GROWTH OF *CANDIDA ALBICANS* ON KERATIN AS SOLE SOURCE OF NITROGEN

by

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I. INTRODUCTION.

1. Clinical manifestations caused by C. albicans and related species.

Candida albicans (Robin, Berkhout, 1923) is the most common pathogenic fungus isolated from clinical material. It was first observed by Langenbeck in 1839 at autopsy. He found this yeast-like fungus in the intestines of a person who had died from typhoid fever. In 1842, David Gruby, the founder of medical mycology, clearly recognized yeast-like fungi involved in thrush of small children. He studied the fungus with his Swedish student Berg. Significantly, Gruby called the fungus "aphthophyte" and wrongly thought it to be a Sporotrichum species. In the first treatise of medical mycology, Robin studied morphology of this fungus and the clinical manifestations caused by it. He already distinguished the two growth-forms as seen in the white patches (unicellular yeasts and filaments) and suspected a relation between the yeast and filamentous forms. The morphology of the fungus induced Robin to call it Oidium albicans, the first scientific name given to Candida albicans. The generic name Oidium paraphrases the yeast phase while the epithet albicans is descriptive of the white patches seen in thrush. Later, Stumpf confirmed Robin's conjecture about the relation between the unicellular yeasts and the filaments seen in the white patches of thrush. The first cultures of the thrush fungus were obtained by Quinquaud and Grawitz. These isolations and cultures were followed by innumerable reports of isolation of the thrush fungus from clinical material. Unfortunately, the subsequent publications on the mycology of the yeast-like fungi did not result in establishing a clear and concise record of the morphology, taxonomy and classification. The descriptions of C. albicans under more than 200 synonyms fully reflect the state of confusion caused so often by early workers in the field of medical mycology. Therefore, our knowledge of the diseases caused by Candida albicans and related species is rather limited.

In 1923, Berkhout reinvestigated the genus Monilia, in which the thrush fungus and related species had been most often placed. She separated these yeast-like fungi, until then called Monilia, from the imperfect forms of the genus Stromatinia. The latter fungi were retained in the genus Monilia while the yeast-like fungi were placed in the genus Candida, which arrangement found the approval of the Third International Congress of Microbiology in 1939. The work of Berkhout induced Langeron and Talice and later Langeron and Guerra to review once more the Candida species. The slide culture method of Rivalier and Seydel proved to be very useful for the study of the filamentization of the yeasts, whereas fermentation of different sugars, assimilation of sugar and of nitrogenous compounds, arbutin splitting etc. were found to be valuable physiological criteria. Diddens and Lodder and later Lodder and Kreger-van Rij extended these studies which resulted in the establishment of a reliable classification of the Candida species. Hereby the basis has been provided for a reinvestigation of the clinical manifestations caused by Candida species.

In relation to diseases of man and animals C. albicans is the most often isolated Candida species as isolated from clinical specimens. The following Candida species have been isolated from 7152 specimens received in this laboratory during the past five years. As the causative organism of diseases of men and animals, C. albicans is the Candida species most commonly isolated from clinical specimens.

Candida	albicans	1603
"	parapsilosis	230
"	Krusei	20
"	guilliermondii	8
"	tropicalis	3
"	pseudotropicalis	2

"	intermedia	2
"	curvata	1

These findings correspond to other reports about the frequency of occurrence of different Candida species (Diddens and Lodder 1942).

The clinical manifestations caused by Candida species are usually referred to as moniliasis, although some authors tend to change the name of such conditions following the taxonomic changes. Therefore, in more recent publications the disease is often called candidiasis or candidosis.

Candida albicans is primarily an invader of the mucous membranes. Infection of the oral cavity, thrush, characterized by white patches of varying sizes on the mucous membranes, is the most common mycotic infection of man. In New Zealand, Marples and diMenna found the oral cavity infected by C. albicans in 37% of children and in 50.5% of adults. Vulvovaginitis is very common too. Severe pruritus, vulvitis, vaginitis or profuse and curdy discharge are usually found in this condition. Jones et al. isolated C. albicans in 91.07% of their 280 cases of mycotic vulvovaginitis; the other C. species encountered were C. stellatoidea, C. parapsilosis, C. Krusei and C. tropicalis. Sexual communicability of the infection affecting the external genitalia, the perianal and anal area of males was reported by Waisman. In each case the condition was acquired from the wife.

In cutaneous moniliasis the infected areas (axillae, infra-mammary regions, umbilicus, groin, anus, interdigital spaces) become reddish, moist and macerated. Occasionally the feet become infected too and present a similar clinical picture as in athlete's foot (Becker and Ritchie; Hopkins; Hopkins and Benham; Nikolowski and Mueller).

