmus milk: litmus turns blue.

Splitting of arbutin : absent.

Ascospores : absent.

There seems little doubt that this isolate is closely related to Candida parapsilosis. Identification was considered tentatively on the basis that the cell shape in liquid media is consistently rounded : there were no oval or pseudomycelial cells which are typical of C. parapsilosis. This feature was constant in 3 different trials.

The fermentation and assimilation patterns, the appear-:ance of the pseudomycelium, and the presence of giant cells on maizemeal agar are all features common to this isolate and Candida parapsilosis, and the organism I 5460 is therefore tentatively referred to this species.

4. I 7094 B Isolated from sputum 25.11.57

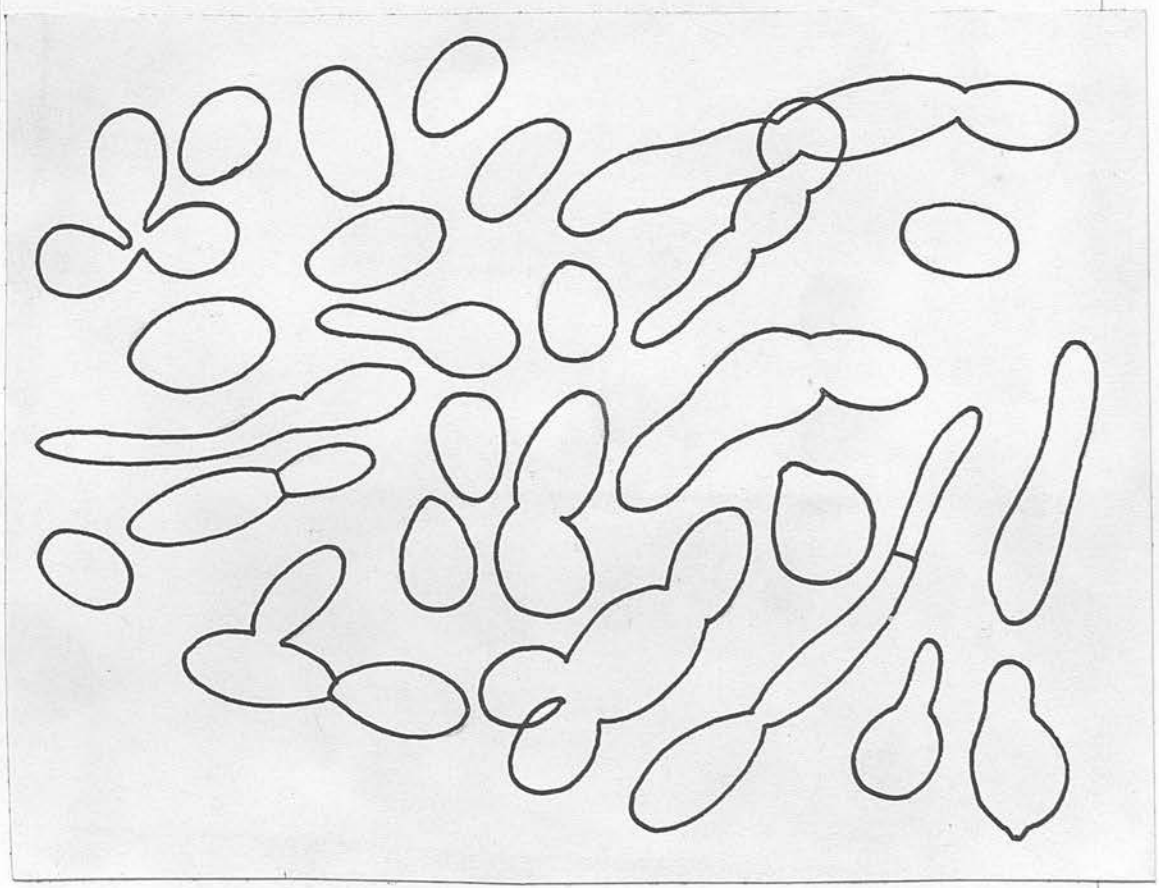
Liquid culture : sediment only; no surface growth.

Maltagar, 1 month : streak tough, shiny, grey, with a faint tinge /

tinge of pink, more or less flat, with a fringe of mycelial growth round the margin.

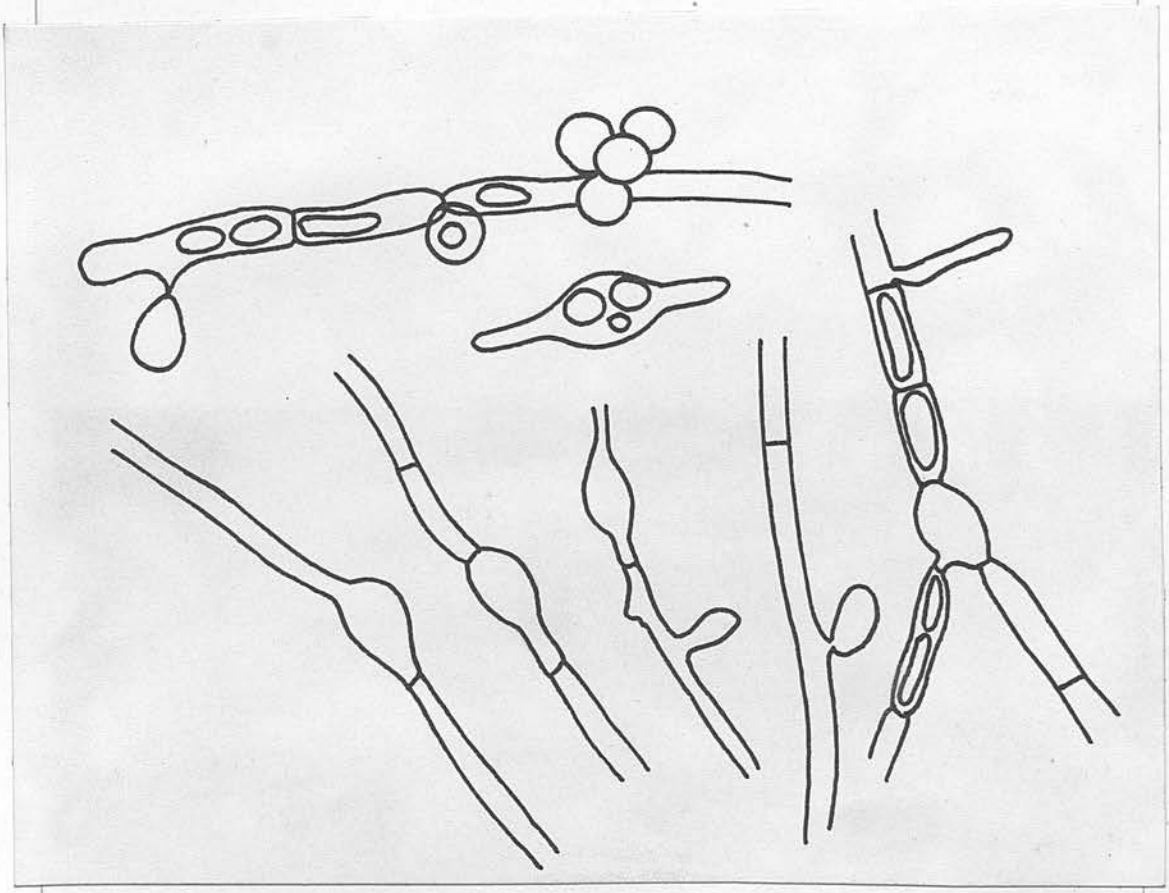
Pseudomycelium : extensive mycelial development. Spores produced in pleurogenous clusters along the length of long, branched, vacuolate, true hyphae.

(Text Figure 6) X 3200



Cells : /

Cells : (Text Figure 7) X 1400



very variable in shape; from short to long-oval, some cells ovoid. Numerous pseudo-:mycelial strands, and also some true mycelium. (3.4-5)μ X (4.5-9)μ, but transitions were observed from long-oval through cylindrical to pseudomycelial.

Fermentation : absent.

Sugar assimilation : 4 auxanogram tests revealed that glucose and galactose (weak) were assimilated, but the /

the results for lactose assimilation were inconsistent. Liquid medium tests showed that glucose, galactose, sucrose, and maltose were assimilated and that lactose was not.

Nitrate assimilation : absent.

Ethanol as sole carbon source : no growth.

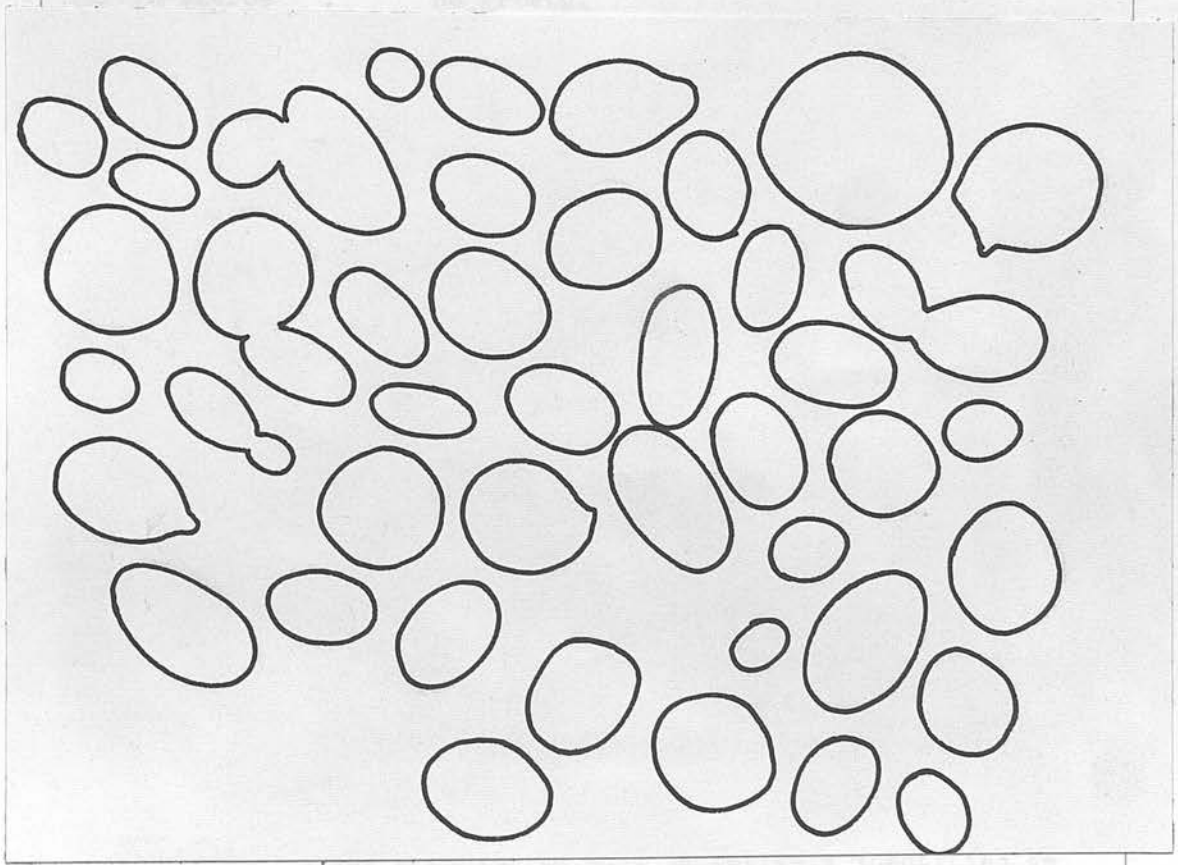
Splitting of arbutin : absent.

Ascospores : doubtful.

The presence of extensive true mycelium with pleurogenous production of budding spores is characteristic of the genus Endomycopsis Dekker. However, this genus is classified in the ascosporogenous Endomycetaceae due to its production of intercalary or terminal asci. The present isolate was examined repeatedly on carrot plugs, gypsum blocks, and in sterile water. The presence of ascospores was never established with any certainty, and only one structure was interpreted as an ascus, which appeared to have 3 crescent-shaped ascospores within. That the isolate should be referred to the yeasts (sensu stricto) is indicated by the presence of budding cells (Text Figure 6), but due to the failure of the tests to demonstrate ascospore production, the organism can be referred to Endomycopsis only on a provisional basis.

5. /

5.	I 7987	Isolated from pus 23.12.57
Liquid culture :		3 days - trace of ring. Moderate sediment.
		1 month - sediment only.
Malt agar, 1 month :		soft, smooth, more or less glistening, spreading, pale brown with a mauve colour-:ation at the centre of the streak; mycelial growth absent.
Pseudomycelium :		absent.
Cells :		(Text Figure 8) X 3200



rather variable : may be small and rounded (2-3)µ, /

(2-3)μ, or short-oval to oval, and measuring (2.5-7.5)μ X (4-9)μ. Some cells are characterised by the presence of small (< 1μ) polar papillae.

Fermentation : none of the sugars tested was fermented.

Sugar assimilation : only glucose assimilated. In the liquid medium test, some growth was noted in maltose.

Nitrate assimilation : absent.

Ethanol as sole carbon source : no growth.

Splitting of arbutin : absent.

Ascospores : absent.

This organism was incapable of growth at 37°C.

This yeast shows certain characteristics of the genus Cryptococcus, viz. the absence of ascospores, of pseudomycelium, and of fermentation. Tests for the production of "starch" were carried out as described by Lodder and Van Rij (39). The test was weakly positive, i.e. when the iodine solution was placed on the yeast growth, colour production was not intense. Since 5 repetitions of this test failed to demonstrate conclusively, the production of "starch", and as none of the species of Cryptococcus already described possess the assimilation pattern characteristic of this isolate, the organism is only tentatively identified as Cryptococcus sp.

6. /

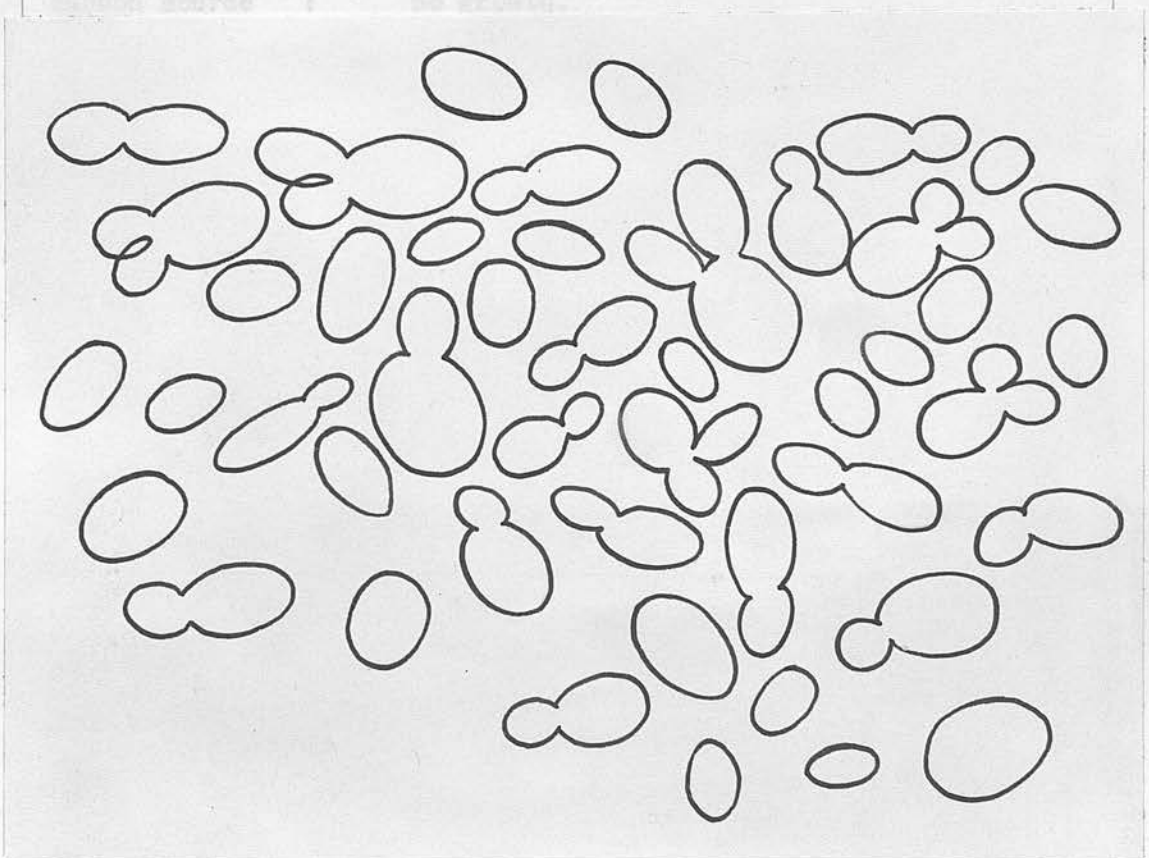
6. J 3104 Isolated from urine 10.3.58

Liquid culture : no surface growth; little sediment.

Malt agar, 1 month : soft, pasty, purple-pink, spreading, matt, covered with narrow wrinkles towards the margins. Mycelial growth absent.

Pseudomycelium : absent.

Cells : (Text Figure 9) X 3200



oval to long-oval; single, in pairs, or small clusters. Multilateral budding.

(2-5)μ X (3-6)μ.

Fermentation : /

Fermentation : none of the sugars tested was fermented.

Sugar assimilation : auxanograms were inconsistent. Liquid medium tests showed that glucose, galactose, sucrose, maltose, and lactose were assimilated.

Nitrate assimilation : absent.

Ethanol as sole carbon source : no growth.

Splitting of arbutin : positive.

Ascospores : absent.

This organism was incapable of growth at 37°C.

The isolate can be referred to the genus Cryptococcus on the basis that ascospores are absent, that no pseudomycelium is produced, and that dissimilation is strictly oxidative. Its presence in this genus was confirmed by the production of "starch". Many of the characters of the isolate are shared with C. laurentii, including the cell size and shape, the inability to assimilate nitrate, and the sugar assimilation pattern. There is however a marked difference in the appearance of the streak culture between C. laurentii and isolate J 3104. The appearance of the purple-pink colour in the latter is atypical of the genus, and it may be considered that this character would warrant the classification of the organism in the genus Rhodotorula. Amongst the members of this genus, R. flava bears a considerable resemblance to the isolate under discussion. This species, however, does not produce /

produce "starch". Another yeast closely resembling R. flava and C. laurentii was R. aurea described by Lodder (104). This organism, which is now considered identical to C. laurentii, differed from R. flava in its slightly reddish tinge on malt agar, and its ability to form a starch-like compound. The writer considers that the close similarity between the present isolate, and C. laurentii justifies its inclusion in this species, and that the colour difference is of secondary importance.

7. J 3145 B — Isolated from sputum 10.3.58

Liquid culture : 3 days - sediment only. One trial showed a trace of a ring.
1 month - sediment only.