Moniliasis of the nails, especially of the finger nails, is quite frequent (Connor; Engelhardt and Brackertz). This disease seems to be more common in women with vulvovaginitis. In such cases the index and middle

finger are most often found to be infected, probably from scratching of the vulva. Pus formation and swelling differentiates moniliasis of the nails from nail infections caused by Trichophyton species. In moniliasis, as in trichophytosis, the nails become yellowish, thickened, brittle and lustreless.

Candida species have been found in the cornea by Mendelblatt, and their isolation from infected ear canals is rather frequent.

The bronchopulmonary form of moniliasis is characterized by a persistent cough and clinical signs of bronchitis (Cohen A.C; Morin, Leblond and Fiset). The sputum often contains gray flakes consisting of yeast cells and hyphae. The pulmonary form is more rarely encountered and is difficult to diagnose. In mild cases, cough and expectoration are the only complaints. Hemoptysis, dyspnea, chest pain, weakness, weight loss, fever and night sweats are additional symptoms in severe cases (Orie; Hamil; Hobby; Oblath et al).

Endocarditis as caused by Candida species has been reported fairly frequently in recent years by Geiger et al; Kunstadter et al; Luttgens; Sachs and Ata; Wikler et al; Wolfe and Henderson; Zimmerman.

Meningitis, more commonly caused by Cryptococcus neoformans, a yeast showing a marked predilection for the central nervous system, is not so often caused by Candida species (Craig and Gates; Halpert and Wilkins; Morris, Kalz and Lotspeich).

Intestinal moniliasis characterized by diarrhoea, burning, excessive gas and flatus, abdominal pains, pains in the rectum, pruritus ani and occasional bleeding from the rectal mucosa is a well defined clinical entity (Brabander et al).

Cases of generalized moniliasis have become more frequent due to the indiscriminate use of newer antibiotics (Aureomycin, Terramycin). In a number of such cases, the Candida species invaded almost every internal organ (Beemer, Pryce and Riddell; Brown et al; Duhig and Mead; Gausewitz et al,

Gutman).

The incidence of moniliasis of the skin and nails has also increased considerably. No satisfactory explanation of this phenomenon can be given although there is no doubt that the low and varying degree of pathogenicity of C. albicans is increased in and on the human and animal body by the use of such antibiotics.

Since the literature does not reveal any facts about the pathogenicity of C. albicans for keratinized tissues and the laboratory reports on the isolations of C. albicans from skin and nails usually meet with skepticism on the part of the clinician, it was decided to investigate the not yet proven keratinolytic activities of C. albicans.

2. Some properties of keratin pertaining to its enzymatic breakdown.

The vertebrate skin keratin is formed by cell division in the non-keratinized Malpighian layers. The cells are displaced outwardly from this layer and during this process their chemical composition is gradually altered. The non-protein constituents such as ribonucleic acid, ascorbic acid, glycogen, glutathione and enzymes become reduced in proportion and most of them disappear completely. The fibrous elements become consolidated, concentrated, rendered insoluble, hard and dry. The fibrous elements in hairs are shifted roughly to the long axis of the hair, while in skin, nails and hoofs they become flattened and remain unorientated. As keratin undergoes the hardening process sulfhydryl groups of cysteine disappear and the formation of disulfide bonds takes place (Giroud and Leblond). This process can be followed by histochemical methods. The sulfhydryl groups (-SH) give a positive, the disulfide groups (-S-S-) give a negative nitroprusside reaction.

Keratins are scleroproteins. Although the amino acid composition of keratins of different origin varies considerably, there is a remarkable

constancy in the molecular ratio of certain amino acids: histidine, lysine and arginine always occur in constant proportions 1:4:12. This fact is often emphasized among the characteristics differentiating keratin from other proteins (Block, 1951).

Ward and Lundgren (1954) give a complete review on the chemistry of keratin from which the following tables have been taken.

TABLE A.

Keratin Composition in Terms of Amino Acid Residues.

	Hair	Horn	Epidermis
Residue	Number per 100 residues		
Glycine	6.4 - 6.3	15.4 - 14.3	10.0 - 23.9
Alanine	3.7 - 3.5	3.4 - 3.1	-
Valine	5.5 - 5.6	5.4 - 5.2	4.5 - 6.4
Leucine	5.8 - 7.1	7.0 - 7.1	8.0 - 8.5
Isoleucine	4.2 - 4.1	3.9 - 4.1	6.7 - 7.0
Phenylalanine	1.7 - 2.4	2.3 - 2.7	2.1 - 2.3
Proline	4.4 - 9.3	8.6 - 7.9	3.5 - 3.7
Serine	8.3 - 11.3	7.8 - 7.2	19.7 - 21.1
Threonine	6.9 - 8.0	6.2 - 5.7	3.6 - 3.8
Tyrosine	1.4 - 1.9	2.5 - 3.4	2.4 - 4.1
Aspartic acid	3.5 - 6.5	7.0 - 6.6	6.0 - 8.2
Glutamic acid	10.9 - 10.8	11.3 - 10.5	7.8 - 14.1
(amide)	9.9 - 9.3	9.8 - 9.1	10.4 - 11.1
Arginine	6.0 - 6.9	4.7 - 6.8	4.3 - 9.0
Lysine	1.5 - 2.4	2.0 - 2.7	2.6 - 6.3
Hydroxylysine	-	-	-
Histidine	0.5 - 0.9	0.5 - 0.7	0.5 - 0.8
Tryptophan	0.2 - 0.7	0.4 - 0.8	0.9 - 1.2
(Half) cystine	16.3 - 16.8	12.1 - 14.6	2.4 - 4.3
Methionine	0.6 - 0.7	0.4 - 1.6	1.0 - 2.3

Analyses of human nails and cattle hoofs are not as numerous as analyses of hair and wool. Block (1951) gives the following composition of human nails in terms of gm. per 16 gm. of N:

Arginine	10.0
Histidine	0.5
Lysine	2.8
Cystine	12.9
Methionine	1.0
Glutamic acid	15.7
Aspartic acid	8.4

Not only does the amino acid composition of keratins vary, but also the occurrence of amino acids as terminal residues varies (Table B).

TABLE B.

N - Terminal Amino Acid Residues of Wool and Hair

Author	Middlebrook	Tibbs	Tibbs	Tibbs	Blackburn
Keratin	Lincoln wool	Romney wool	Merino wool	Human hair	N.Z. 56's wool
		In gram Equivalents			
Glycine	5.2	4.5	4.7	3.9	0.5
Alanine	1.3	1.2	1.2	1.0	1.4
Valine	2.4	2.4	2.4	4.0	4.0
Serine	1.3	1.2	1.2	1.0	0.2
Threonine	4.8	4.9	4.9	4.0	2.2
Aspartic acid	0.6	0.6	0.6	0.5	0.3
Glutamic "	1.3	1.2	1.2	1.0	0.8

Two types of keratin can be distinguished on the basis of physical character and chemical composition: they are soft keratins and hard keratins.

The soft keratins, typically represented by human skin, thickened parts of cows' noses and lips, and horse burrs, have a low degree of consolidation and are low in sulphur content, which constitutes only about 1% of dry weight. The sulphur in soft keratins is evenly distributed between cystine and methionine. On the other hand, hard keratins which are found in hairs, nails, horns, hoofs, feathers and wool, have a high sulphur content, from 2-5%, predominantly in the form of cystine; they have a much more coherent structure and high tensile strength.

Up to 20 different amino acids have been repeatedly identified in keratin hydrolysates (Ward and Lundgren; Lang and Lucas; and Meyer). The high cystine content of keratin is widely agreed to be its most significant analytical feature. It is of fundamental importance to the stabilization of the structure of keratin and yet it is quantitatively a surprisingly variable factor. The range of cystine content is most significant in distinguishing hard keratins from soft keratins (Giroud and Leblond, 1951); it also denotes the nutritional status and the age of the individual; it may further differentiate follicles on an individual, and even the two sides of a hair follicle may each have a different sulphur content. Hence the difficulty in obtaining identical samples of keratin from two different sources. Moreover, the determinations of cystine are often complicated through losses suffered on hydrolysis and erroneous quantities are obtained in relation to the original keratin sample (Lucas and Beveridge, 1940; Barrit 1934).

Keratinized tissues are not homogeneous in their chemical composition. An average sample of wool or hair upon simple water extraction yields substances that are not proteins. Bolliger, 1951, Bolliger and Gross, (1952) extracted from hair a number of compounds such as uric acid, urea, ammonia, creatine, creatinine, phenolic substances, pentose, desoxyribose and unidentified reducing substances. Fatty acids were found associated normally with human hairs and

were wrongly believed to have fungistatic action in hairs of adults (Rothman et al; Kligman and Ginsberg).

Spectographic analysis of human skin, nails and hairs by Goldblum et al; and Dutcher and Rothman revealed the presence of metallic ions, 14 of which were identified. Among them were Zn, Mg, Cu, Fe, and Mn.