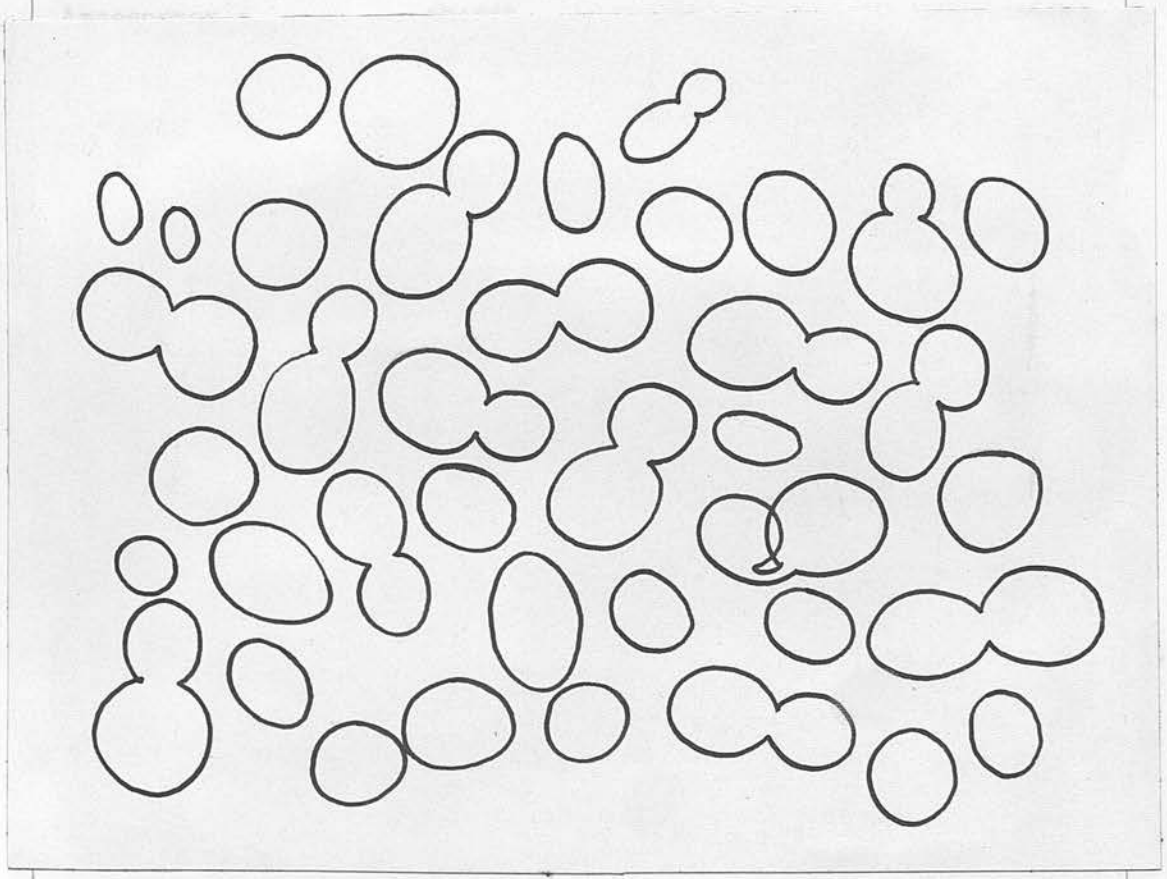
Malt agar, 1 month : streak soft, pasty, spreading, flat. Surface covered with numerous small transverse ridges. Cream coloured. Mycelial growth absent.

Pseudomycelium : absent.

Cells : (Text Figure 10)

/

Cells : (Text Figure 10) X 3200



rounded to short oval. May be single, but more commonly in pairs. Multilateral budding. (2-5)μ X (2.5-6)μ.

Fermentation : none of the sugars tested was fermented.

Sugar assimilation : glucose, galactose (weak), sucrose, and maltose were assimilated.

Nitrate assimilation : absent.

Ethanol as sole carbon source : trace of growth.

Splitting /

Splitting of arbutin : positive.

Ascospores : absent.

This organism was incapable of growth at 37°C.

The absence of ascospores, pseudomycelium, and ferment-:ing ability suggest that the isolate is a member of the genus Cryptococcus. This is confirmed by its ability to synthesize "starch". The assimilation pattern is similar to that of C. neoformans, and other similarities include growth in ethanol, the splitting of arbutin, and the size and shape of the cells. The streak culture, however, with its lack of a mucoid consistency, and its cream colour, differs considerably from that of C. neoformans. Furthermore, the presence of cell capsules on old cultures was not noted. Wolfram and Zach (105) have described a yeast which is similar to the present isolate. This is Eutorulopsis uniguttulata Zach (Wolfram et Zach), which is now considered by Lodder and Van Rij (39) to be a variety of C. neoformans, viz. C. neoformans var. uniguttulatus (Wolfram et Zach) Lodder and Van Rij. This organism is characterised by very reduced capsules. In consequence, the appearance of the streak is not mucoid and yellowish-brown, but pasty and cream-coloured. As this is the only difference between species and variety, it is considered that the isolate may be tentatively identified as C. neoformans var. uniguttulatus.

8. /

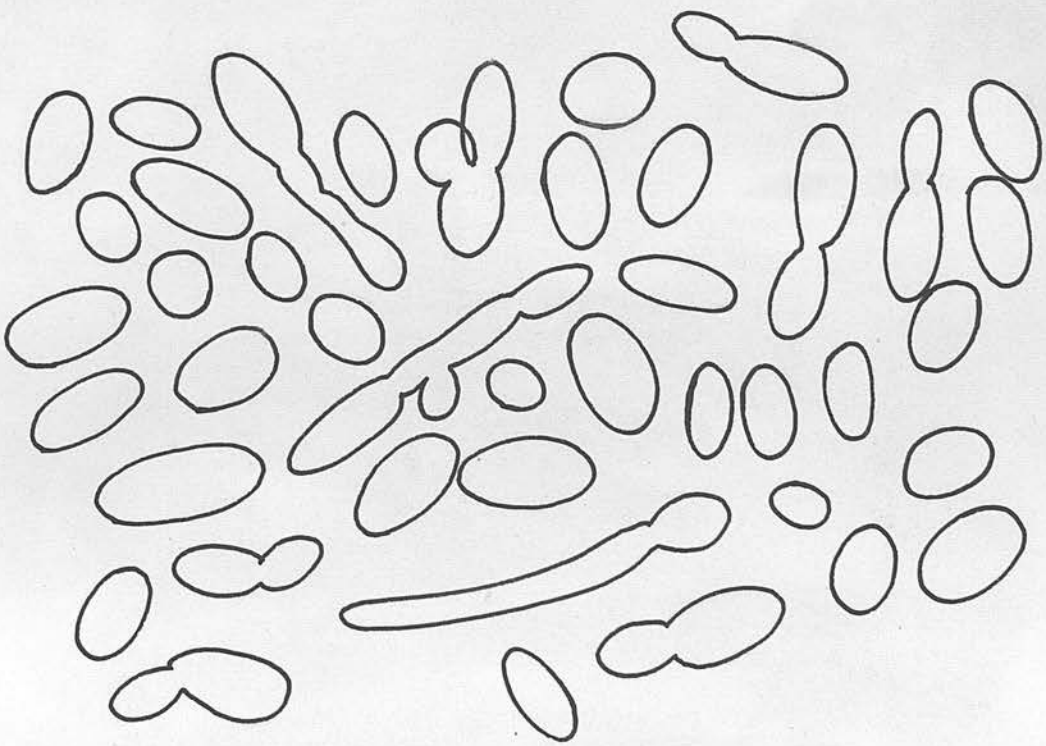
8. J 4463 — Isolated from a submaxillary gland 1.5.58

Liquid culture : 3 days - trace of ring. Poor growth.
1 month - sediment in the form of a thin, crumpled membrane (Plate I, Figure 3).

Malt agar, 1 month : streak soft, spreading, dull, pink-brown, covered with small transverse ridges. Mycelial growth absent.

Pseudomycelium : a primitive pseudomycelium consisting of branched chains of blastospores was observed on a single occasion.

Cells : (Text Figure 11) X 3200



oval /

oval to long-oval or elongate, rarely short-oval. Multilateral budding. (2-3.5)μ X (2.5-7.5)μ, elongate cells may be up to 15μ in length.

Fermentation : absent.

Sugar assimilation : glucose, galactose, sucrose, and maltose were assimilated.

Nitrate assimilation : absent.

Ethanol as sole carbon source : no growth.

Splitting of arbutin : weakly positive.

Ascospores : absent.

This organism was incapable of growth at 37°C.

The classification of this isolate is dependent on the nature of the pigment which was recorded on malt agar. This colouration was less pronounced than that observed in other isolates of Rhodotorula and corresponded to Daphne Red in the Ridgeway colour code (106). The presence of carotenoid pigments, although not confined to the genus Rhodotorula is nevertheless a convenient and practicable criterion for classifying the red or pink yeasts into a single genus. Several tests have been described for the chemical determination of the carotenoid nature of these pigments, usually based on the removal of the pigment from the cell, its crystallisa-:tion, and subsequent colour reaction with concentrated sulphuric acid. /

acid. Lodder and Van Rij (39), however, have concluded after a study of some of these methods that a practicable method was not available for the confirmation of the carotenoid nature of a pigmented yeast. In their classification, they relied on visual observation alone.

The reddish colouration of the present isolate is considered to be sufficiently pronounced to suggest that a member of the genus Rhodotorula is involved. Many of the features of the present isolate are similar to those of R. rubra (Demme) Lodder, including, the size and shape of the cells, the presence of a primitive pseudomycelium, the sugar assimilation pattern, the failure to assimilate potassium nitrate, and the absence of a fermentive ability. Both organisms split arbutin, the present isolate only weakly. It differs in its failure to grow in a liquid medium with ethanol as the sole source of carbon, and in the appearance of the streak, which is not mucoid. Growth of this organism is characteristically "clumped" in all tests involving liquid media, being particularly obvious in the test for production of ring or pellicle (Plate II, Figure 3).

The similarities mentioned above, and the undoubted presence of a reddish pigment have led to the conclusion that this isolate can be tentatively referred to R. rubra.

9. /

9. J 4854 Isolated from a wound swab 20.5.58

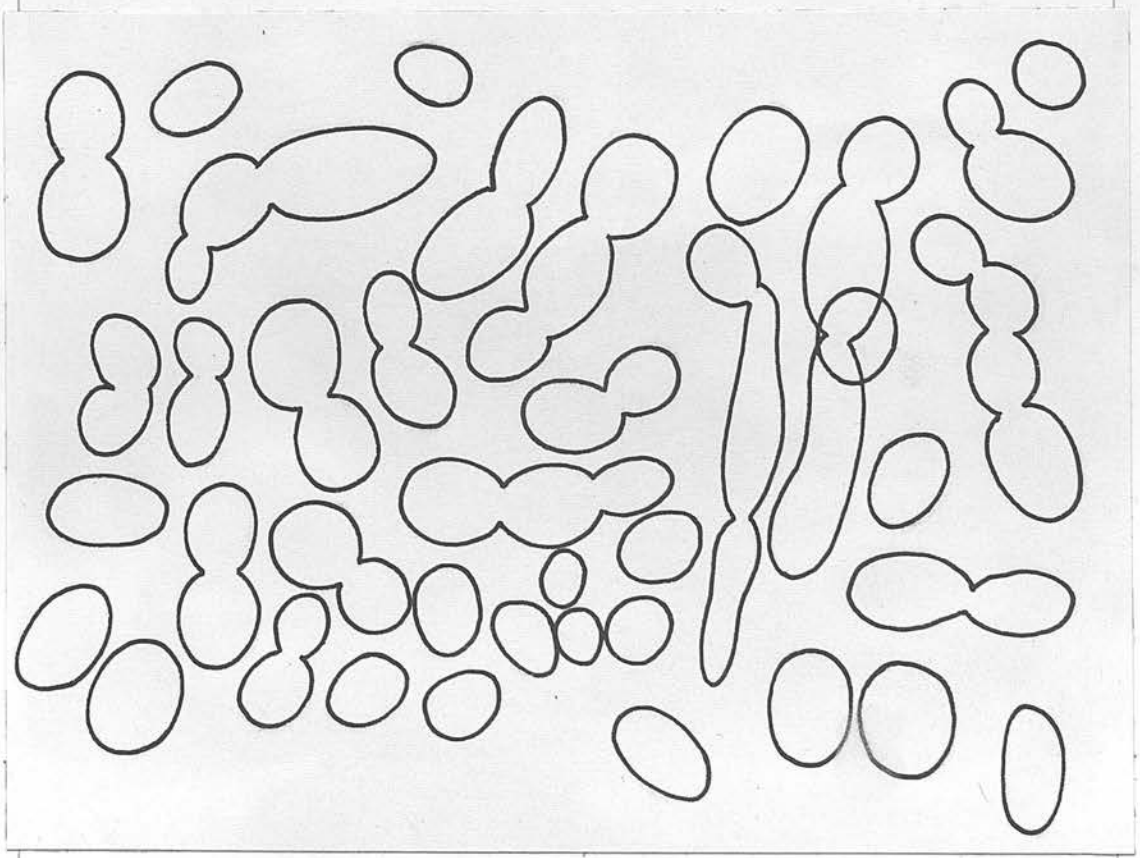
Liquid culture : 3 days - poorly-defined ring. Heavy sediment. Some trace of surface growth.

1 month - sediment only.

Malt agar, 1 month : streak soft, smooth, more or less shiny, grey. Narrow pseudomycelial fringe present to margin.

Pseudomycelium : extensive, forming a conspicuous narrow fringe to the streak. Mycotoruloides. Also uniaxial pseudomycelial strands bearing compact verticils of blastospores.

Cells : (Text Figure 12) X 3200



short-oval. May be single but are more commonly in short chains. A few pseudomycelial cells are present. Multilateral budding. (2-4)μ X (2.5-9)μ.

Fermentation : only glucose is fermented.

Sugar assimilation : only glucose is assimilated.

Nitrate assimilation : absent.

Ethanol as sole carbon source : trace of a ring.

Splitting of arbutin : absent.

Ascospores : absent.

Absence of ascospores and the production of pseudomycelium indicate that this isolate can be regarded as a species of Candida. Fermentation and assimilation of glucose only is found in C. krusei, and other characters in common with the present isolate include the failure to assimilate potassium nitrate, and an inability to split arbutin. Significant differences between the two yeasts under consideration do occur, however, firstly, the isolate J 4854 does not produce a pellicle in liquid media; and secondly the cells are predominantly short-oval. C. krusei is characterised by the formation of a well-marked pellicle on liquid media, and by the shape of its cells which are usually elongate to cylindrical, with only a small number of short-oval. Furthermore, in the ethanol test, C. krusei produces a thin, dull pellicle, whilst J 4854 forms only /

only a trace of surface growth. Lodder and Van Rij (39) have pointed out that the absence of a pellicle is not necessarily significant, and cite the case of the change occurring in the authentic strain of Torula monosa Kluyver. This strain was maintained as a separate species because although closely related to C. krusei it lacked the ability to form a pellicle, and formed a less differentiated pseudomycelium. Over a number of years, however, the streak culture changed from a soft to a tough consistency, and the ability to form a pellicle which was at first absent or much reduced was greatly increased. These authors now consider that T. monosa is a synonym of C. krusei. If, therefore, the ability to form a pellicle is not necessarily a character of fundamental importance, its absence cannot prevent any one isolate from being referred to this species, providing that other characters are similar. As a trace of surface growth was noted for J 4854 in liquid culture, and as an occasional pseudomycelial cell is found when 3 day old cultures are examined, the isolate is tentatively referred to C. krusei.

10. J 5069 Isolated from sputum 26.5.58

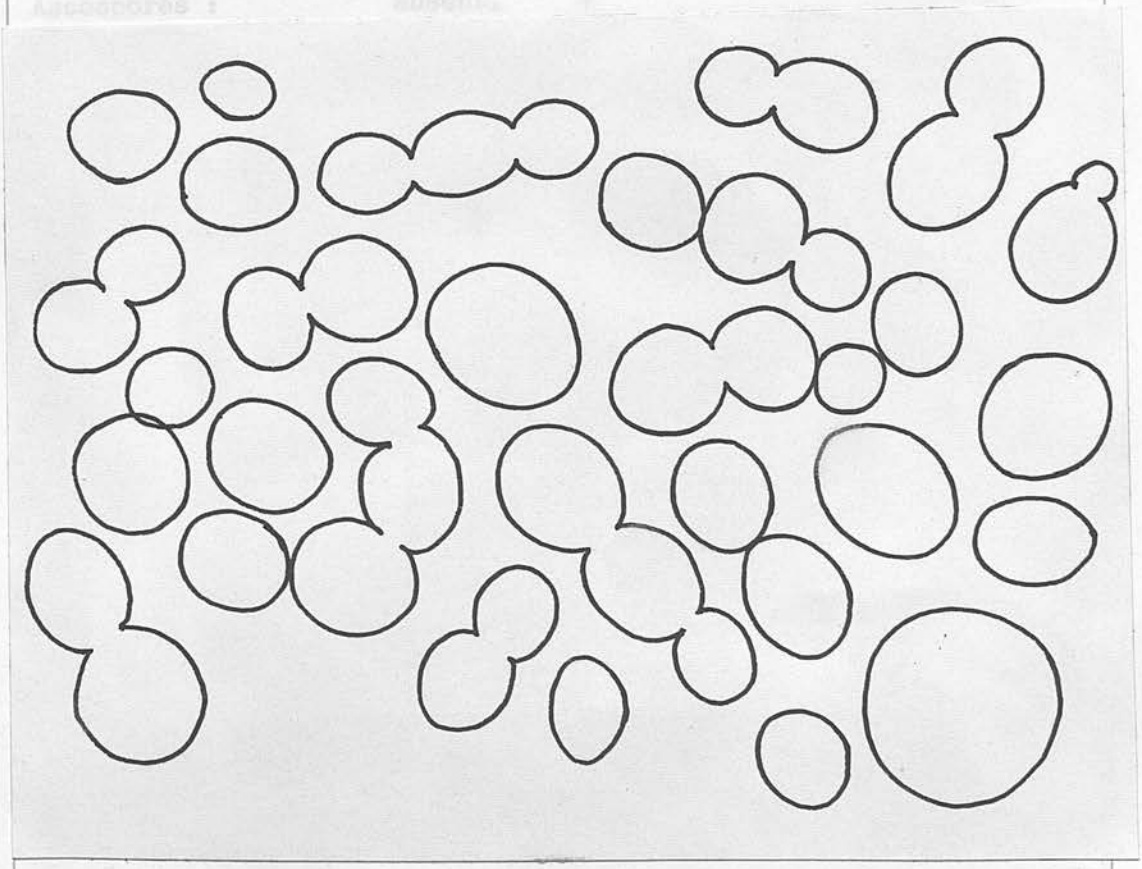
Liquid culture : 3 days - thin trace of ring. Heavy sediment.
1 month - heavy sediment; no surface growth.

Malt agar, 1 month : streak soft, white, somewhat spreading; surface matt, with numerous small warts. One or two pseudomycelial strands arise from /

from the margins of the streak. Submerged growth is present.

Pseudomycelium : well-developed; mycotoruloides, loose verticils on a normally unbranched axis.

Cells : (Text Figure 13) X 3200



rounded : single, in pairs, chains, or clusters. (2-9)μ in diameter.

Fermentation : glucose and galactose fermented.

Sugar assimilation : glucose, galactose, sucrose, and maltose assimilated.

Nitrate /

Nitrate assimilation : absent.

Ethanol as sole carbon source : narrow, incomplete ring; islets.

Growth in litmus milk : no change noted.

Splitting of arbutin : absent.

Ascospores : absent.

The absence of ascospores, and the development of pseudomycelium indicate a species of Candida. The fermentation and assimilation patterns, together with the absence of a red, non-carotenoid pigment suggest that the isolate should be referred to C. parapsilosis. This species is characterised by having round, oval, and long pseudomycelial cells in young cultures. Furthermore, the production of giant cells in the pseudomycelium was not noted in the present isolate. Nevertheless, it is considered that the absence of oval or elongate cells does not prevent the isolate from being considered as C. parapsilosis.