Goddard and Michaelis (1934) drew attention to the existence of two cross linkages in the keratin molecule other than the disulfide bond: a salt-like bridge and hydrogen bond. It is known that chemical agents e.g. thioglycollate, used to dissolve keratin require a higher alkalinity than is needed to reduce a disulfide such as cystine. Yet, an alkaline solution of the same pH without a reducing agent would not be able to dissolve keratin in comparable time. The following hypothesis was offered by the authors: there might be in the keratin molecule a bridge formed by electrostatic attraction of the $-NH_3^+$ group of diaminoacids for the carboxyl group of dicarboxylic acids. Such salt-like bridges would be broken in alkaline solution by removal of a proton from the amino group. Should this apply to keratin then it would explain how the higher pH level actually effects the breaking of such a bond: moreover the completion of such a reaction is necessary before the reducing agent could act on the disulfide cross linkage. A third bond is a hydrogen bond between the polar side groups of residue of amino acids. The hydrogen bonds are of great value in stabilizing a variety of crystals (D. F. Waugh, 1954; Woodin 1954; Fearnley, 1950) and likewise they add to the toughness of keratins. The reduced keratin shows a tendency to reoxidation. In order to stabilize in the reduced form, the hydrogen bond has to be broken. This can be effected by the action of chemical agents known to act specifically upon hydrogen bonds: alkali, concentrated solution of urea, guamidine salts, phenols and amides. If cross linkages determine the resistance of keratin to enzymatic digestion, then the number of cross linkages would determine the relative susceptibility of

keratin to biological attack. Barlow and Chattaway (1955) proved that this was actually the case. The reduction of keratin by treatment of hairs with thioglycolic acid or with sodium metabisulphite opened the disulfide bonds, while refluxing with lithium bromide caused the breaking of hydrogen bonds. Such modified keratin, with a reduced number of cross linkages provided a much better medium than native keratin for growth of Microsporum canis, M. audouini, Trichophyton rubrum, Epidermophyton floccosum. The increase in the number of cross linkages by binding some of the existing free amino groups to the free carboxyl groups, on the other hand, resulted in an increased resistance of thus altered keratin to fungal attack. Keratin can also react with oxidizing agents, but solubilisation is brought about in this way more slowly than when reducing agents are used. The disulphide bond of cystine is one of the most reactive centres in the keratin molecule on which both oxidizing and reducing agents preferentially act. H_2O_2 effects oxidation of these bonds slowly, whereas fluorine acts faster. H_2O_2 can oxidize cystine in wool partly to cysteic acid and partly to cystine disulphoxide; it also attacks free amino groups of basic amino acids and peptide bonds. All these reactions effected by absorbed H_2O_2 are marked only at elevated temperatures, while at room temperature the absorption is slow and no oxidation can be detected, except in very alkaline solutions over a period of several hours. (Alexander, Hudson and Fox 1950; Alexander, Carter, Earland 1950). Chlorine at pH below 8, and hypochlorous acid can also effect oxidation of wool keratin. In connection with these studies, recent morphological research on wool fibres has established the existence of two membranes, one of which, the epicuticle is situated on the extreme outer surface, whilst the other forms part of the cuticle and is referred to as subcuticle. It has been shown that the presence of the epicuticle does not affect the rate of diffusion of the hypochlorite ion, while in alkaline solutions the negatively charged subcuticle retards the penetration of anions and diffusion

through this membrane determines the overall reaction (Alexander, Gough and Hudson, 1951). The oxidation of wool tyrosine probably yields an indole derivative, while permanganate oxidizes tyrosine to a quinone (Alexander and Gough, 1951).

The rupture of these resistant cross linkages can be effected under much milder conditions by enzymes present in microorganisms, larvae of insects and certain birds. These enzymes and their actions will be discussed later.

Much work on the submicroscopic structure of keratins has been done by means of X-ray diffraction (Astbury; Meyer; Bear and Hugo; Giroud and Leblond). However, the different stages of contraction of keratin as revealed by this method failed to show any correlation with its susceptibility to enzymatic breakdown (Stahl et al*) and therefore will not be discussed in detail here.

3. Studies on metabolic utilisation of keratin.

(a) Morphological observations.

Microscopic observations on fungi parasitizing keratinaceous structures such as hair and skin were made by Gruby in 1842. His work was forgotten for a number of years and resumed by Sabouraud who studied in great detail the invasion of hairs by a highly specialized group of pathogenic fungi, the dermatophytes. The results of his clinical and mycological investigations were published in his most famous and unique treatise "Les Teignes" in 1910. This work covers the whole field of ringworm of the scalp, skin and nails. Sabouraud already recognized that the dermatophytes only invade and attack keratinized tissues and that, as in the example of hairs, the fungus stops growing at the borderline between the keratinized and non-keratinized part of the hair. Although at that time little was known about the chemical and physical properties of keratin, Sabauraud quickly realized that the main difficulty in the treatment of infections of hair lies in the physical properties of keratin which covers

the parasite and makes it inaccessible to therapeutic agents.

The dermatophytes invade only the living keratin. In the parasitic phase, they are characterized by a morphologically reduced appearance (Langeron). Plaut placed infected skin pieces and infected hairs in a moist chamber and observed the formation of morphological structures never seen in the parasitic phase, in scrapings or hairs. Davidson and Gregory repeated these experiments in 1934. They placed detached hairs, naturally infected by Microsporum audouini, M. felineum, and Trichophyton gypseum in a van Tieghem cell without any nutrient medium and observed all the characteristic morphological structures (Micro- and macroconidia and others) developing around the hairs in a humid atmosphere.

If the dermatophytes are induced to grow on detached hairs in a humid atmosphere they do not grow in the parasitic phase characterized by reduced morphology. In that case the invasion of the hair is performed in quite a different way. Furthermore, the dermatophytes growing on hairs "in vitro" often develop many of the morphological features known from their growth on Sabouraud media such as micro- and macroconidia. However, fluorescence as developed in hairs in vivo infected by certain dermatophytes cannot be observed in cultures (Felsher; Gregory). These important observations of Plaut, Davidson and Gregory, and also Vanbreuseghem 1949 indicate the marked difference in the growth forms of the dermatophytes on living, non-detached keratin (parasitic phase) and on detached, dead keratin (saprophytic phase).

The dermatophytes are characterized by a highly developed host-specificity. Epidermophyton floccosum invades only the keratin of the smooth skin, while e.g. Microsporum canis will parasitize the hair and skin. Neither of these attacks nails, whereas some Trichophyton species (T. violaceum, T. Schonleini) can infect skin, hairs, and nails. Microsporum audouini

attacks the hairs of children only, M. canis, a closely related species, infects the scalp of children and adults. It might also be mentioned that some dermatophytes parasitize only on human keratinized tissues (Trichophyton interdigitale, Microsporum audouini), while others can be isolated from ringworm of man and animals, e.g. M. canis, T. discoides and T. gallinae causes favus in poultry only. So far, no satisfactory explanation of these phenomena has been offered. However, it is safe to assume that the different forms of keratin may play a decisive role in determining the host-parasite relationship.

The invasion of detached hairs by dermatophytes differs from that of non-detached hairs so thoroughly studied by Sabouraud. The hyphae originating from a "herpes circinatus" of the scalp epidermis pass along the hair to the mouth of the hair follicle. Here a mycelial plug is formed from which large hyphae push downwards between the wall of follicle and the surface of the hair. Some of these hyphae penetrate through the cuticle and grow downwards stopping short of the bulb in a fringe of delicate hyphae because the hyphae have reached the end of the keratinized part of the hair (Adamson's fringe). In detached hairs, Mandels et al (1948) observed that invasion was effected most quickly through fiber ends or else through breaks in the cuticle. The substance cementing the cortical cells was dissolved first, by the action of the invading organism (M. gypseum and Pseudomonas species), thus loosening the cortical cells which were subsequently digested, while the cuticle remained undissolved and apparently was not attacked at all. Vanbreuseghem (1949, 1952), on the contrary, observed that Trichophyton rubrum attacked the cuticle readily which was followed by the progressive digestion of the cortex. When the digestion proceeded deep enough into the hair it would eventually break. This author also noted that species of animal origin, e.g. Microsporum canis, possess stronger keratinolytic action than species of human origin such as M. audouini.

Page made a careful study of the growth of M. gypseum on fragments of

horn keratin. Coarsely powdered horn keratin was scattered over the solidifying surface of a non-nutrient agar plate, and a colony of M. gypseum was then inoculated in the center of the plate. The first hyphae originating from this colony did not seem to be influenced by the surrounding keratin particles. However, after these hyphae grew past the particular keratin fragment under observation numerous side branches were put out which grew toward it and penetrated it. Within ten days the keratin particle was dissolved by the enzymatic action of this dermatophyte. Page concluded from these observations that keratinolytic enzymes diffuse from the fungal elements very slowly and only do so over a short distance, or that some inactivation of the enzyme takes place gradually as the diffusion proceeds.

Microorganisms other than dermatophytes were also found to possess keratinolytic activities. Burgess (1928) observed the destruction of wool fibres by bacteria and fungi. Epithelial cells were attacked and dissolved first. He also was able to grow these organisms which he had isolated from "mildewed cloth" on sterilized woollen fabric.

The presence of keratinolytic enzymes by Karling was discovered in another group of fungi, the chytrids. This author could cultivate these fungi outside their natural habitats only on media containing keratin as sole source of nutrient. When hair was used the fungus invaded it through a single point of entry in the cuticle and then spread by means of rhizoids through the cortex and medulla. Zoosporangia were formed inside the hair shaft and the pressure thus developed in the hair burst the cuticle setting free the zoospores.