11. J 5595 Isolated from stool 19.6.58

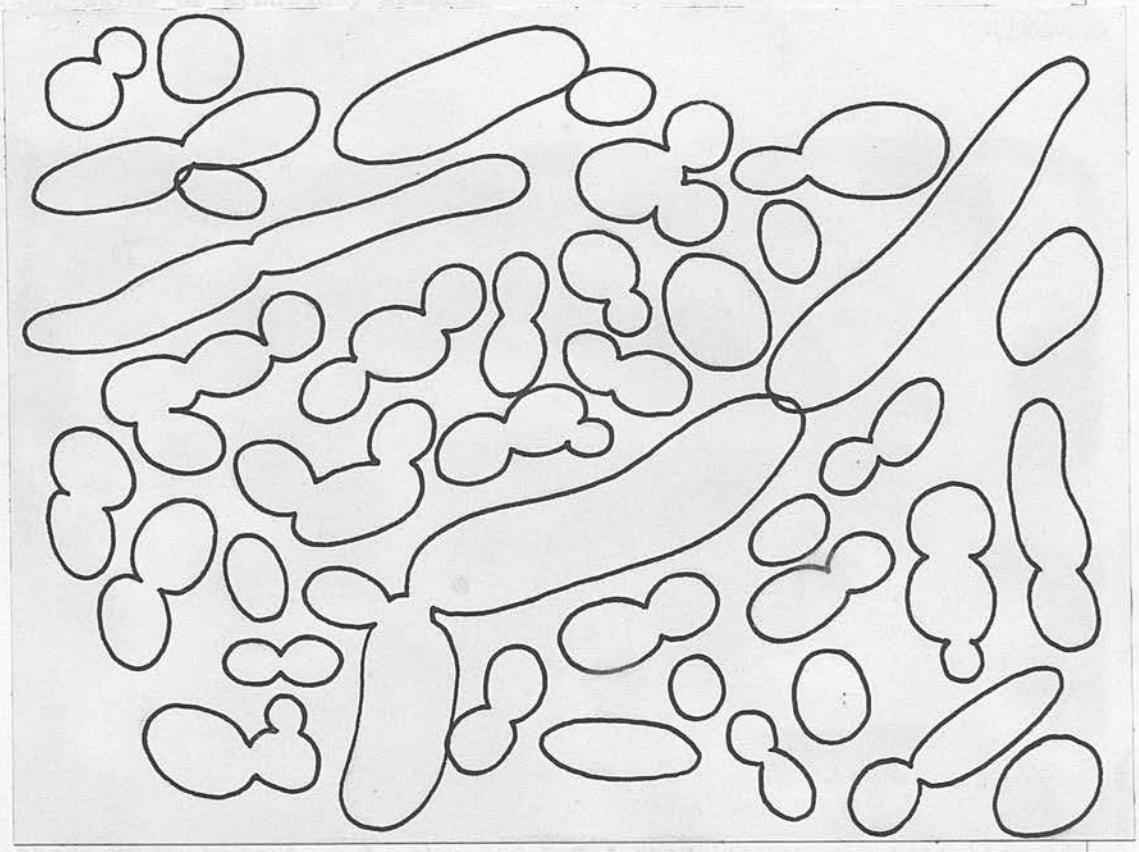
Liquid culture : 3 days - creamy ring. No pellicle or islets. Heavy sediment.
1 month - compact sediment; ring absent.

Malt agar, 1 month : streak soft, smooth, more or less shiny, pale brown; margin smooth. Submerged growth present.

Pseudomycelium : well-developed, forming a dense fringe to the /

the inoculated streak. Mycotoruloides - loose verticils on uniaxial axes.

Cells : (Text figure 14) X 3200



rounded to short-oval. Pseudomycelial cells present, often in chains. (2-4.5)μ X (3.5-8)μ. Pseudomycelial cells may be up to 20μ in length.

Fermentation : a weak fermentation of glucose only.

Sugar assimilation : only glucose assimilated.

Nitrate assimilation : absent.

Ethanol /

Ethanol as sole carbon source : trace of ring; islet present.

Growth in litmus milk : colour changes to blue.

Splitting of arbutin : absent.

Ascospores : absent.

Absence of ascospores and the production of pseudomycelium indicate that the isolate belongs to the genus Candida. Species determination in this genus is based primarily on the presence or absence of a fermentive ability. The ability to ferment and assimilate glucose only is a characteristic of C. krusei. The present isolate differs from this species, however, in lacking the characteristic pellicle, whilst the appearance of the streak, and the size of the cells in young liquid culture also differs. It has already been pointed out (p. 79) that the absence of this feature need not necessarily prevent the inclusion of an organism in this species, provided that the other characters are consistent with those of C. krusei. In the case of J 5595, there are indications that this is so, and similarities are seen in cell shape, the abundant production of pseudomycelium, the inability to assimilate potassium nitrate, and the failure to split arbutin. Lodder and Van Rij consider that fermentation and assimilation characteristics are more stable and consequently of more diagnostic value than other properties, and this would suggest that the isolate could be referred to C. krusei. Classification of the organism in this species, /

species, however, can only be considered provisional, the absence of a pellicle preventing a more positive determination.

Filamentous Fungi

These organisms were not included in the present survey, and their occurrence was not made a subject for special study. It has already been pointed out that in such an investigation, more stringent precautions would have to be taken to present contamination from airborne spores. In the examination of yeasts, only a small number of species are distributed by means of airborne spores, and the problem of aerial contamination is not so pronounced. It was observed that the thermophilic mould Aspergillus fumigatus was present in the atmosphere of both laboratories concerned with the present survey. In this species it would be difficult, if not impossible to determine whether or not it was associated with the sample, or occurring as a contaminant. Other species of mould which were apparently associated with the inoculated surface of the plate were Mucor racemosus Fresen., Aspergillus niger Van Tieghem, and Geotrichum candidum Link., which was isolated from a stool sample of a 63 year old female patient suffering from ulcerative colitis. This organism, which resembles a yeast in culture has been reported from several sources in the human, including an association with the stools of hospital patients suffering from several disorders of the alimentary tract (56). It was noted that the incidence of Geotrichum in one group ("food upsets") was as high as 56.3%. Only one example of this organism was observed, and its presence /

presence was noted in a plate which also yielded a heavy growth of C. albicans.

One plate inoculated with the aural discharge from a child showed a heavy growth of Stemphylium ilicis Tengwall which covered and was confined to the inoculated portion of the plate. It is probable that this organism was existing as a saprophyte in the ear passage. The fungus was incapable of growth at 37°C and there is no suggestion that it was actively associated with the complaint.

Yeasts from sources other than human and atmospheric

In the course of the survey which constitutes the main portion of this paper, the examination of atmospheric plates led to the isolation of Cryptococcus laurentii, C. albidus, Candida guilliermondii, and Rhodotorula mucilaginosa, all of which also were reported from the human body. The scarcity of records of the extra-human occurrence of some yeasts normally associated with an animal source, i.e. Candida albicans (81, 82, 83, 84, 85) and Torulopsis glabrata (90, 91) led the writer to examine a number of samples of vegetable and other material to determine whether or not a further contribution to these records could be made. Up to the time of writing, 100 samples have been examined in the following manner :

The samples were collected in small metal boxes (57 X 38 mm) fitted with a snap-lid. Before use they were sterilised in the autoclave. Material was collected by means of a sterile scalpel or tweezers, and 2-3 g transferred into a tube containing 10 ml of sterile water. This was shaken vigorously and allowed to stand for 30 minutes. Approximately 0.5 ml of the supernatant liquid was withdrawn by means of a sterile pipette and placed on the surface of a 2.5% malt agar plate incorporating antibiotics (see p 11). The inoculum was spread over the surface with a flamed inoculating needle, and the plate incubated at 37°C.

Examination /

Examinations of the plates was made daily for a period of four days.

Sources examined included compost, soils from various sites, stagnant water, leaf litter, moss, grass clippings, and portions of decaying vegetation. Only 2 yeasts were obtained, viz. Rhodotorula mucilaginosa isolated from compost derived exclusively from grass clippings; Candida guilliermondii from soil beneath a climbing ivy (Hedera colchica dentata variegata).

These two species were both isolated from the atmosphere and both occurred in the human body. R. mucilaginosa is therefore the most widely distributed species occurring in the sources examined by the writer, although numerically less frequent than either C. albicans or Torulopsis glabrata.

Neither C. albicans nor T. glabrata were isolated from soil or vegetation, but it is not suggested that the number of samples examined in the time were sufficient to demonstrate their presence. The ability of C. albicans to survive for several months in soil (82), and its presence in the alimentary canal of several types of animal (96) would suggest that its presence could be expected in soil with which animal faeces might come in contact. Nothing is known of the ability of Torulopsis glabrata to survive in the soil, and little is known of the number of animals which harbour this yeast. The fact that it has been reported from garden soil (91) might suggest that it is capable of surviving saprophytically in the soil in the same manner as C. albicans

Further /

Further studies on the biology of these organisms in relation to their occurrence and survival in sources outside the animal were not carried out in the present study.

Identification of fungi submitted to the Mycoses Unit

Ten yeasts were submitted to the Experimental Mycoses Unit from the Northern General Hospital, Edinburgh, for identification. Using the procedure and tests described in pp 14-27 the organisms were classified as follows :

1) CHR Isolated from blood sample. Received 23.9.57. Identified as *Candida tropicalis*.

2) M 23337 Isolated from sputum. Received 28.2.58. Identified as *Torulopsis glabrata*.

3) M 23366 Isolated from sputum. Received 28.2.58. Identified as *Torulopsis glabrata*.

4) M 23750 Isolated from sputum. Received 28.2.58. Identified as *Saccharomyces cerevisiae*.

5) U 13634 Isolated from urine (catheter specimen). Received 25.7.58. Identified as *Candida tropicalis*.

6) M 23754 Isolated from sputum. Received 25.7.58 Identified as *Torulopsis glabrata*.

7) M 26057 Isolated from a throat swab. Received 12.8.58. Identified as *Cryptococcus albidus*.

8) M 27934 Isolated from vagina. Received 16.8.58. Identified as *Rhodotorula mucilaginosa*.

9) R 27877 Isolated from Bronchi (trap specimen from a bronchoscope). Received 27.8.58.

10) M 28568 Isolated from throat, gastric washings and faeces. Received 7.10.58.

Identification of isolates R 27877 and M 28568 was not made before completion of the present study.

The isolate CHR was received for identification from a fatal /

fatal case of septicaemia which occurred at the Western General Hospital, Edinburgh. This case has been described in detail from the clinical aspect (107) and evidence of its pathogenicity towards several strains of mice has been obtained by Dr. W. Blyth (108) of the Experimental Mycoses Unit. Identification of this organism was carried out by the writer, by means of the procedure described in pages 14-27. In view of the exceptional interest of this isolate, which was made from one of the few cases of yeast sept-:icaemia on record, an account of the morphological and physiolog-:ical tests carried out are described below. The isolate was received from Dr. Maccabe of the Northern General Hospital, Edinburgh, on September 23rd, 1957. A culture of this organism has been sent to the culture collection at the Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine.

Liquid culture : After 3 days at 25°C, cells are rounded or short-oval, occasionally oval, measuring 3.5-6μ X 4-9μ. Cells are usually single but may occur in pairs or short chains. Some development of elongated pseudomycelial cells occurs: a ring, some islets, and a coarse sediment are produced. After 1 month, traces of a ring, and some surface growth can be seen.

Malt /

Malt agar, 1 month : after 1 month at 17°C, the streak is tough, rather matt, raised, with transverse ridges. It is greyish-white in colour and there is an abundant production of mycelial growth which forms a conspicuous fringe to the margin of the streak. Some submerged growth is present.

Pseudomycelium : well-developed on maizemeal agar, forming extensive branched outgrowths from the inoculum streak. True mycelium is present. Blastospores are not produced in abundance on the pseudomycelial strands after 6 days, but where present are arranged according to both mycocandida (pseudomycelium strongly branched, blastospores few, in small verticils) and mycotoruloides (blastospores in loose, branched verticils).

Fermentation : glucose, galactose, sucrose, and maltose are fermented. Lactose and raffinose are not fermented.

Sugar assimilation : glucose, galactose, sucrose, and maltose are assimilated. Lactose is not assimilated.

Nitrate assimilation: absent.

Ethanol as sole carbon source : growth moderate, with the formation of small islets on the surface.

Splitting /

Splitting of arbutin : absent.

Growth in litmus milk : the colour changes to blue.

Ascospores : absent.

This description is in almost complete agreement with the standard description of Candida tropicalis given by Lodder and Van Rij (39). The differences between their description and the appearance of the present isolate are a slightly smaller size of cell, and the presence of growth on a liquid medium containing 3% ethanol as the sole carbon source. These differences are of a minor nature, and there is little doubt that this isolate can be identified with certainty as C. tropicalis. It should be noted that all other isolates of this species made in the course of the survey described above were also capable of growth with ethanol as the sole carbon source.

The early stages of growth of this isolate when inoculated into mice are described in a separate section of the present work.

Evaluation of the methods used in the identification

A practical classification of the yeasts can only be obtained by the use of a number of characters. No one character is absolute, and final identification is achieved by reference to the combined morphological and physiological characters of any particular organism. From this it follows that a scheme of classification becomes more accurate by the incorporation of a larger number of tests. From the practical aspect, however, the value of any classification is determined partly by the validity of the criteria which are employed, and partly be its ease of application. The system proposed by Lodder and Van Rij (39) is an important step towards achieving such a system : the criteria used are sufficiently varied to avoid overemphasis of any one character, and it is possible to base identification on general rather than particular characteristics. This system is based on the recognition of some characters as primary and others as secondary, the latter being confirmatory rather than indicative. Features of primary importance include the presence of ascospores, fermentation and assimilation patterns, pellicle formation, cell size and shape, method of budding, and characters of vegetative reproduction : those of secondary significance include growth on a liquid medium containing ethanol, splitting of arbutin, appearance of the streak on malt agar, and growth in litmus milk. Several /

Several of these tests have been discussed above (pp57-83) but a consideration of the methods used in the process of identification is given below.

Liquid culture : From this test, the presence or absence of a pellicle and details of cell morphology are determined. The absence of pellicle formation in 2 of the isolates described above (J 4854 and J 5595) calls for special attention. Both have been referred to a species characterised by the production of a pellicle. If the tentative identification is valid, the method employed for the detection of this feature may be inadequate. The production of a pellicle, however, is dependent on a number of factors, including the age and vigour of the inoculum, the size and shape of the vessel, and the composition of the medium. The synthetic liquid medium described on p 15 was employed as the results obtained by the use of malt extract were not consistent. Both media were tested against organisms known to form pellicles on liquid media, and it was noted that the synthetic medium supported a greater degree of pellicle development. Organisms used in this preliminary trial included Candida pelliculosa, Hansenula anomola, C. krusei, Pichia membranaefaciens, and C. mycoderma. (C. pelliculosa and C. krusei were obtained from the culture collection maintained at the London School of Hygiene and Tropical Medicine; the remainder were obtained from the National Collection of Yeast Cultures at the Brewing Industry Research Foundation, Nutfield, Surrey). In all cases /

cases growth and pellicle formation were abundant. It was noted that an organism capable of forming a pellicle frequently, but not invariably produced a strong surface growth on other liquid media, e.g. fermentation tubes or liquid media for assimilation tests; in some cases a pellicle was produced on the glass surface at the base of the stock culture bottle. The ability to produce a pellicle does not appear to be invariable, and it is well known that prolonged subculturing of a yeast may alter this ability. It is concluded therefore that the method and medium used in the above survey are adequate for the demonstration of a pellicle.

The size and shape of a yeast cell vary considerably, but may be of considerable taxonomic importance. The majority of organisms studied in the present work were characterised by the production of cells that were rounded or oval : since the number of rounded to oval cells varies considerably in each of the isolates, it was concluded that the appearance of the cells is only of secondary importance. It must be emphasised that this conclusion refers only to those isolates made during the above survey.

Malt agar : The appearance of a streak culture on malt agar may be of assistance in the identification of an unknown isolate, but as it is subject to a considerable degree of variation its taxonomic significance must be considered secondary. The agar medium used for the examination of a streak culture (p 11) was considered to be satisfactory. Results were consistent and

in /

in agreement with the descriptions given by Lodder and Van Rij. Some isolates appear to be more stable in appearance than others, and certain characters (including texture, colour, and the prod-:uction of a pseudomycelial fringe to the margin) were often of considerable assistance in the identification of an unknown. Certain species showed very little variation in their appearance on malt agar, Torulopsis glabrata in particular being stable in this respect. In contrast, Rhodotorula mucilaginosa showed considerable variation in colour and texture, even in successive subcultures of the same isolate.

Pseudomycelium : This character is of primary importance in the anascosporogenous yeasts and is the basis for separating Torulopsis from Candida. Nevertheless, it is often difficult to determine whether or not pseudomycelium (i.e. the concrescence of elongated cells) is present. Transitions between the presence and absence of pseudomycelium occur, and in such cases the criterion becomes of secondary importance. When repeated examination of maizemeal agar streaks failed to establish conclusively whether or not pseudomycelium was present, identifica-:tion was achieved by consideration of the other characters of the isolate. The medium and method used in examining pseudomycelial production were considered adequate for an accurate assessment. Over 90% of the yeasts subsequently identified as C. albicans produced chlamydospores using this method, and although special methods /

methods for the demonstration of this feature have been described (109, 110, 111), it is considered that the identification of this species can be achieved satisfactorily by consideration of all the characters, particularly fermentation and assimilation.

Fermentation : In the fermentation tests described on pp. 18-21, no attempt was made to determine whether or not any fermentable carbohydrate was present in the peptone solution. None of the isolates showed fermentation of all the sugars tested, and it is considered that the tube containing the non-fermented sugar acted as a blank. The fermentation tubes were examined for a period of not less than 15 days as it was found that a positive result was occasionally delayed until 12 or 13 days after inoculation.

The ability to ferment certain sugars appears to be a stable character, and by employing the media and methods described above, reproducible results were obtained. The practice of replicating fermentation tests which was adopted by the writer has resulted in an accurate assessment of this test, and it is considered that the results obtained were valid.

Sugar assimilation : The combination of auxanographic and liquid media tests is considered to provide sufficient accuracy. In most cases replicated tests with auxanograms were sufficient to obtain the assimilation pattern, and this method was very convenient where growth of the organism was moderately rapid : Slow growing yeasts often gave inconclusive results, /

results, but assessments were obtained using the liquid medium test (43). The replication plate method described by Shifrine et al. (112) was tested, but it was decided that the auxanographic and liquid medium tests gave more accurate results.

Nitrate assimilation : Assessment of this test was carried out by the exclusive use of auxanograms. Liquid medium tests were not employed as it was found that even the slow-growing yeasts showed a result when the plate was incubated for 2 to 3 days before inoculating the surface with the substances under test. The possibility therefore exists that the application of a liquid medium test might have revealed the presence of a slight assimilation of nitrate which might not have been demonstrated in the auxanogram.

Growth in ethanol : It was noted that growth in a 3% ethanol medium did not always conform to the description given by Lodder and Van Rij, and this was particularly evident in the case of C. tropicalis. Although this species is considered to be more or less incapable of growth in 3% ethanol, it was found that all isolates of this organism showed conspicuous growth in this medium. The method used was that described by Lodder and Van Rij, with no modifications : although the majority of the species isolated agreed with the published descriptions, it is considered that the result of an ethanol test is not of any great significance in the classification of any organism /

organism examined in the present survey.

Both splitting of arbutin and growth in litmus milk were found to give dubious or inconsistent results when tested against stock cultures, and are considered to be of limited taxonomic significance.

Ascospore production : The ability of an organism to form ascospores is of fundamental importance in the classification of a yeast. In the course of a routine examination of a number of yeasts, however, certain difficulties are encountered. The factors influencing ascospore production are numerous, and no single test medium can be employed to obtain consistent results. A number of sporulation media exist, and Lodder and Van Rij list no fewer than 10 different media which have been used in testing for the production of ascospores. Only 3 test media were used by the writer, and the possibility therefore exists that ascospore production may have been overlooked. For practical reasons alone the number of tests for ascospore prod-:uction cannot exceed a certain number, and this figure was arbitrarily limited by the writer to 3, viz. carrot plugs, gypsum blocks, and malt agar. All isolates were tested for ascospore production, but replications were never carried out on those which had produced chlamydospores, together with an ability to ferment glucose, galactose, and maltose, and assimilate glucose, galactose, sucrose, and maltose. These were considered to be C. albicans, and /

and no further tests for ascospore production were made.

Identification of any particular organism was often facilitated by the comparison with a named culture obtained from The London School of Hygiene and Tropical Medicine or the Brewing Industry Research Foundation.

P A R T II

STUDIES ON THE MORPHOLOGY OF YEAST CELLS IN VIVO

Introduction

Examination of any pathogenic organism is greatly facilitated by its ability to exist and reproduce when isolated from its host. An understanding of the biology of a pathogen can be obtained from in vitro studies by the accumulation of information on its morphology, physiology, taxonomy, and biochemistry. To obtain a greater knowledge of the biology of any pathogen, however, studies must be made of the organism in relation to its host. In the case of human pathogens this is often carried out by the use of laboratory animals in which the symptoms of disease occurring in man are reproduced. Studies are frequently made of the effect of the pathogen on its host in terms of symptomatology or pathology (both gross and microscopic).