Keratin of both human and animal origin eventually finds its way into soil where in course of time it disappears, presumably broken down by the action of soil microorganisms. Jensen (1932) in his review of the literature points out that as early as 1899 Pfeiffer observed that horn meal was a good source of nitrogen for plants, an indication of the existence of soil organisms capable of

degrading keratin. Jensen followed this lead and mixed samples of garden soil with horn meal predigested with pepsin and trypsin. Periodically, determinations of nitrate were made together with an investigation of microflora. Within twenty days there was a marked increase of nitrates in the sample mixtures while colonies of the Actinomycetales became more numerous. The fungus flora did not appear to be influenced by the presence of keratin in the soil. Two Actinomyces species were repeatedly isolated and successfully cultured on media with keratin as sole source of nitrogen. Jensen described one of these strains as Actinomyces citreus.

(b) Biochemical investigations.

MacFayden (1896) found that culture filtrates of Trichophyton tonsurans contained proteolytic enzymes. He was able to demonstrate the liquefaction of gelatin by such culture filtrates, however these filtrates did not show any action on hairs. When he grew this dermatophyte on granulated keratin he could not observe any perceptible digestion but the tested keratin became more soluble in alkali than native keratin. Apparently the enzymatic action of T. tonsurans induced alteration of the keratin molecule. Tate (1929) examined five dermatophytes, one yeast and two Aspergillus species for the presence of enzymes in their mycelia. Proteases, similar to trypsin, were found in all organisms tested. In addition, the presence of urease, lipase, maltase, diastase, and amygdalase was demonstrated but tests for keratin-splitting enzymes were negative.

Deaminase activity in the mycelia of Microsporum and Trichophyton species grown on standard laboratory media was studied by Bentley (1953) and Chattaway et al. (1954) The rate of reaction of the powder prepared from acetone dried mycelium was low. The authors concluded the deaminases formed in these mycelia were of no essential importance in the metabolism of amino acids.

Stahl and co-workers (1950**) studied the degradation of wool by the

action of Microsporum gypseum. The dermatophyte was grown on a medium containing only wool and mineral salts. The maximum digestion of the wool was reached in 14 days while the maximum liberation of soluble organic nitrogen into the filtrate occurred in 11 days. The total nitrogen in the wool residue at various stages of degradation by M. gypseum does not vary appreciably with time, whereas the sulphur values showed a constant increase with time of digestion, indicating a differential hydrolysis of wool protein. Wool is not significantly deaminated until the keratin molecule has been degraded to polypeptides and amino acids. The metabolic filtrate consisted of 36% organic and 60.4% inorganic nitrogen. The latter was found to be ammonia.

Daniels (1953) grew Microsporum canis on media with keratin as sole sourse of nitrogen or as the sole nutrient. The digestion of keratin was proved by paper chromatography of the hydrolized culture filtrates. Fifteen amino-acids normally occurring in keratin hydrolisate were identified in the first case. When the dermatophyte was grown on keratin alone the number of amino acids was smaller, only aspartic acid, glutamic acid, glycine, alanine, threonine, histidine, arginine, lysine, leucine, valine, and serine were found. It may be assumed that under these unfavourable conditions the fungus metabolized an additional number of amino acids.

Birds of prey such as hawks and vultures feed on smaller birds. They devour the entire prey, flesh, bones, feathers, claws and beaks digesting it relatively quickly. Stankovic et al (1929) fed a hawk with sparrows and crows. They pumped its crop several hours after feeding. A small volume of a yellowish liquid of pH 4.0 was obtained which was incubated with keratin particles. In 24 hours, the authors reported,visible digestion of keratin took place. Quantitative determinations showed considerable increase in amino nitrogen. Unfortunately their interesting work was not continued.

The most thorough research into the nature of keratinolytic action

was done on moths' larvae, Tineola biselliela, by Linderstrøm-Lang and Duspiva (1935). The larvae of the clothes moth feed voraciously on wool and pelts. A microscopic examination of periodically excised intestines of the larvae after they had fed on wool revealed all stages of gradual disintegration and dissolution of the consumed wool fibres in the middle intestine. The cortex of the hairs was the first to break up into smaller fragments and individual cells, all of which would eventually dissolve. The enveloping cuticle, being more resistant to digestion, collapsed last of all and finally could be traced no more. Examination of the excreta did not reveal any cuticle or for that matter any undigested keratin structures. A glycerine extract of the intestines was tested for presence of keratinase; the pH of the intestinal juice was found to be between 9.6 and 10.2. Although in vitro the intestinal juice had marked activity against casein, it had none against native wool. Colour tests made with the intestines of larvae that had just fed on wool gave a strong positive nitroprusside reaction thus indicating the presence of -SH groups. A negative reaction was obtained when the intestinal tract was empty. With full intestines, however, after their exposure for a few minutes to the air, the nitroprusside reaction was also negative; this suggested disappearance of -SH groups or their possible oxidation to -S-S- groups. These observations led the investigators to believe that in the larval intestines keratin is reduced at one stage of the digestion, the reduction being expressed by the appearance of sulfhydryl groups. Since the nitroprusside reaction was negative or only very weakly positive in the absence of keratin it was clear that the reducing agent does not contain an -SH group; or it could be possible that the reducing agent is formed in the presence of keratin only. The first assumption was thought to be correct since methylene blue, injected into the intestine, turned colourless in the middle intestine only and remained in its oxidized state in its other parts. This observation together with the negative