Tissue culture techniques (122, 123) have been extensively used in morphological and physiological studies of a number of human and animal tissues : they have contributed greatly towards an understanding of the form and function of healthy and diseased cellular systems, and have been responsible for great advances in the knowledge of viruses (124). In the study of certain pathogens which are incapable of growth outside the host body, tissue culture affords a valuable laboratory method /

method for a study of their characters.

The isolate of Candida tropicalis which was considered to be responsible for the death of a 61 year old woman (pp 90-93) was made the subject of further investigation. It was shown that none of the morphological or physiological characters observed in the course of its identification distinguished this isolate from any of the other isolates of C. tropicalis made in the course of the survey. The association of this species with various human sources has already been noted (p 56). It appears to be a normal and non-pathogenic inhabitant of several human sources, and it is only infrequently reported as the causal organism of diseases in the human (57, 98, 99, 100, 116, 121). Descriptions of its morphology in vitro have been given by Martin et al (41) and Benham (125), but records of its appearance in morbid material have usually been confined to its isolation rather than its morphological description in vivo. Hill and Gebhardt (113) included a species of C. tropicalis in their examination of the morphological changes of a yeast when introduced subcutaneously into mice. No alteration from the yeast-like phase was noted. The lack of pseudomycelial development in vivo was also noted by Stovall and Pessin (130)

Many of the organisms causing major systemic mycoses are blastosporic in the parasitic phase, although a mycelial phase may be produced in culture. This phenomenon is found in /

in Sporotrichum schenki (Hektoen et Perkins) Matruchot, Blastomyces dermatitidis Gilchrist et Stokes, Paracoccidiodes brasiliensis (Splendore) Almeida, and Histoplasma capsulatum Darling. It was originally believed that C. albicans was similar to these species in having the parasitic phase blastosporic (126, 127, 128). This view has been queried by Hill and Gebhardt (113) who suggested that for this fungus, the reverse may be true, viz. that the filamentous form is better adapted for growth in animal tissues. Blyth (132) has demonstrated the presence of pseudo-:mycelium in the kidneys of mice inoculated with this species. The same writer (108) has demonstrated the pathogenicity to mice of the C. tropicalis (Isolate CHR) isolated from the fatal case of septicaemia. In view of the pathogenic nature of the CHR strain, it was decided to examine the morphology of this isolate in vivo, to determine if the parasitic habit was accompanied by any morphological chages. An initial experiment was carried out using a tissue culture technique for the examination of phagocytosis. For reasons described below, this method was replaced by the examination of subcutaneous tissues into which the yeast cells had been introduced. In the latter experiments 3 other isolates of C. tropicalis were examined for comparison with the CHR strain, together with a single isolate of C. albicans whose pathogenicity towards the strain of mice used had already been demonstrated (108). Also examined were isolates of Torulopsis glabrata, Saccharomyces fragilis, and S. carlsbergensis.

Material and Methods

a) Tissue culture: The method described below is a modific-:ation of the technique adopted by Drysdale (129) for the study of a bacterial disease of sheep. The method involved the production of monocytes in the peritoneal cavity of guinea pigs following the inoculation of a substance which acted as a mild irritant. The leukocytes were maintained in a sterile Ringer's solution and used in the study of phagocytosis of the yeast cells.

Adult female guinea pigs weighing approximately 350 g were obtained from the Small Animal Breeding Unit, The Bush Estate, Midlothian.

All glassware used in this series of tests was specially cleansed before being sterilised. This was necessary since the monocyte cells do not remain viable unless the glass-:ware is free from all surface impurities. The method of cleaning was as follows :

1) heated to boiling in "Calgon" (an inorganic detergent)
2) rinsed in tap water
3) heated to boiling in 4% hydrochloric acid
4) rinsed in tap water
5) heated to boiling in 4% sodium hydroxide
6) /

6) rinsed in tap water

7) heated to boiling in distilled water

8) rinsed with 2 changes of distilled water.

After drying in air, the individual portions of glass-:ware were wrapped in greaseproof paper and autoclaved at 15 atmos for 15 minutes. Although the final heating and rinsing is normally carried out with water that has been triple distilled, it was found that water obtained from an "Elgastat" portable deioniser was sufficiently pure and that monocytes remained viable for the duration of the test, viz. 5 days.

A 1% solution of glycogen was prepared and Seitz filtered. Under aseptic conditions 1 ml of the sterile solution was transferred to a tube containing 3 ml of sterile water. After shaking, 2 ml of the resultant solution, containing 5 mgm of glycogen were injected intraperitoneally into a guinea pig. After the lapse of 5 days, the monocytes were obtained in the following manner : the animal was killed by means of ether. 30 ml of a buffered sterile Ringer's solution containing $NaHCO_3$ and an anticoagulant (heparin) were inoculated intraperitoneally and the abdomen kneaded gently to ensure that the monocytes went into suspension. The Ringer's solution was made up as follows :

sodium chloride	2.25 g
calcium chloride	0.05 g
potassium chloride	0.1 g
de-ionised water	250 ml

The solution was autoclaved at 15 atmos for 15 minutes. /

minutes. Before use, 5 ml of a dilute (1.4%) solution of potassium bicarbonate and 0.1 ml of a 1% sterile solution of heparin was added.

After the kneading process, the abdominal wall was opened, the peritoneal contents transferred by means of a pipette into centrifuge tubes and centrifuged at 3,000 r.p.m. for 3 minutes. The supernatant liquid was decanted off, some fresh Ringer's solution was added, and the monocytes resuspended by gentle mixing with a pipette. After centrifuging again, the cells were washed, and this process was repeated 3 times. After the final wash the Ringer's solution containing the suspended monocytes were poured into a Petri dish which contained up to 15 coverslips measuring 23 X 7 mm. The dish was allowed to stand for 1 hour at 37°C, by which time the monocytes had become attached to the coverslips. Excess fluid was drained off, each coverslip was placed in a small glass corked tube (40 X 9 mm) containing a yeast suspension in Ringer's solution of the organism under test. Incubation was carried out at 37°C. To provide a constant irrigation of the coverslip, a form of roller tube culture was devised using a laboratory clinostat (Plate II, Figure 4). The rotating disc had been modified to accommodate a cylindrical tin measuring 10 X 7.5 cm. The tubes containing the monocytes were attached horizontally to the sides of the tin and kept in position by elastic bands. 24 tubes could be accommodated round the margin of the drum. When examinations were /

were to be made, the coverslip was withdrawn and placed on a sterile slide, after ensuring that the side bearing the monocytes was placed against the slide. The margins of the slide were sealed with vaseline, and the upper surface of the coverslip gently cleaned by means of a fine camelhair brush. The slide was placed on the microscope stage which had been fitted with a thermostatically controlled stage heater set at 37°C. Obser-:vations of the living material were made by means of phase contrast microscopy.

The organisms examined were 1) Candida tropicalis isolate CHR, see pp 90-93. 2) C. albicans isolate I 5115 isolated from the urine of a 69 year old male patient suffering from a urinary infection. Both of these isolates were maintained on 2.5% malt agar : the cell suspensions used in the tests were made from 12 hour cultures on malt agar incubated at 37°C. Coverslips bearing the living monocytes were introduced to each of 10 tubes containing a suspension of the yeast under examin-:ation in Ringer's solution. Observations were carried out after intervals of 1, 2, 4, 8, 12, and 24 hours, and thereafter daily for a total period of 5 days.

An attempt was made to adapt the technique to the isolation and examination of mouse monocytes, using 5 ml of Ringer's solution to obtain the cell suspension, but it was found that an insufficient number of white blood cells was obtained.

b) /

b) Subcutaneous inoculation: The technique was a modification of Selye's procedure for the demonstration of granuloma production in mice (114) : this method was modified by Higginbotham et al (115) and described by Hill and Gebhardt (116). The technique involves the injection of 1 ml of air subcutaneously into the nape of the neck of mice. This produces a pouch into which the organisms are injected in 0.2 ml of inoculum. After inoculation, the animals are sacrificed at varying intervals, portions of connective tissue dissected out from the walls of the bubble, and mounted on a slide for staining and observation. The procedure adopted in the present work was as follows : 9 adult male white mice (University of Birmingham strain) weighing approximately 30 g each, were selected for each trial. The hair covering a region approximately 1.5 cm in diameter was removed from the area around the nape of the neck. This was accomplished by removing the hair as far as possible with scissors, and clearing the remaining hairs by means of chemical depilants ("Veet" or "But-O"). The inoculum was prepared by suspending cells from a young (18 hour) colony grown on a malt agar slant at 37°C. In the case of C. albicans and C. tropicalis it was shown that a small number of pseudomycelial cells were present in these colonies. Their presence was not desirable in the inoculum, and they were eliminated by making a second subculture and incubating for 24 hours /

hours. The inoculum prepared from the second subculture was exclusively blastosporic in nature, and no pseudomycelial cells were observed. Cells from the second subculture were added to tubes containing 4 ml of sterile 0.85% saline, to give a suspension which was standardised by reference to an absorptio-:meter. The number of yeast cells administered to each animal was determined by the use of a Thoma haemacytometer slide, and was approximately 4,000,000.

1 ml of air was introduced into the subcutaneous tissue at the region of depilation, following surface sterilis-:ation of the skin with ether. This was effected by means of a sterile 1 ml glass hypodermic syringe fitted with a number 20 needle. 0.2 ml of the inoculum was then introduced into the pouch. Animals were sacrificed after 1, 2, 4, 8, 12, 24, and 48 hours, whilst the remaining 2 animals were retained for a period of up to 24 days. The animals were killed by exposure to coal gas fumes, and the air bubble dissected partially free. Portions of connective tissue were removed from the walls and floor of the bubble, spread over the surface of a glass slide and air dried. The saline yeast suspension from which the inoculum had been obtained was retained and incubated at 37°C. Examinations of cell shape were made at intervals corresponding to the removal of sheets of connective tissue from the animal. Swabs were made from the base of the bubble at each dissection and /

and streaked onto the surface of a malt agar slant. This was incubated at 37°C and, together with microscopical examination of the animal tissue gave an indication of the presence and viability of yeast cells within the bubble. Two slides were made at each dissection, and these were stained with

1) Periodic-Acid-Schiff stain (Kligman's modification) as described by Conant et al (4).

2) May Grunwald stain (131)

The former stain (P.A.S.) was employed to demonstrate the morphology of the fungal structures, and gave no indication of the relation of the yeast cells to the host cells. This was obtained by means of the second stain, where the relationship was clearly observed.

The organisms studied by means of this technique were

1. Candida albicans : isolate I 5115, obtained from the urine of a 69 year old male patient suffering from a urinary infection. The pathogenicity of this organism towards the strain of mice used in the experiment had been previously demonstrated by Blyth (108).

2. Candida tropicalis : isolate CHR, obtained from the blood of a case of fatal septicaemia.

Both of these organisms are characterised by the production of pseudomycelium in vitro.

3. Torulopsis glabrata : isolate I 7358, obtained from the sputum /

sputum of a 69 year old female who had been admitted to hospital with a pulmonary embolism. This species is characterised by its inability to produce pseudomycelium.

4. Saccharomyces fragilis : isolate H 7981, obtained from the stool of an 83 year old female patient suffering from abdominal pain. This species produces pseudomycelium but is not regarded as a pathogen.

5. Saccharomyces carlsbergensis : isolate Y 379, obtained from Dr. L.J. Wickerham for testing the quantitative fermentation of raffinose (pp 20-21). This species does not produce pseudomycelium, and is not pathogenic.

6. Candida tropicalis : isolate J 3495, obtained from the sputum of a 40 year old male patient suffering from bronchitis.

7. Candida tropicalis : isolate I 5599, obtained from an abdominal wound on a 62 year old female.

8. Candida tropicalis : isolate I 6133, obtained from the sputum of a 77 year old female patient suffering /

suffering from chronic bronchitis.

All of these organisms were capable of growth at 37°C.

Organisms 6, 7, and 8 were studied to give a comparison with the CHR strain.

Hill and Gebhardt washed the cells used in their tests in a 0.85% saline solution before inoculation. In a preliminary trial with isolates I 5115 and CHR, the morphological changes of unwashed and washed cells was compared. Inoculations made directly from the yeast suspension described above constituted the "unwashed" cells. A similar suspension was centrifuged and resuspended in a fresh quantity of sterile saline. This was carried out 3 times, and the final suspension constituted the "washed" cells. The course of development of the yeast, and the host reaction were identical in the 2 groups, hence the process of washing was not considered necessary.

Results

a) Tissue culture :

1. Candida albicans : isolate I 5115

No phagocytosis was observed after 13 hours. The yeast cells were budding, but no pseudomycelial cells were produced. Monocytes and yeast cells were seen in close proximity to one another, but no ingestion could be observed. After 42 hours pseudomycelium was moderately extensive and several monocytes were noted containing yeast cells. Some blastospores were irregular in out-:line, empty or with amorphous cell contents, and were apparently undergoing digestion. After 5 days several large distended yeast cells similar to those developing in old malt agar cultures were seen; these cells measuring up to 14µ in diameter.

2. Candida tropicalis : isolate CHR

After 90 hours, monocytes containing apparently viable cells were observed (Plate II, Figure 5). None of the observations made up to and including 5 days showed the presence of pseudomycelial strands, and no distended blastospores were seen.

b) /

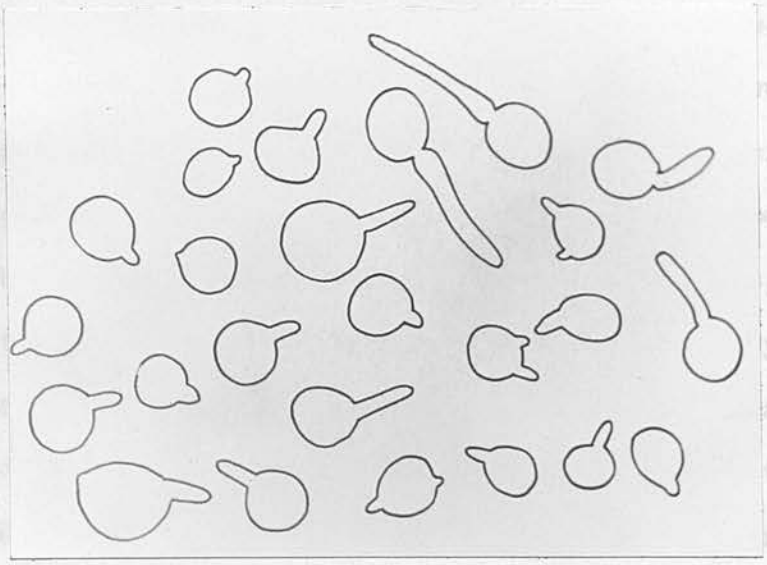
b) Subcutaneous inoculation :

1. Candida albicans : isolate I 5115

1 hour : An examination of 500 cells showed that 305 (61%) had germinated to produce short cylindrical out-:growths 0.6-1.2μ in breadth, and up to 8.4μ in length (Plate III, Figures 6,7; Plate IV, Figures 8,9). None of these structures was produced in the inoculum where the majority of the cells remained entire, only occasionally showing a rounded bud. These cylindrical outgrowths occurring in vivo were produced at any part of the surface of the cell. The majority of cells showing this characteristic form of germination produced a single outgrowth, but 2, 3, and occasionally 4 were formed. The first indication of germination was the appearance of regions in the cell wall which were less deeply stained than the remainder of the cell. The wall then bulged outwards in this region, forming a thin walled, narrow bud, which rapidly grew to form the cylindrical outgrowths so typical of this species. (Text Figure 15).

/

(Text Figure 15). X 2200



No septum was formed between the new growth and parent cell, protoplasmic continuity being maintained. Both protoplasm and wall of the newly formed growth were less deeply stained than the parent cell. The process greatly resembles the formation of a "germ tube" which is seen in so many fungal spores. The point of origin of this "germ tube" was occasionally marked by a constriction (Plate IV, Figure 9) which reduced the diameter of the outgrowth by approximately half. Growth by extension was rapid, and by the end of the first hour, the tube had frequently reached a length of 12μ. Budding of the blastospores appeared to have been completely suppressed, without a reduction of cell growth /

growth, hence the only form of growth of the yeast was the production of these thread-like pseudohyphae. The appearance of the yeast cells in this phase is very characteristic, and unlike any of the other species examined. On maizemeal agar, many cells do produce buds which elongate to form pseudomycelial cells, but these are invariably more robust, and are usually regular in outline. The "germ tubes" were straight or curved, and were invariably unbranched at this stage. By the end of the first hour, a considerable number of the original blastospores had been ingested by phagocytic cells of the host : even at this early stage the protoplasm of some of these phagocytosed cells was paler than those which were free : the protoplasm was somewhat irregular in outline, and the cells were evidently undergoing digestion. The majority of the cells, however, were free in the matrix of the connective tissue, and the proportion of phagocytosed cells was small. The phagocytes appeared to be monocytes; polymorphonuclear leucocytes were not present.

Some cells with well-developed "germ tubes" had been engulfed by a phagocyte, and it was noted in a number of instances that whilst the blastospore itself was intracellular, the actively growing pseudohypha was not /

not contained within the phagocyte (Plate III, Figure 7). This confirmed the observation made by Hill and Gebhardt (113) who have suggested that this may be the mechanism which hinders ingestion of the yeast by the host phagocytes, and consequently permits survival of the fungus within the host.

2 hours : Two hours after inoculation the number of cells which had produced an elongated pseudomycelial growth had increased to 97%. The "germ tube" had now reached 22.7µ or more in length (Plate V, Figures 10, 11; Plate VI, Figure 12). It differed from any pseudomycelial development observed in vitro by its sinuous appearance, often curving back on itself then resuming its original or a different direction of growth. At this stage, the pseudomycelial thread was still unbranched, deeply staining, and typically non-septate, although occasional septa were observed near the point of origin from the parent cell. This pseudomycelial strand varied in thickness from 0.2µ to 1.6µ, the majority being approximately 0.7µ in diameter. The strand gradually increased in thickness from its point of origin, finally becoming constant in breadth and remaining so. Although it was observed that the early stages of germination frequently showed the presence of more than one pseudomycelial initial, it was rare to find more than one thread /

thread growing from any one blastospore. There appeared to be a definite relationship between the size of the blastospore and the thickness of the pseudomycelial thread to which it gave rise. Visual observation of the cells associated with the larger threads (1-1.6μ) showed that they were invariably associated with blastospores which were larger than normal.

Phagocytosed cells were again in evidence, but only a small proportion of the yeast cells were found within host phagocytes. The amount of digestion which they had undergone was indicated by their inability to take up the basophilic stain. Some ingested cells showed a similar staining appearance to those which were free, and were apparently newly ingested. Others showed a progressive reduction in their ability to react to the stain, becoming pale pink in colour, irregular in out-:line, and ultimately devoid of protoplasm. The final stages in digestion were shown by the appearance in the phagocyte cytoplasm of a clear space which retained the outline of the original yeast cell. Polymorphs (polymorphonuclear leucocytes) were seen occasionally, but at this stage there was apparently little host response.