nitroprusside reaction indicated that a reduction potential exists in the middle intestine. Apparently the H atom is transferred from a donor to the disulfide bridge of the cross linkage of cystine in the keratin molecule. The reducing system was regarded by the authors as a particular activator of the Tineola keratinase without which this keratinase cannot function. Duspiva (1936) examined the larvae of Galleria mellonella which normally does not feed on wool. The author made the larvae consume wool. However, it was found that the wool was not digested and keratinolytic activity could not be induced.

Canizares and Shatin found that 13 species of the dermatophytes and Candida albicans possess dehydrogenase activity as revealed by tests with 2, 3, 5-triphenyl tetrazolium chloride.

Blackburn digested wool with papain in sodium bisulphite solution. The latter reduced the keratin and made it more accessible to the action of papain. The undissolved residue consisted mostly of cortical cells and had a higher sulphur content than the intact wool from which it was derived, an observation also made by Stahl et al in 1949 and 1950** This observation suggests that the "intercellular" material contains less sulphur and is preferentially removed. Hydrolysis of the parts made soluble by the action of papain yielded nine amino acids: leucine, isoleucine, valine, alanine, glycine, threonine, serine, glutamic and aspartic acid.

The observed facts on the metabolic utilization of keratin allow the designation of certain features which appear to be characteristic of the process:

1. Keratin, with its normal quota of trace compounds, can support growth as the only source of nutrient or as the sole source of nitrogen. Organisms capable of utilizing keratin in this way were found among dermatophytic fungi, actinomycetes and chytrids and bacteria; also among birds of prey

and moth larvae.

2. The breakdown of keratin observed under experimental conditions is generally a slow one. Days and weeks may elapse before any products of keratin hydrolysis appear in measurable amounts. This applies chiefly to the action of microorganisms while animal breakdown of keratin is a faster process.
3. The pH level at which keratin utilization has been observed was at pH 4 in the case of birds of prey and around 10 in the case of clothes moth larvae. In fungal cultures the pH is usually between 5 and 6.
4. There are indications that the crucial point of keratinolytic activity centres around the reduction of the disulfide bonds of cystine in the keratin molecule. A chemically reduced keratin was found susceptible to action by papain.
5. Keratinolytic activities appear to be restricted to certain organisms only; attempts to induce them in other organisms not normally utilizing keratin, proved unsuccessful.

4. Studies on the metabolism of Candida albicans.

Diddens and Lodder studied 79 strains of C. albicans they had received from all parts of the world. All these strains were isolated from the human or animal body. Therefore, they regarded this species as a true parasite. Negroni and Fischer isolated C. albicans from vegetable matter and most recently, diMenna isolated this species twice from soil in New Zealand. She also found that C. albicans survived in experimental plots for a period of at least nine months. However, attempts to isolate C. albicans from the air by Nilsby and Norden were unsuccessful. They were only able to find the fungus near the bedside of a patient from whom throat swabs and sputa had grown C. albicans. Ashford isolated C. albicans from a loaf of bread in an endemic area of sprue in Porto Rico and believed that that fungus was associated with this condition.

Most of the work on yeast metabolism has been carried out with yeasts used in industry. Investigation of the metabolism of pathogenic yeasts has only been started in recent years as the result of greater incidence of mycotic infections by such pathogenic fungi.

It is generally accepted that Candida albicans does not ferment sucrose. However, van Niel and Cohen (1942) found that sucrose is fermented but much more slowly and less completely than glucose; there is only a quantitative difference in the fermentation of these two substrates. In all cases the gas produced is exclusively CO_2, more gas being produced in bicarbonate than in phosphate media, indicating the formation of acidic products in the former. Production of acids has never been reported. Under aerobic conditions C. albicans grows well on sucrose. When suspensions of C. albicans which have fermented glucose are subsequently exposed to air, there ensues a rapid O_2 uptake; apparently the ethanol, formed during the anaerobic phase is being used as oxidation substrate. While the oxygen uptake was observed to be high and constant over the entire period of ethanol oxidation, the rate of CO_2 production was subject to rather large fluctuations increasing with time. This suggests that oxidation of ethanol proceeds with accumulation of small amounts of an intermediate oxidation product. A temporary accumulation of acetic acid was observed. Further experiments showed that ethanol and acetic acid can be oxidized simultaneously by C. albicans but only after addition of thiamin. In this process, acetic acid formation may be observed.