4 hours : Growth of the threads had increased considerably, and /

and some of the pseudohyphae were now 80μ or more in length, although the majority did not exceed 40μ. The appearance of the yeast was now mycelial (Plate VI, Figure 13), with distinct septa and branches present. Blastospore production had ceased entirely, and all growth was still in the nature of elongation. The branches were produced at a septum, where blastospore production normally occurs, but the bud rapidly underwent elongation and formed a pseudomycelial strand similar to the one produced from the original blastospore. Verticil-:late branching did not occur. The pseudomycelial cells were 12-34μ in length and 0.6-1.3μ in breadth. The strands were interwoven with one another and tended to form rather dense tangled networks which were local-:ised in certain regions in the connective tissue. Progressive differentiation and elaboration of many of the pseudomycelial strands was also accompanied by an increase in the number of cells which had apparently lost their viability. Phagocytosis was still in evidence and polymorphs were more numerous but were still not abundant. Many of the original blastospores had become irregular in outline, somewhat distended, and had failed to take up the stain. These cells had not been phagocytosed and were apparently derived from the original /

original blastospores. They measured from 4.4μ to 9.7μ in diameter, in contrast to the cells of the original inoculum which measured from between 3-4μ. It seems likely that these cells were in a moribund condition, and are perhaps comparable to the giant cells found in old cultures of C. albicans and other species. Their derivation from the original inoculum cells was indicated by their occurrence at the point of origin of a pseudo-:mycelial strand. They were only observed in portions of the connective tissue where a particularly heavy growth of the fungus occurred. When only a few strands of pseudomycelium were present, these distended cells were not noted. This suggests that the production of these cells might not have been attributed directly to host reaction but may be correlated with unfavourable localised conditions occurring at the centre of a develop-:ing colony. It is postulated that these conditions may have been generated by the fungus rather than the host. Blyth (132) has described the appearance of "bladder cells" in the abscesses produced by C. albicans in certain organs of the mouse : it is not known whether the distended cells described above represent an early stage in the formation of "bladder cells".

8 hours : Little difference was observed in the general appearance /

appearance when compared with the observations made at 4 hours. Pseudomycelial threads again occurred in tangled masses. A certain amount of pus was present. The yeast elements were less deeply stained. Numerous distended and collapsed cells 8-10μ in diameter, were present, and these were normally unstained. Many of the pseudomycelial cells were also noticeably distended, occasionally up to 3μ in breadth. Only the original blastospores remained deeply stained. Colonies were characterised by the presence of disintegration of both host and yeast cells, failure to take up the basic dye, and the distension of individual cells. Polymorphs were still not abundant, although a large number of large phagocytes, possibly monocytes, were associated with the yeast colonies. The appearance of individual strands was still similar to that described above at 4 hours, viz. consisting of the original rounded, deeply staining blastospore, with a narrow, normally unbranched curved pseudohypha consisting of 2 or 3 elongate cells (Plate VII, Figure 14). This thread occasionally branched, to produce another pseudomycelial growth of elongated cells, but no rounded blastospores were formed. The diameter of the strand was normally constant, but occasionally the newly formed pseudomycelial cell /

cell was swollen at its origin, tapering again to the original width. This gave the strands a characteristic beaded appearance. In some cases the strands were very long, measuring up to 145μ. Phagocytosis was still active, and many digested cells were visible. Polymorphs were seen in quantity around the margin of a pseudomycelial colony, but some were also observed amongst the threads.

After 8 hours, observation was made more difficult by the production of pus, which tended to mask the included pseudomycelium. The network of threads became infiltrated with dead yeast cells, and with both living and dead phagocytes : this resulted in the formation of a rather thick, semi-opaque cushion of infected material where considerable difficulty was experienced in recording fine details.

12 hours : The yeast cells were now almost exclusively mycelial in appearance, and few of the original blastos-:pores could be seen. When present they were always associated with a pseudohypha, and were apparently the cells which had originally give rise to these strands (Plate VII, Figure 15). Few of the pseudomycelial threads showed any marked increase in length when compared with the previous series of observations, but a number of them have produced verticils. Whether or not these /

these verticils were produced was apparently dependent on the freedom or absence of the particular pseudomycelial growth from one of the extensive pus-infiltrated colonies described above. When the pseudomycelial strands were more or less detached from one of these colonies, and occurred as small discrete units, the individual cells were normally shorter, and verticillate branching was found (Plate VIII, Figures 16,17; Plate IX, Figures 18,19) : each verticil consisted of 1 or 2 elongate cells, or a single, rounded blastospore which exceeded the diameter of the pseudomycelial cell from which it originated. Strands occurring within an extensive colony were longer, unbranched, and of uniform thickness. The appearance of blastospores in the former group marks the first occasion on which their presence was observed since inoculation. The reduction in length of the pseudo-:mycelial cells was in some cases very pronounced, and on occasion, the pseudopypha appeared as little more than a chain of oval cells (Plate IX, Figure 18). Concomitant with the decrease in length was an increase in width. These shorter cells varied considerably in their dimensions, but the length was occasionally reduced to 3.4μ, and the breadth increased to 2.5μ, i.e. proportions which were not dissimilar to the cells of the original inoculum. /

inoculum. Pseudomycelial cells in the extensive colonies were normally up to 40μ in length, and approximately 1μ wide. Polymorphs were now abundant, and phagocytosis was still active. Many of the phagocytes had become aligned alongside the short pseudomycelial elements.

24 hours : Fungal elements were much more uncommon, and when present, almost invariably occurred as short, branched discrete units. Verticillation was conspicuous, and the cells were much shorter than those noted after 4 or 8 hours. (1-2.7μ X 3.4-23μ). (Plates X-XIII, Figures 20-27). The individual cells were still in an active state of growth, and it was observed that the most recently formed growth stained more deeply than the older portions. (Plate X, Figure 21). Some pseudo-:mycelia were growing apparently without any host response (Plate X, Figure 21), whilst others (Plate X, Figure 20) were associated with phagocyte cells. Under the low power of the microscope, it was often possible to observe the position of a pseudomycelium by the aggregation of deeply staining polymorphs. Various stages in the dis-:integration of the yeast cells could be seen at this stage, always in association with the clusters of phagocytes. The cytoplasm of the pseudomycelial cells became progressively less deeply stained, ultimately becoming /

becoming colourless, although the original outline could still be seen (Plate XII, Figure 25). The final stage is shown in Plate XIII, Figure 26, where the position of the original pseudomycelium was indicated by the accumul-:ation of phagocytes, polymorphs, and cell debris. In a number of cases, phagocytes could be seen attached to the pseudomycelial cells (Plate XII, Figure 24). When phagocytes were associated with the pseudomycelia, increase in length appeared to be inhibited, and the pseudomycelial cells became shorter and thicker (Plate X, Figure 20). This might afford an explanation for the differences in morphology noted between "clumped" and "discrete" pseudomycelia noted after 12 hours, the difference being related to the relative scarcity of phagocytes within the extensive colonies. The overall tendency at this stage was for the reappearance of blastospores, the change in growth apparently being related to the presence of phagocytes.

After 24 hours, the presence of pus had a tend-:ency to mask the presence of the fungus. A few strands could still be detected, but their staining reaction was weak, and morphological details were not visible. No yeast cells could be observed after 96 hours, although cultures made from the pus contained in the subcutaneous bubble were positive.

All /

All cultures up to and including 96 hours were positive. Lesions were noted on the kidney after 48 hours, and on the liver after 96 hours, but no yeast was recovered from cultures inoculated with this material. One animal died after 9 days, but no post-mortem was possible (due to cannibalism).

One animal was sacrificed after 24 days, but no yeast cells were recovered from the subcutaneous site of inoculation, or from any of the organs tested, viz. heart, lungs, spleen, kidney, liver, and bladder.

2. Candida tropicalis : isolate CHR

1 hour : Many of the cells had germinated to form buds which were indistinguishable from those produced in vitro (Plate XIV, Figure 28). Budding did occur in the cells of the inoculum but this was rare and most of the blastospores remained entire throughout the period of the experiment. Of 500 cells examined, 145 (29%) had produced this primary bud, which was unlike that produced by C. albicans : it was spherical rather than tubular, and was usually constricted at its origin. The bud was approximately 1.3µ-1.7µ in width, originating from a base 0.6-0.8µ across, and measuring up to 3.5µ in length. Cells of C. tropicalis are oval rather than rounded, and this primary bud was produced at or near the polar region.

In /

In no case was more than one bud observed on each cell, in contrast to C. albicans where 2 or more outgrowths were relatively common. Unlike C. albicans elongation of the blastospore itself may precede bud formation. Growth was not so pronounced as that which took place in C. albicans and the appearance lacked any morphological modifications which could be regarded as characteristic. Phagocytosis was present (Plate XIV, Figure 29) and a number of cells were observed which were weakly stained and apparently undergoing digestion.

1½ hours : bud elongation was much more pronounced and the primary outgrowth was already assuming a cylindrical appearance. The number of blastospores producing a bud had increased slightly to 40%. The length in some cases extended to 11μ, but the majority showed an average cell length of approximately 6.5μ. In one or two cases a secondary bud had already appeared at the distal end of the primary one (Plate XV, Figure 30). The width of the elongating bud was between 2-3.1μ, compared with 0.6-1.2μ which was recorded for the homologous structure in C. albicans. Occasionally, a second bud was observed forming on the parent cell, usually occurring on the opposite pole to the one which had given rise to the initial bud : normally, however, the cells had a single outgrowth, which /

which differed from that produced by C. albicans both in its greater thickness and in being straight or only slightly curved.

2 hours : The outgrowths from the blastospores were now definitely cylindrical (Plate XV, Figure 31), and in many case had given rise to elongated pseudomycelial strands consisting of 1, 2, or occasionally 3 elongated cells. The individual cells were frequently 15-21μ long. There appeared to be a fundamental difference in the manner of growth of the two species at this stage. In C. albicans the "germ tube" was at least in the early phase, apparently mycelial in nature, and there was little indication of a pseudomycelial origin : the growth was rapid and was typically non-septate. In C. tropicalis, the pseudomycelial nature of the outgrowth was evident from its initial appearance, and the production of new cells by budding was always conspicuous; furthermore, the number of cells producing a primary cell were proportionately much lower than those producing "germ tubes". Thus, production of primary buds, cells, or pseudomycelial strands was observed in 53% of the cells examined, compared with the figure of 97% obtained for the number of blastospores of C. albicans producing "germ tubes" (both assessments made on a count of 500 cells).

The /

The two species could be distinguished from one another by their appearance, and the comparatively thick pseudomycel-:ial strands of C. tropicalis with a straight or only slightly curved outline were in contrast to the narrower strands of C. albicans with their sinuous outlines. The pseudomycelial strands of C. tropicalis showed occasional branching, the branch arising by budding from the junction of 2 pseudomycelial cells, and itself showing elongation. Phagocytosis was active, many of the yeast cells showing progressive loss in their ability to take up the stain. Many of the blastospores were neither contained within phagocytes, nor had they formed pseudomycelium. They were lying apparently free in the matrix of the connective tissue and were deeply stained. Free cells of this type were rarely noted in the case of C. albicans. In one trial numerous polymorphs were observed, and many of the yeast cells were undergoing phagocytosis by host cells other than the polymorphs.

4 hours : Strands consisting of as many as 6 pseudomycel-:ial cells were occasionally seen, but this was exceptional. Normally, the strand contained 1-3 cells. Many of the original blastospores were by this stage irregular in outline, rather swollen, and in some cases had collapsed. They were more readily seen in material which had been stained /

stained with the Periodic Acid-Schiff technique. The tendency, as with C. albicans was for the suppression of blastospore production, and for the increase in pseudo-:mycelial production. This was now conspicuous (Plate XVI, Figures 32, 33, 34) and already the individual strands were forming an interwoven network. In some respects, this resembled the growth of C. albicans, differing, as described above by their more robust nature and more regular outline. These strands were consistently 2μ across, compared with 0.7-1.2μ for the comparable structure in C. albicans. Phagocytosis was very conspicuous (Plate XVII, Figure 35) and a large number of cells, both blastospores and elongate buds had apparently been digested. In these cells, only the empty cell wall remained, which was colourless and in some cases irregular in outline. Pseudomycelium was generally less deeply staining than that of C. albicans. The majority were unbranched, but a number of them bore a single oval blastospore at the junction between 2 pseudomycelial cells. This varied in width from 2.5-3.5μ, and showed varying degrees of increase in length. Blastospore production was very much reduced, and most of the oval or rounded deeply staining cells were those presumably introduced with the inoculum. A large number of polymorphs and other phagocytes were present, although /

although not necessarily in association with the yeast cells. There was again a tendency to form "clumped" colonies, which consisted of interwoven strands, unchanged blastospores, digested cells, phagocytes, and pus cells.

The aspect of the yeast at this stage was less characteristic than that of C. albicans. The pseudomycel-:ial cells, for example were not unduly long, nor did they differ from pseudomycelial growth which appears in vitro. Although blastospore production was reduced, it was not entirely absent, and the appearance was not almost exclusively mycelial as it tended to be with C. albicans.

8 hours : Polymorphs were abundant, and many of the yeast cells were undergoing phagocytosis. The pseudomycelial strands were never longer than 100μ and a large number of blastospores still remained, many of them apparently arrested at the stage where the primary bud was 6-15μ in length. There appeared to have been a general check on the growth of the yeast, and there was little difference in the appearance when compared with the observations made at 4 hours. As with C. albicans, the yeast often occurred in extensive plaques which consisted of yeast cells, phagocytes and pus cells. The individual threads, however, were noticeably shorter than those occurring in C. /

C. albicans, and normally measured from 10-35µ in length, although, as mentioned above, this may exceptionally extend to a length of 100µ. These strands were rarely composed of more than 3 cells. Branching and blastospore production was still observed, but the majority of cells showed the arrested development described above. Many of the cells were apparently moribund, being unstained and irregular in outline.

12 hours : The general aspect had changed very little. Sections stained by the Periodic Acid-Shiff reaction showed the presence of blastospores and short pseudomycelial strands up to 60µ in length (Plate XVIII, Figure 37). Material stained with the May-Grunwald stain showed the presence of a large number of phagocytes, and with this stain, the yeast cells were weakly staining. In the centre of some of the more extensive colonies, the strands occasionally attained a length in excess of 100µ, these threads being narrow (1-1.4µ), and consisting of up to 4 or 5 cells (Plate XVII, Figure 36). Numerous blastospores were present in various stages of disintegration. The ability to form pseudomycelium was clearly much less than C. albicans, where after the same lapse of time, growth was almost exclusively mycelial. In the material of C. tropicalis, long pseudomycelial strands were rarely observed /

observed outwith one of the colonies.

Examination of the 12 hour material revealed little that had not already been noted after 4 hours. Differences from the earlier stage were seen primarily in the production of pus, and an increase in the number of phagocytosed cells. It would therefore appear that growth of the yeast cells occurs during the first 4 hours, after which there is an almost complete cessation of any further growth or differentiation.

Observations made on material obtained after 24, 48, and 72 hours failed to reveal any further elaboration of the yeast pseudomycelia, and any changes noted concerned the increasing production of pus. Single blastospores were observed in the connective tissue until the conclusion of the experiment, a period of 9 days.

Malt agar slants inoculated with swabs made from the floor of the bubble showed development of yeast cells up to and including 5 days. Post mortems failed to reveal any gross pathological changes, although the kidneys of an animal sacrificed after 9 days were pale, and had white diffuse lesions. No yeast cells were recovered. No yeast cells were recovered from the faeces, urine, or heart blood after 5 days, nor were they recovered at any time before that, from those sources.

3. /

3. Torulopsis glabrata : isolate I 7358

The cells remained blastosporic throughout the duration of the experiment. There was a slight increase in the proportion of cells producing buds, and counts made at 1 and 2 hours showed an increase from 23-41% of the total number examined. A corresponding increase in the number of bud-forming cells in the suspension originally used for the inoculum raised the total from 8-25%. Phagocytosis was again seen after 1 hour, and this increased with increasing pus formation. Polymorphs were present after 4 hours, and abundant at 8 hours. At 12 hours most of the blastospores which were visible were contained within phagocytes : a single phagocyte occasionally contained as many as 20 blastospores. These intracellular yeast cells were well-stained and few of them showed any signs of digestion (Plate XVIII, Figure 38). Budding was rarely observed, and the cells were almost exclusively entire. The number of blastospores occurring outwith a phagocyte was very small. When examined after 7 days, blastospores were rarely observed. Swab cultures made from the floor of the bubble after this time were positive, and a malt agar slant inoculated with a swab from the bladder also gave rise to yeast colonies. Blood and lung cultures made at this time did not give rise to yeast colonies.

4. /

4. Saccharomyces fragilis : isolate H 7981

After 1 hour, the blastospores showed a considerable degree of budding and elongation (Plate XIX, Figures 39, 40), when compared with the inoculum suspension. 23% of the cells had formed buds, and occasionally (Plate XX, Figure 41) long pseudomycelial strands up to 50µ in length were produced, consisting of up to 3 cells. In one case a single lateral blastospore was seen. The breadth of the pseudomycelium varied from 0.7-1.1µ. A certain amount of blastospore elongation or pseudomycelial formation did occur in the yeast cells retained in the inoculum suspension, but not to the same extent as that which took place in vivo. This early formation of elongated cells was quickly checked, and later observations failed to show any further development. At 24 hours, no pseudomycelial strands could be seen, and the number of yeast cells present was very small. Polymorphs again made their appearance gradually, the number of yeast cells undergoing digestion increased, and pus was formed. Single blastospores could still be seen after 48 hours.

This organism is not normally considered pathogenic, but one animal died after 48 hours. Discrete white lesions 1-2 mm across were observed on the liver, but it was considered unlikely that this was the result of inoculation /

inoculation of the animal with the yeast. No organism was obtained from the lesions, or from swabs from any of the organs examined, viz. heart, lungs, kidney, bladder, or spleen.

5. Saccharomyces carlsbergensis : isolate Y379 (Plate XX, Figure 42)

Unlike any of the other species tested, there appeared to be a delay in the increase in budding of the original blastospores when introduced into the air bubble. After 4 hours more than 50% of the free cells had formed buds, but the majority of blastospores were contained within phagocytes and were apparently undergoing digestion. Pus formation accompanied the phagocytosis, and by 12 hours no blastospores could be seen, although the swab culture made from the floor of the bubble was positive.

6. Candida tropicalis : isolate J 3495 (Plate XXI, Figure 43)

7. Candida tropicalis : isolate I 5599 (Plate XXI, Figure 44)

8. Candida tropicalis : isolate I 6133 (Plate XXII, Figures 46, 47; Plate XXI, Figure 45; Plate XXIII, Figure 48)

These 3 isolates all underwent the same morphological changes and induced the same host response as seen in the case of the CHR strain described above : the number of blastospores producing pseudomycelial growth after 1 or 2 hourse was much higher than occurred in the CHR strain. Isolate /

Isolate J 3495, for example, showed cell elongation in 13% of the cells examined after 1 hour, but this had risen by the end of the second hour to 90%. Nevertheless, the sequence of development of the pseudomycelium, its check after 4 hours, and the manifestations of host response were identical to those described above for the CHR strain.

Discussion

Two methods were used in the examination of yeast cells, and it is considered that the more significant results were obtained from subcutaneous inoculation. The tissue culture method was discarded in favour of the air bubble technique for a number of reasons, amongst which the following may be considered : the isolation of monocytes was laborious and their maintenance difficult. Reproducible results were more readily obtained by the air bubble technique, which also provided a more natural environment. Phagocytosis was examined in relation to a single type of phagocyte, and the possible effect of other white blood cells on the morphology of yeast cells could not be examined. Finally, the risk of contamination is considerably reduced in the air bubble technique, and observation by preliminary drying and staining of the tissue was more convenient than the examination of living material. Nevertheless, tissue culture affords a means of making direct observations of living processes, and it is considered that the results obtained are of importance in gaining an understanding of the behaviour of yeast cells *in vivo*.

Of the species examined, only *C. albicans* and *C. tropicalis* showed any significant growth *in vivo*. *C. albicans* in particular underwent a rapid and fundamental change of morphology, blastospore development being entirely suppressed, and filamentation enhanced. /

enhanced. The appearance of the yeast after 4 hours was essentially mycelial, and after 12 hours almost exclusively so. There was a rapid growth of the pseudohyphae up to and including 4 hours, but this became less obvious in later stages. Growth up to 12 hours was by extension only, and branching was exceptional : verticill-:ation, however, was observed for the first time although this only occurred in the "plaques" (pus infiltrated colonies). It appeared that the reduction of cell length and formation of compact verticil-:late branches was connected with the immediate presence of polymorphonuclear leukocytes. This was particularly obvious after 24 hours. Blyth (132) has described the appearance and develop-:ment of pseudomycelium by C. albicans in the kidney of the mouse. He noted the presence of basic pseudomycelium which occurred at the surface membrane, and was essentially mycelial in nature. Follow-:ing penetration, the growth became "transitional" where cell division was increased at the expense of cell elongation, with consequent production of strong verticillation. Later stages were characterised by the ultimate reversion to blastospore prod-:uction. It seems possible that the pseudomycelium developed in the first 4 hours in C. albicans corresponds morphologically to the "basic" pseudomycelium occurring in the surface membranes. The apparent inhibition of cell elongation and increase in verticillation noted after 24 hours may be identified with the "transitional" pseudomycelium. The third stage, i.e. the reversion to /

to blastospore production was not noted in the present work. The apparent association between polymorphs and the production of verticils would seem to confirm Blyth's observations. Although a tentative comparison is made between the results obtained by Blyth and by the present writer, it must be pointed out that the environment of the air bubble differs considerably from that of the kidney. The growth of C. albicans in the present experiments was in a subcutaneous cavity, where a comparison could perhaps be drawn with the peritoneal cavity. It may seem neither reasonable nor justifiable to compare morphological changes occurring in this exceptional and artifical environment with growth inside the kidney. Nevertheless, the similarity between the developmental morphology in the two sites is striking, and a close agreement is apparent in the early stages of growth.

Hill and Gebhardt (113) have figured the change in morphology of C. albicans in vivo, and consider that the change in morphology is related to the pathogenic nature of the yeast. It is pointed out that the production of a pseudomycelium might hinder ingestion by phagocytes. They reported that of 6 species of Candida studied, only C. albicans and C. stellatoidea (Jones et Martin) Langeron et Guerra underwent morphological changes when inoculated into the mouse. None of the other species showed any alteration and remained in the form of blastospores : amongst these species was C. tropicalis. From the results reported and figured above, /

above, it is clear that C. tropicalis does undergo certain morphological changes although these are not so well marked or so persistent as those observed in C. albicans. The tendency is also towards filamentation, and the increase in length of the pseudomycel-:ial cells is very rapid up to the fourth hour. The possibility that the CHR strain of C. tropicalis differed in this respect from any other members of the same species was discounted when it was shown that other isolates produced identical morphological trans-:formations. It is of interest to note that of the 4 strains of C. tropicalis examined by this technique, the CHR strain showed least development of pseudomycelium, when assessed by the percent-:age of blastospores which had formed elongated pseudomycelial cells. After 2 hours, 53% of the cells of CHR examined had produced pseudomycelium or pseudomycelial initials. This contrasted with the figure of 90% obtained for isolate I 3495 after a similar time. In all of the isolates of C. tropicalis cell elongation was checked after 4-8 hours, and in no cases was further growth or elaboration noted.

Observations made on the remaining species of yeast (Torulopsis glabrata, Saccharomyces fragilis, S. carlsbergensis) suggested that although none of these formed pseudomycelium, there was nevertheless an initial stimulation of budding following introduction to the bubble. This was reflected by a count of the number of budding cells when compared with the cells suspended in the /

the saline solution. This budding, however, did not bring about any build up of the number of cells contained within the bubble, and after 4-8 hours, many of the cells were undergoing digestion. T. glabrata was rather exceptional amongst all the species examined in the localisation of blastospores within phagocytes after 12 hours. The majority of blastospores were intracellular and apparently viable when judged by their staining reaction. The presence of the cells of this species within macrophages has been recorded by Lopez-Fernandez (92).

The air bubble technique has certain advantages in studying early stages of development of yeast cells in vivo. Its ease of manipulation, and the rapidity with which results can be obtained are important practical considerations, and it is clear that the method can be of considerable value in examining early stages of infection.

In a recent paper by Young (119), dealing with the invasion and persistence of C. albicans injected into mice, the early stages of development from the blastospore are briefly described. The percentage of cells showing production of "germ tubes" obtained in the present work was almost identical to that reported by Young. He reports the phagocytosis of cells by monocytes, the absence of polymorphs and an increase of the number of cells forming pseudomycelial growth after 2 hours. These observations were also made by the present writer, and by means of

a /

a different sampling and inoculation procedure. Young's observations were made following intra-peritoneal inoculation and the yeast cells were reisolated by removal with sterile swabs. It is known that the introduction of cells of C. albicans by the intraperitoneal route leads to the production of more pathological changes than by subcutaneous inoculation. Nevertheless, on the basis that the observations were concerned primarily with the morphology of the yeasts rather than the pathology of the host, it was concluded that the method and observations recorded in the present work have afforded a valid evaluation of the morphological changes occurring in vivo. Young's description of the early stages of growth of C. albicans (119) in the peritoneal cavity suggest that the initial changes in morphology are similar to those recorded above in the subcutaneous cavity. Growth of C. tropicalis under the conditions of the experiment described above has not been reported before. The pseudomycelial development in the first few hours following inoculation is marked, and this observation is at variance with the findings of Hill and Gebhardt (113). The failure of these authors to observe pseudomycelial production of this species may have been caused by the employment of a non-pathogenic strain of the fungus, a non-susceptible strain of mouse, or perhaps the utilisation of an insufficient density of inoculum.

The emphasis of the present work was primarily on the morphology of the yeasts studied, and any pathological changes occurring /

occurring in the host animal were regarded as of secondary importance. Observations were seldom extended beyond 10 days and this was considered inadequate for the establishment or demonstration of any pathological symptoms. Out of 117 animals inoculated, 99 (84.6%) were sacrificed within the 48 hours following inoculation. In the remaining 18 animals which were maintained for a longer period, two deaths were recorded, but there was no evidence in either case that the yeast under test was responsible. One of the mortalities occurred after 9 days in the series inoculated with C. albicans. The other death occurred in the series inoculated with S. fragilis and took place after 48 hours. It is considered unlikely that the yeast was directly involved in bringing about this death, as it was not found possible to demonstrate the presence of a yeast in any of the organs examined. Particularly significant in this respect was the failure to isolate a yeast from the lesions on the liver.

The increase in bud production noted in the non-mycelial yeasts (Torulopsis glabrata, Saccharomyces fragilis, and, to a lesser extent S. carlsbergensis) is not considered to be a valid criterion, since a similar increase in budding is also observed when the cells are transferred from the yeast suspension to a suitable culture medium. Pseudomycelial development of some species may also occur in vitro, but not with the rapidity or the abundance seen in vivo. The marked elongation of both C. albicans and C. tropicalis within the /

the animal body is considered to be significant in relation to the morphological studies, and can possibly be regarded as a specific adaptation to growth *in vivo*

The experiments described above were intended to provide a clearer understanding of the early development of yeast cells in the animal body. The studies have shown that the early stages are apparently marked by a stimulation of growth, which is evidenced in both *C. albicans* and *C. tropicalis* by conspicuous and rapid elong-:ation of pseudomycelial cells. Observations were confined to the early stages of growth immediately following inoculation and revealed a significant morphological alteration in both of these species when compared with the non-mycelial forms. A significant difference in the degree of development of each of the two species was also demonstrated. It is possible that this may be a morphological expression of a fundamental difference in pathogenicity.

The exceptionally rapid production of mycelial develop-:ment might eventually find application in the establishment of a routine clinical identification of *C. albicans* : it is suggested that the early growth *in vivo* is no less characteristic than the combination of morphological or physiological characters described for this species. An accurate assessment of these transformations however, was considered to be outside the scope of the present work.

Summary of Part I and Part II

1. An introduction to the subject of medical mycology is given with a brief indication of its scope and the organisms concerned. The nature of the Experimental Mycoses Unit is described and the objects of the present work defined.

2. The first part of the work is a survey of the fungi occurring in a variety of human sources, with especial reference to the yeasts. 1,004 samples were examined from urine, sputum, throat, high vaginal, wound, stool and other sources. 289 fungi (28.8%) were obtained; 248 (86%) of the isolates were yeasts.

3. Candida albicans was the most common species and was isolated on 141 occasions (56% of the total number of yeasts). Other species were Torulopsis glabrata (31); Rhodotorula mucilaginosa (16); Candida tropicalis (12); Candida parapsilosis (7); Saccharomyces cerevisiae (4); Candida zeylanoides (4); Cryptococcus albidus (4); Saccharomyces fragilis (3); Candida krusei (3); Hansenula anomola (2); Pichia membranaefaciens (2); Saccharomyces carlsbergensis (1); Trichosporon cutaneum (1); Candida robusta (1); Candida guilliermondii (1); Torulopsis famata (1); Torulopsis candida (1); Cryptococcus laurentii (1); Cryptococcus diffluens (1).

4. Torulopsis glabrata is shown to be a significant member of the flora of the sources examined.

5. A description of 11 isolates which were atypical is given with /

with a discussion on their taxonomic affinities.

6. Rhodotorula mucilaginosa was obtained from decaying vegetation; Candida guilliermondii was isolated from soil.

7. A description is given of a strain of Candida tropicalis which was responsible for a fatal septicaemia.

8. An evaluation of the methods used in the survey is provided.

9. Tissue culture techniques were used to demonstrate phagocytosis of Candida albicans and Candida tropicalis.

10. In vivo experiments showed that Candida albicans rapidly forms pseudomycelium. After 12 hours verticillate branching occurs. An association between branching and the presence of polymorpho-:nuclear leukocytes is indicated. Candida tropicalis also forms pseudomycelium in vivo, but a check is evident after 4 hours. Pseudomycelial strands are branched and production of blastospores is not entirely suppressed.

11. Blastospores of Torulopsis glabrata were predominantly intracellular within host phagocytes after 12 hours.

12. Abortive pseudomycelium is demonstrated in Saccharomyces fragilis.

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P L A T E S

PLATE I

PLATE I

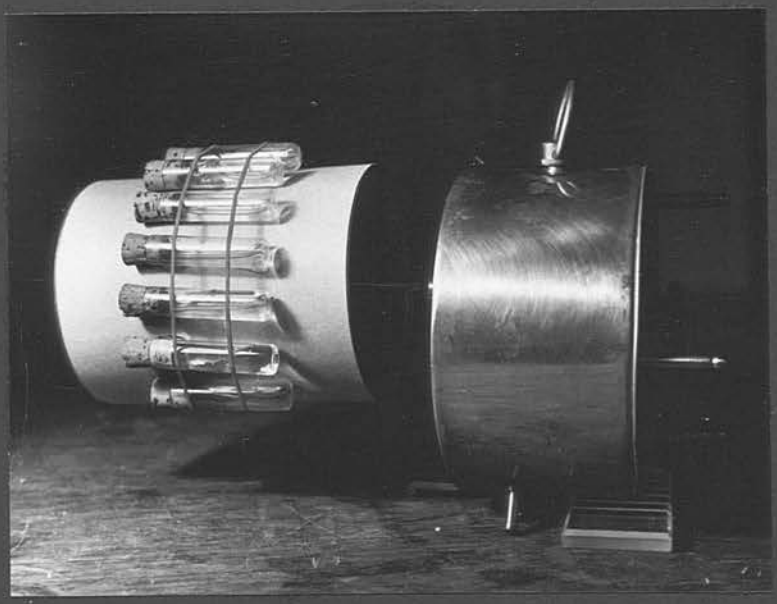
Fig. 1

Fig. 2

Fig. 3

p l a t e II

PLATE II



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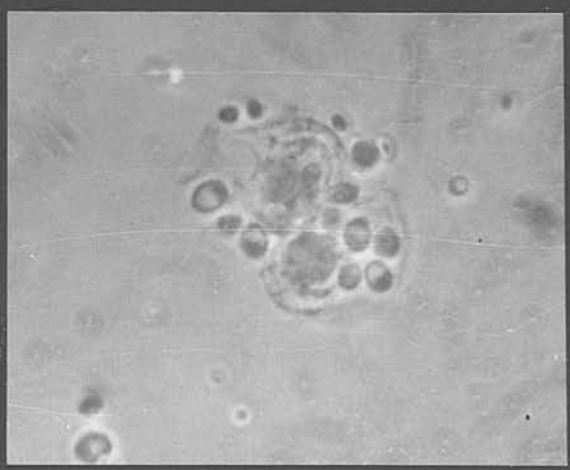
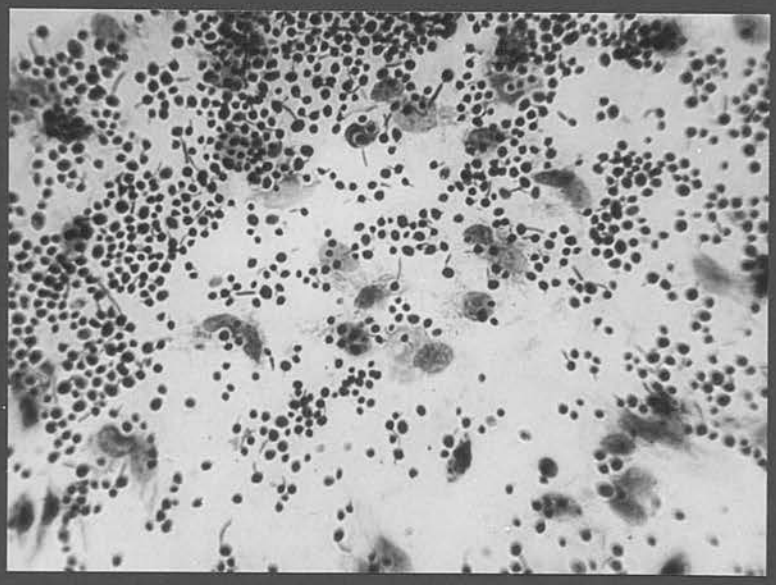


Fig. 5

P L A T E III

PLATE III



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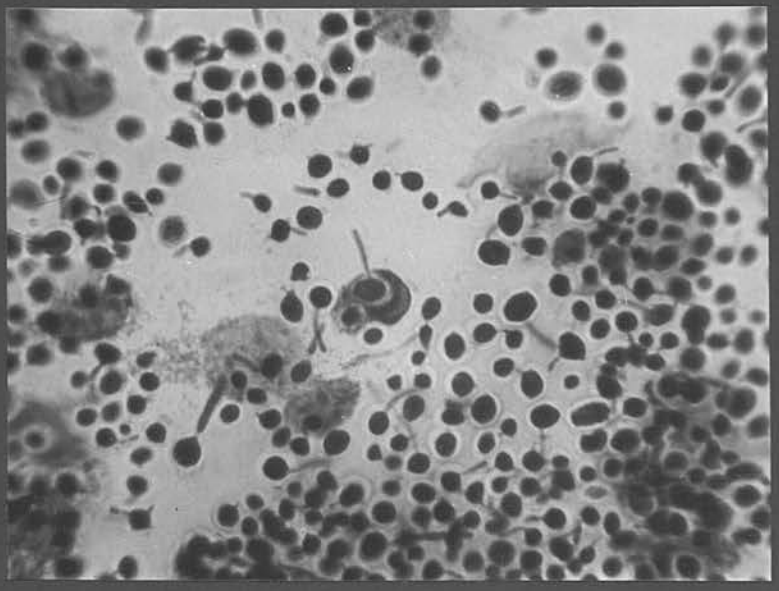
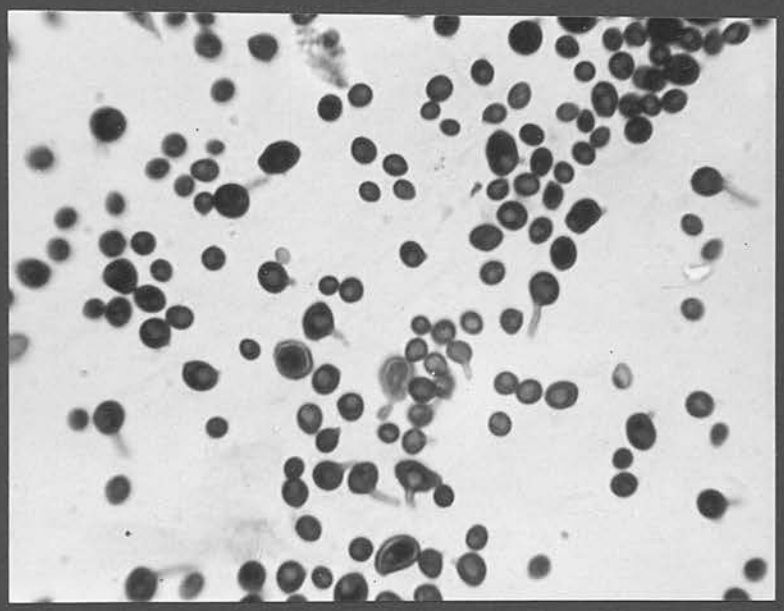


Fig. 7

P L A T E IV

PLATE IV



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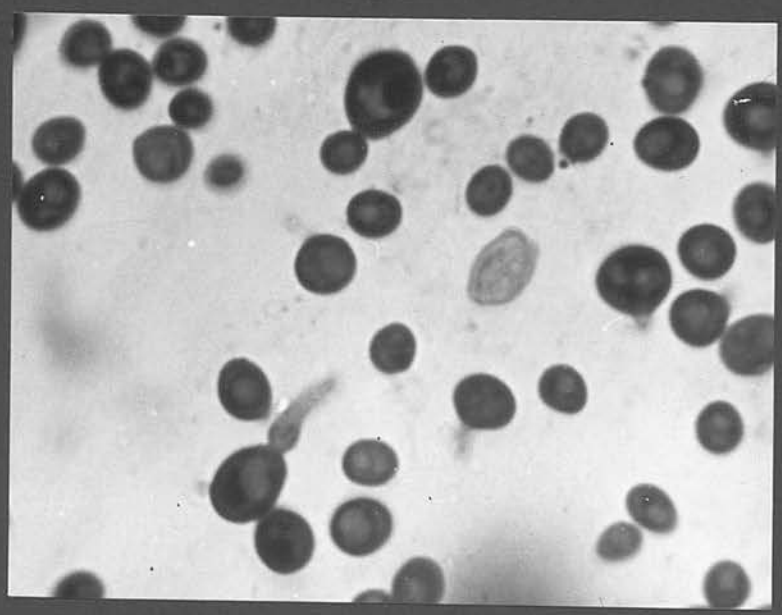


Fig. 9

P L A T E V

PLATE V

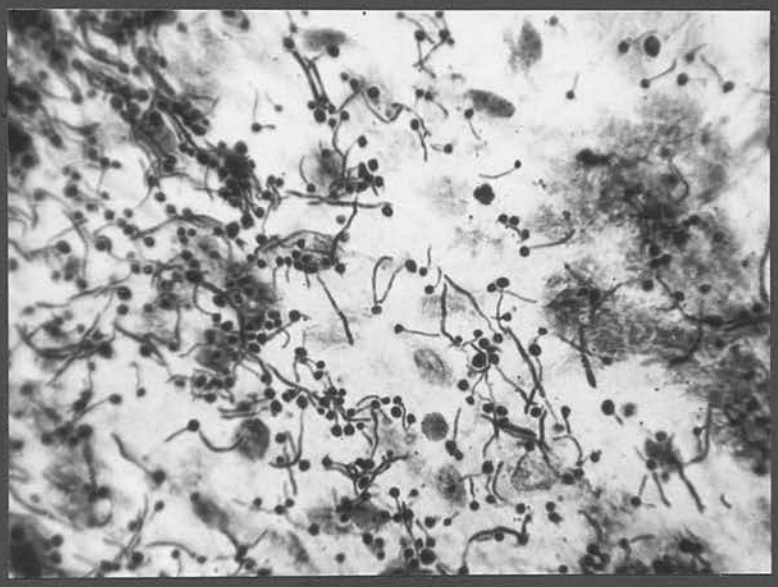


Fig. 10

Fig. 11

P L A T E VII

Fig. 14

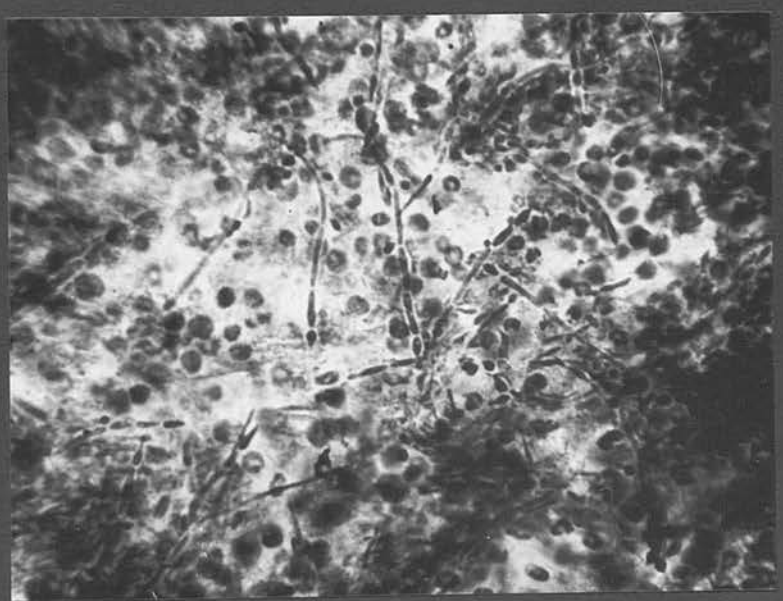
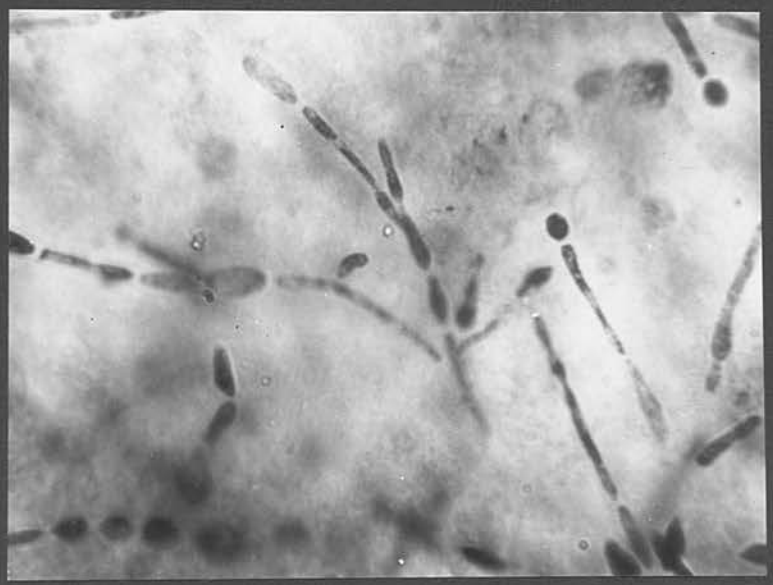


Fig. 15

P L A T E VIII

PLATE VIII



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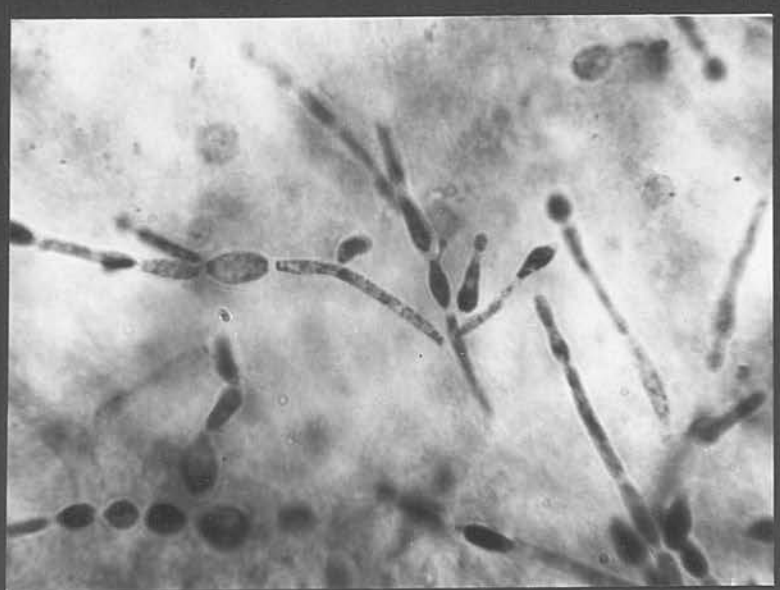


Fig. 16

PLATE IX

PLATE IX

Fig. 18

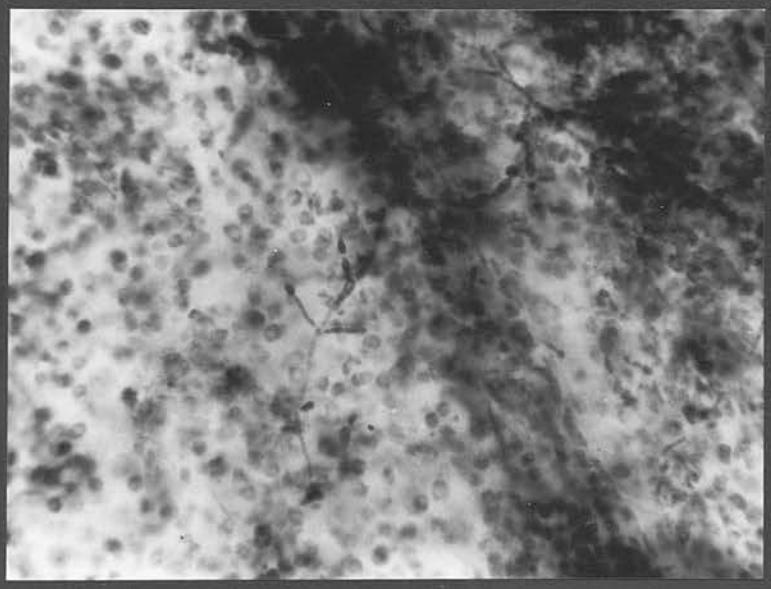


Fig. 19

PLATE X

PLATE X

Fig. 20

Fig. 21

P L A T E XI

PLATE XI

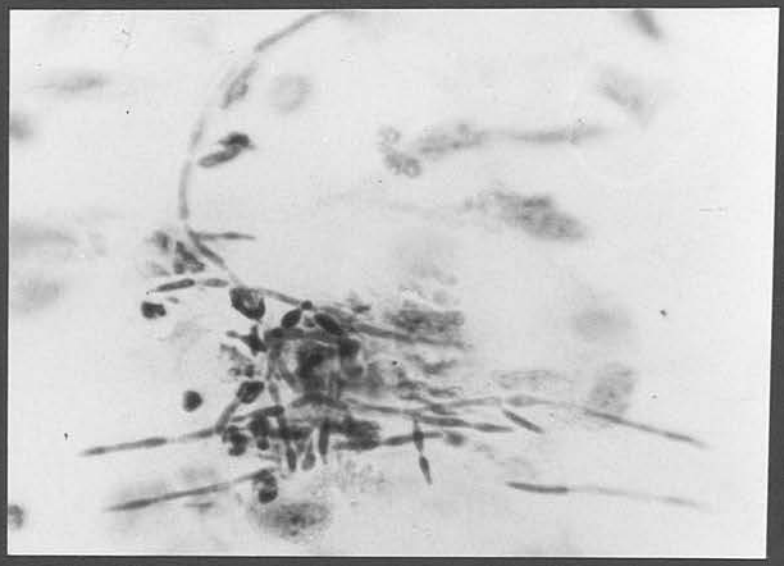
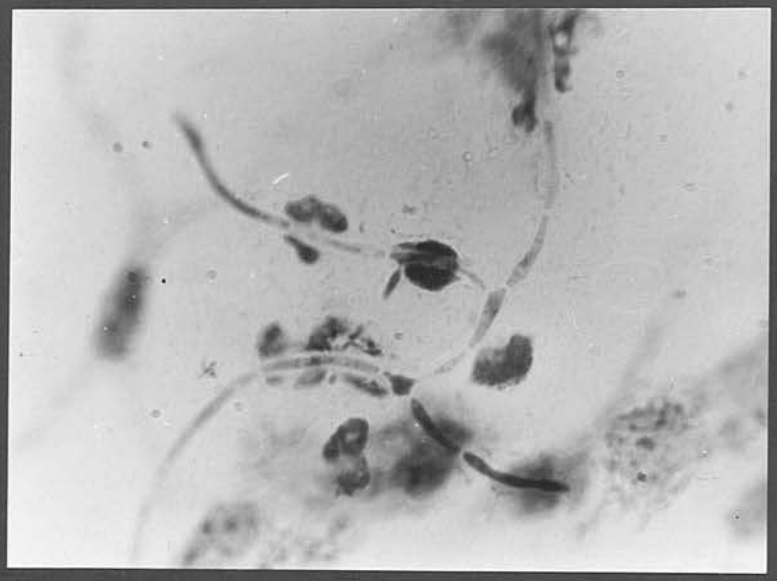


Fig. 22

Fig. 23

PLATE XII

PLATE XII



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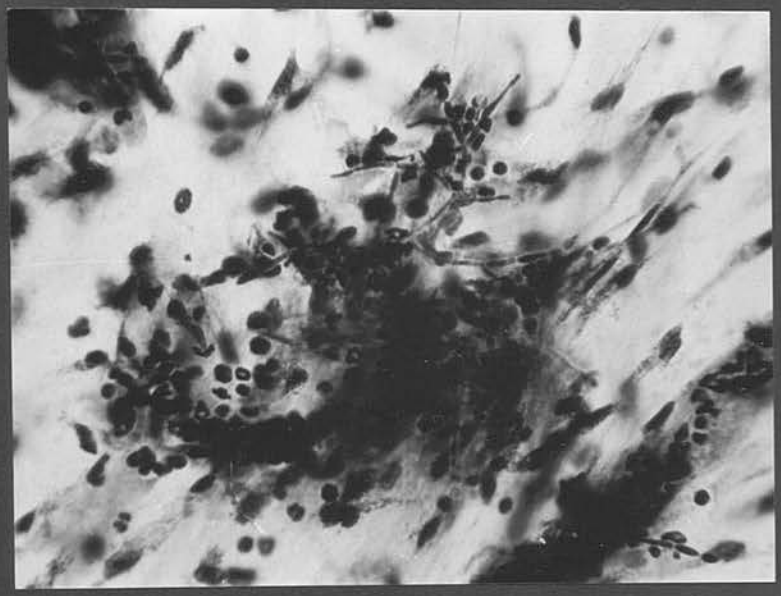
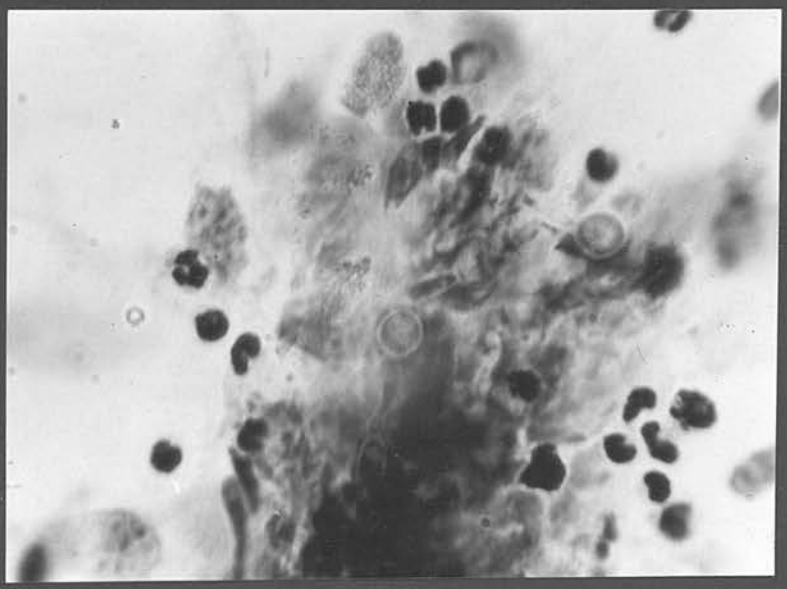


Fig. 24a



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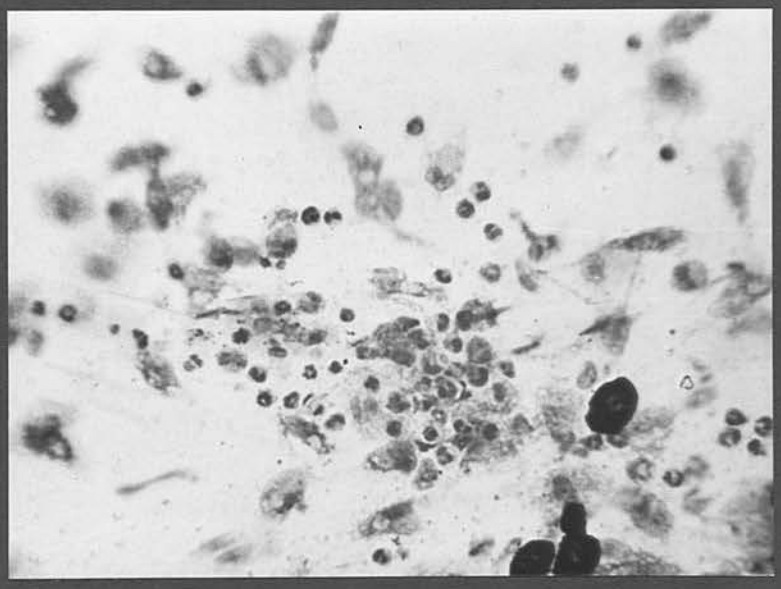

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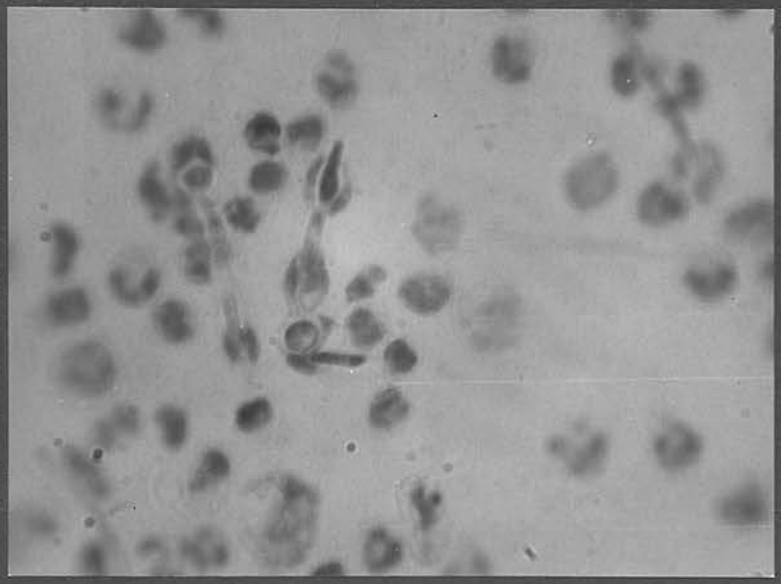
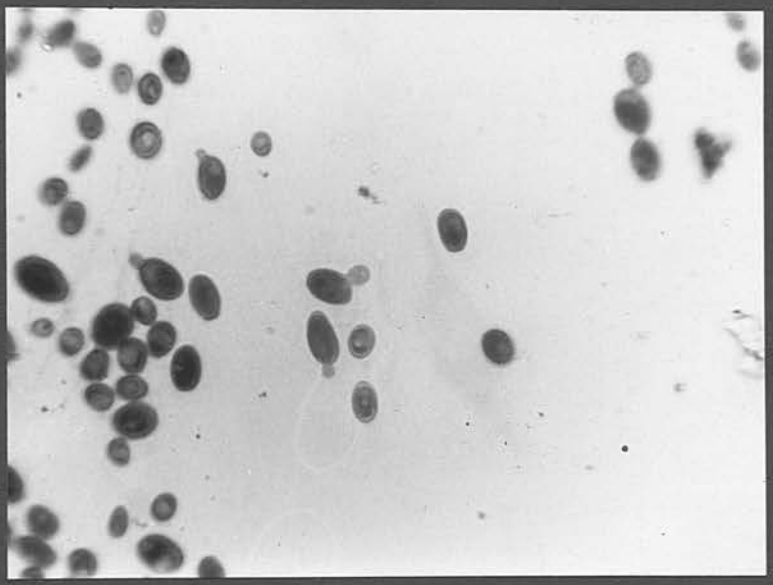


Fig. 27

P L A T E XIV



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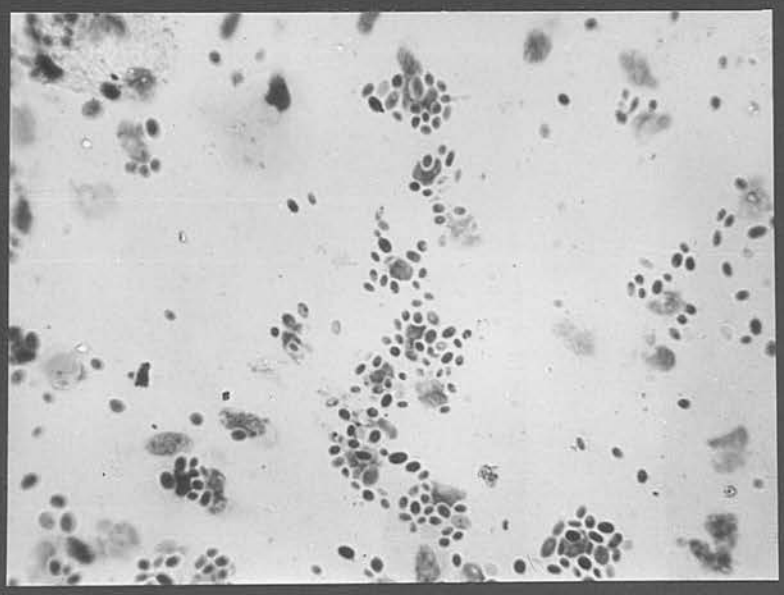

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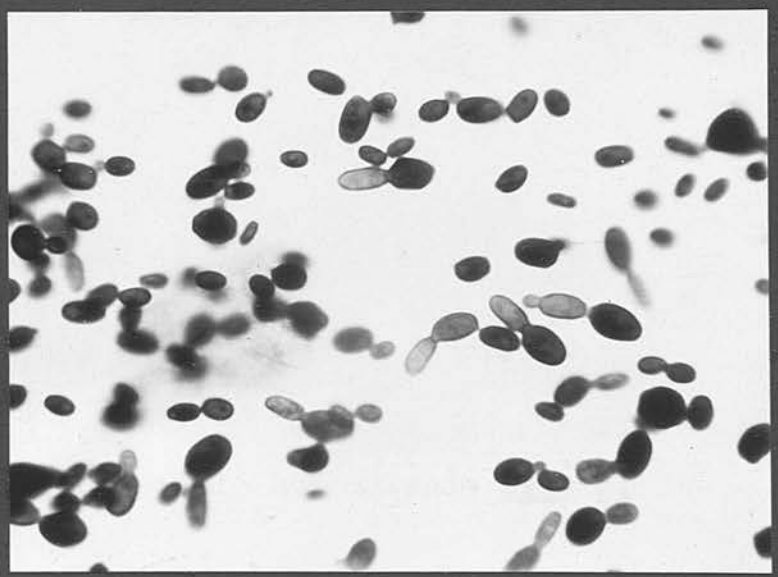
P L A T E XV



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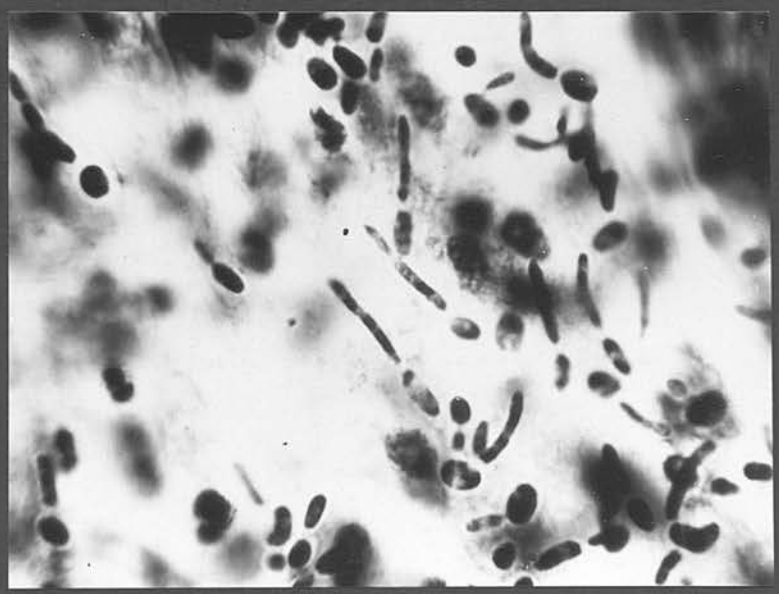

Fig. 31

P L A T E XVI

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Fig. 32

Fig. 33

Fig. 34

P L A T E XVII

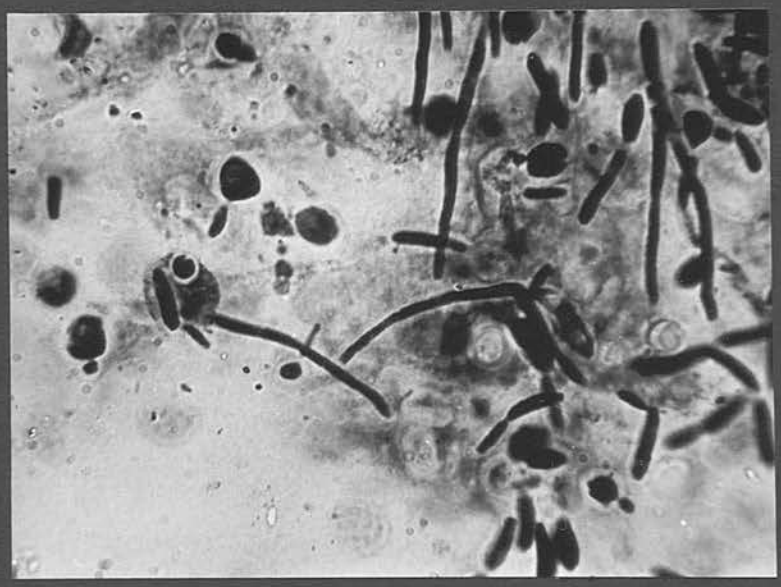
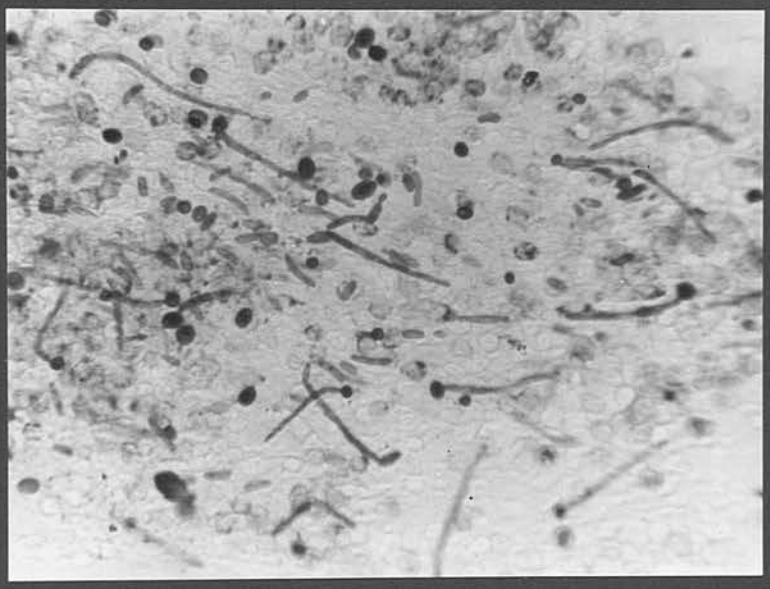


Fig. 35

Fig. 36

P L A T E XVIII

PLATE XVIII



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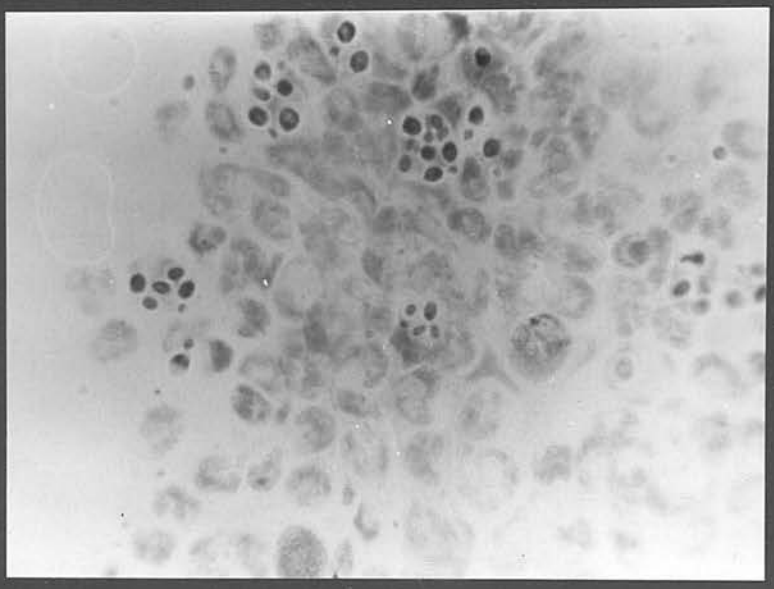
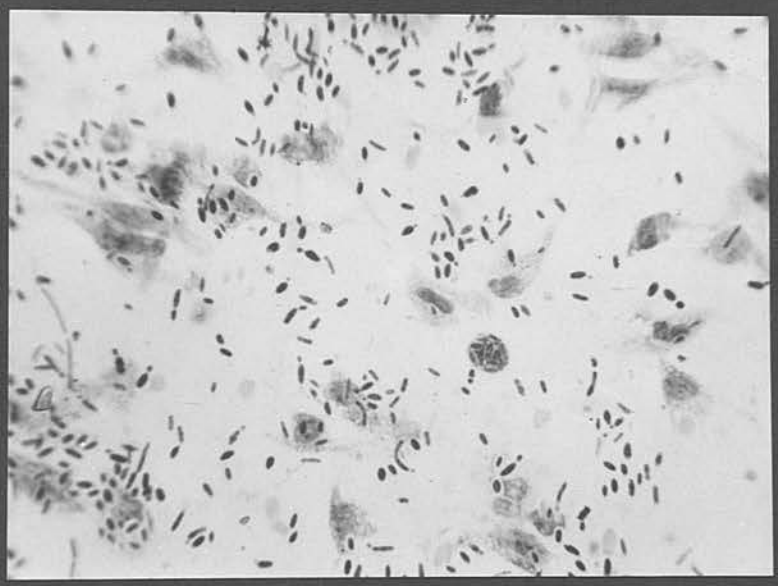


Fig. 38

P L A T E XIX

PLATE XIX



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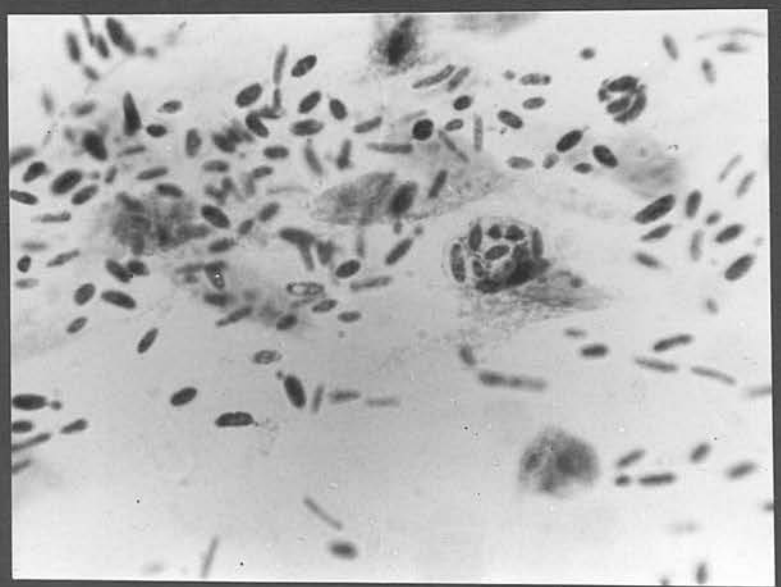
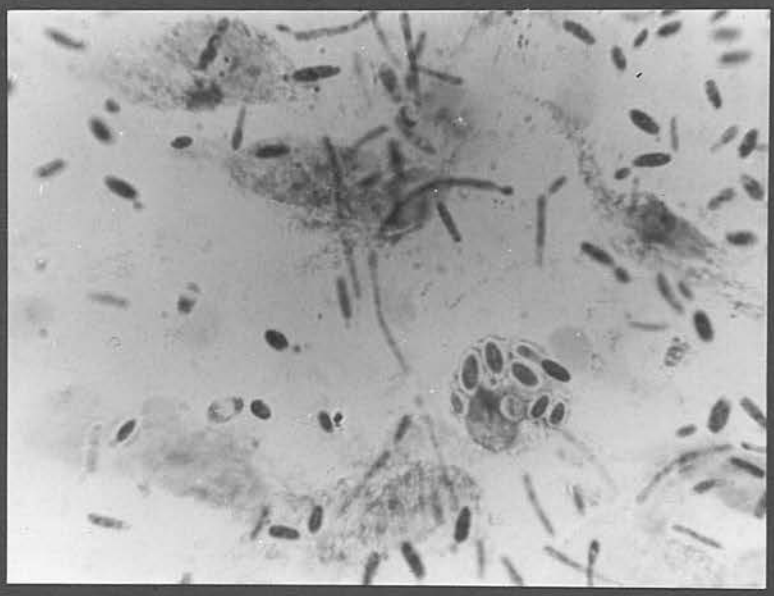


Fig. 40

P L A T E XX

PLATE XX



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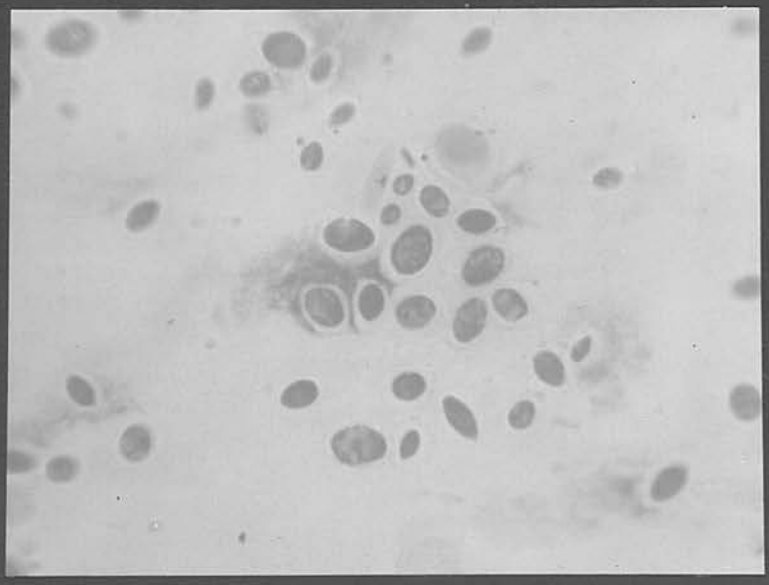
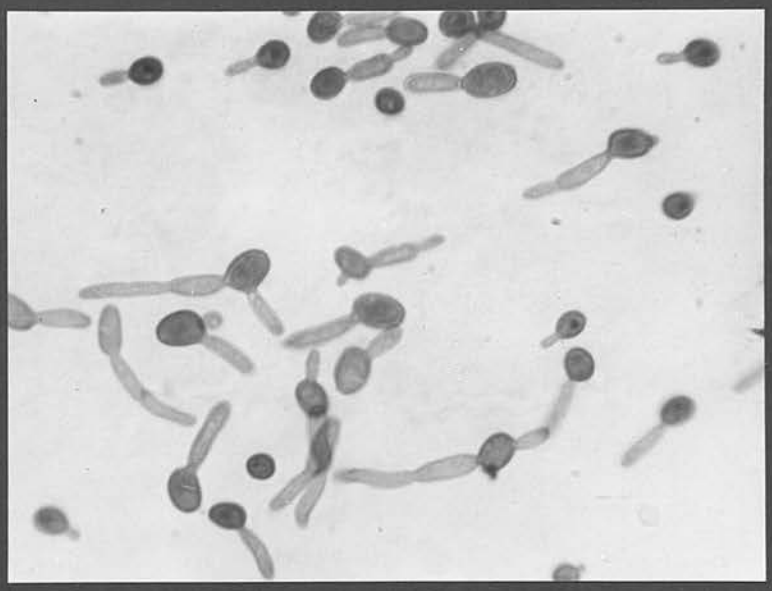


Fig. 42

P L A T E XXI

PLATE XXI



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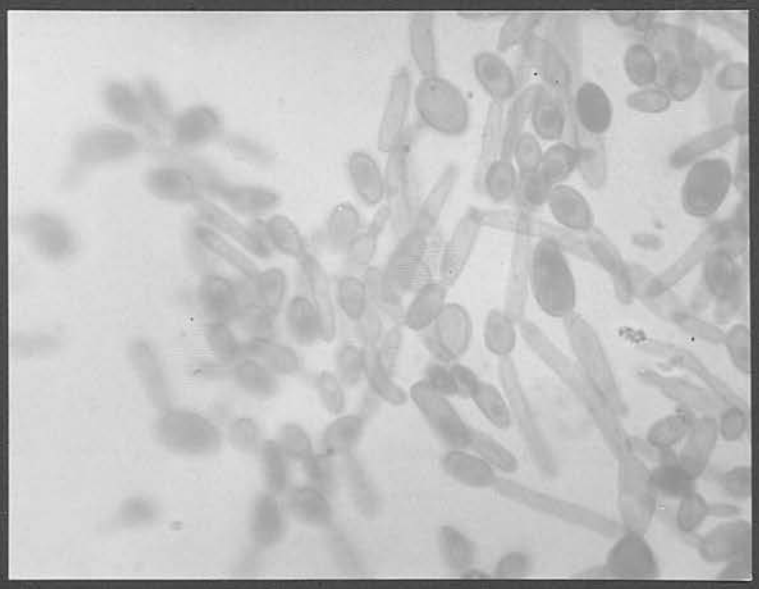

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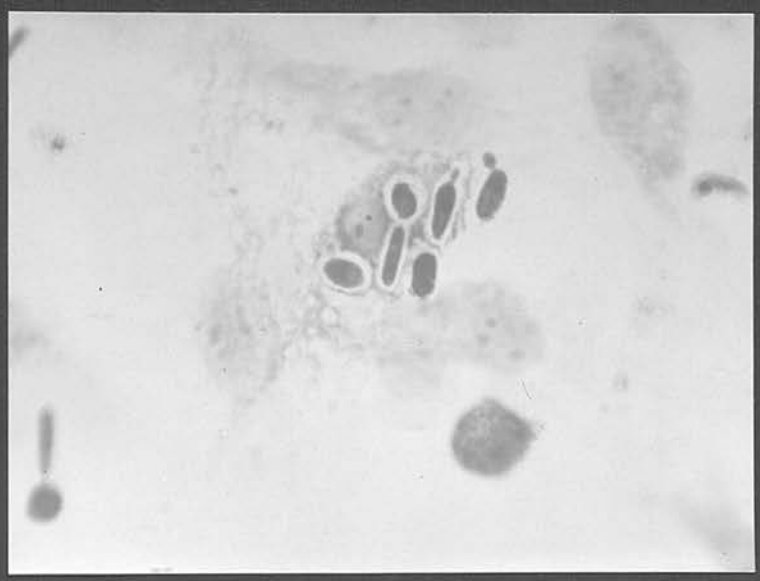
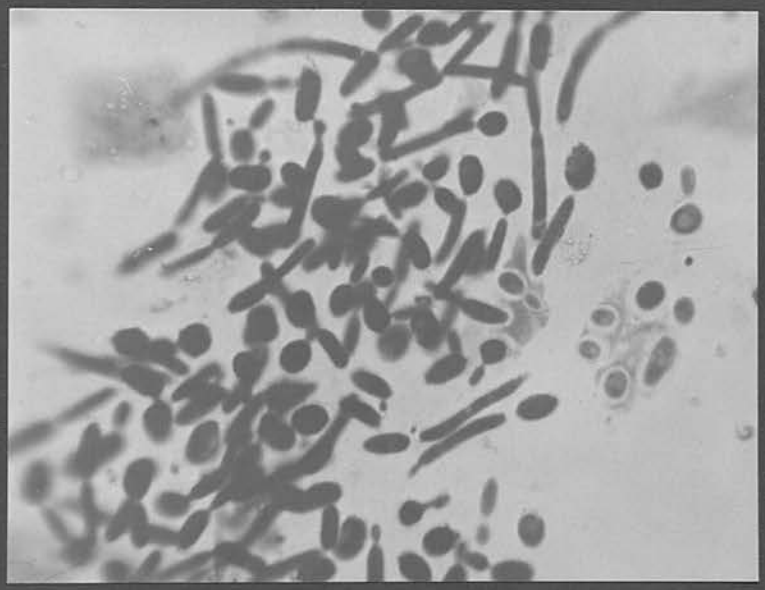


Fig. 45

P L A T E XXII

PLATE XXII



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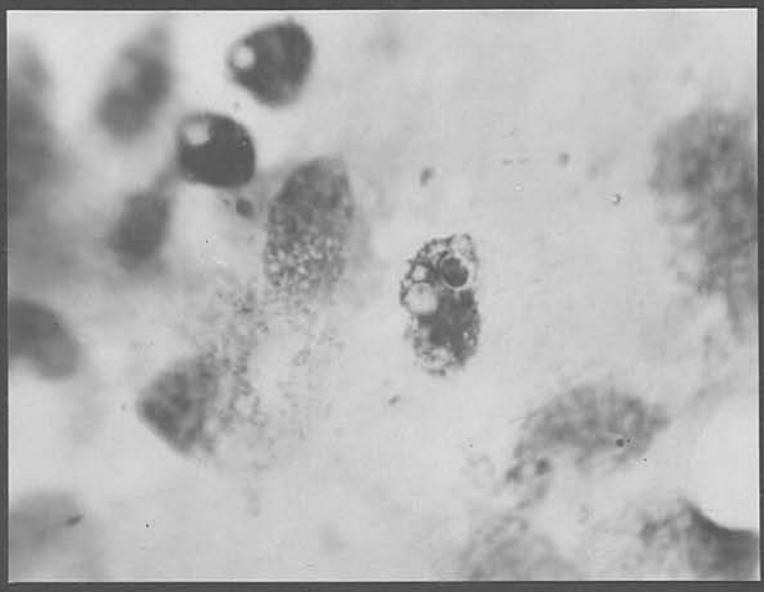

Fig. 47

P L A T E XXIII

PLATE XXIII

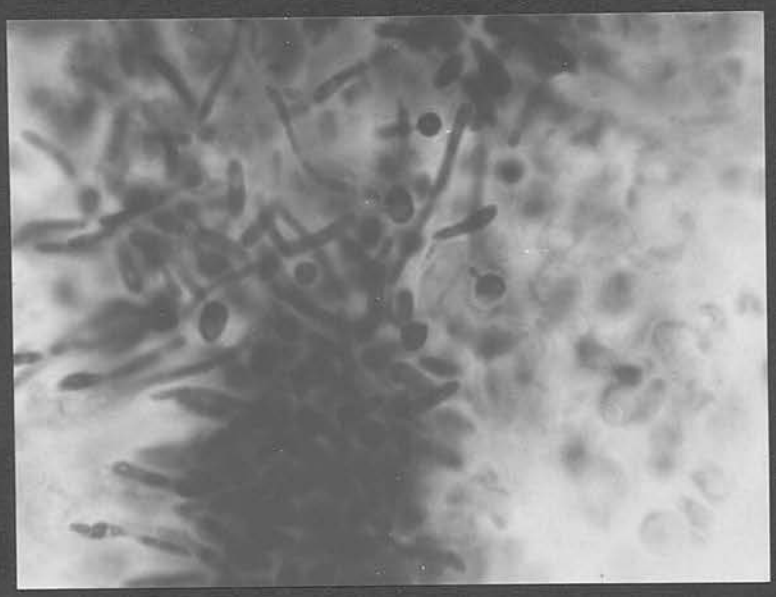

Fig. 48

Acknowledgements

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