The trend of the pH in a glucose-asparagine medium was followed by Norbring-Hertz. She found that the pH dropped from 5.5 to 4 and then returned to its original value. The author assumes that the turning point coincides with the exhaustion of glucose from the medium; however, determinations of glucose were not made.

Schopfer (1945) found C. albicans dependent on bios factors for the

utilization of some forms of organic nitrogen. He did not obtain growth on a synthetic medium consisting of glucose, asparagine and mineral salts. Addition of biotin enabled growth on this medium. Thiamin could not replace biotin but had a synergistic effect. Wickerham not only confirmed Schopfer's findings but also found the dependence on bios factors in nine other Candida species. This author also observed that ammonium sulphate and urea may only serve as a source of nitrogen when the bios factors are present. Urea was found to be toxic if present in high concentrations.

McVeigh and Bell investigated the nutritional requirements of one strain of C. albicans. This strain (Y 475) failed to grow on media containing asparagine and ammonium sulphate as sources of nitrogen even in the presence of vitamins. It was clearly deficient in methionine since it produced poor growth on a medium containing eighteen amino acids except methionine. Sulphur compounds could not replace methionine. However, methionine was able to replace casein hydrolysate. The observations that a strain of C. albicans is deficient in methionine contradicts Foster's general statement that no fungus has an absolute requirement for a specific amino acid. In his opinion, such deficiency could only occur in absence of vitamins which, however, would not hold true in this case.

These observations are not in agreement with the results obtained by Johnson working with another strain of C. albicans. The yeast was grown in Czapek solution in which sodium nitrate was substituted by single amino acids. Twenty-three amino acids, including asparagine, were tested. Growth was measured photometrically and obtained with all 23 compounds although no vitamins or trace elements were added. The mixture of all twenty-three compounds produced the best growth. Of the single amino acids phenylalanine, arginine, glycine, glutamic acid, tyrosine, leucine and asparagine induced the best growth. Hydroxyproline in large amounts did not produce any inhibitory effect.

Norbring-Hertz studied the growth of C. albicans in a glucose-asparagine medium in respect of vitamin and metallic ion requirements. Her strain could utilize asparagine as the sole source of nitrogen in the presence of biotin, iron, manganese and zinc only.

The two growth forms of C. albicans as seen by Robin in the white patches of thrush are also found on culture. No complete explanation of the dimorphism of the fungus can be given yet. However, the investigations of many authors indicate that different ecological factors can induce C. albicans to form filaments instead of producing unicellular structures only. Anaerobiasis, CO_2 atmosphere, starvation, replacement of sugars by poly-saccharides as C- sources, elevated temperatures, high pH values of the medium, low surface tension are some of the ecologic factors which have a morphogenetic effect on the growth of C. albicans. Williams tested C. albicans for its keratinolytic activity. He included C. albicans and Scopulariopsis brevicaulis in his experiments with dermatophytes and claimed that he obtained growth of C. albicans on hairs placed in sterile water.

Vanbreuseghem (1949) also tested C. albicans for its keratinolytic activities but could not confirm the findings of Williams. He placed a piece of agar bearing a colony of C. albicans on a stretched hair. Growth of the yeast phase was observed at a small distance from the point of inoculation only. However, the yeast failed to invade the hair, nor did it produce any damage to the hair.

Two researchers from Vienna, Ehrmann and Wiedmann report a survey in 1927 which revealed that diseases of the nails presented 0.13% of all cases in dermatological clinics. During the years 1946-1950 fungus infections of the nails alone amounted to 0.85% of all skin cases seen in the University Clinic in Vienna. Ehrmann and Wiedmann isolated C. albicans from almost all these cases. These figures correspond closely with observations made in this

laboratory. Ehrmann and Wiedmann were aware of the fact that the pathogenicity of C. albicans for keratinized tissues had never been proved and therefore decided to investigate this question by animal experiments. Suspensions of strains of C. albicans isolated from nail infections were injected into the nail bed of 97 rats. Although a number of animals were put on vitamin free diet and dystrophy had been caused by severing interruption of nerves only 19 or 97 rats showed alterations of their nails. Since these experiments were regarded as inconclusive channels were drilled into the nails of 24 guinea pigs and injected with suspensions of C. albicans. Six weeks after the injections, alterations in the nails of 15 guinea pigs were observed. The nails became lustreless, yellowish and brittle and resembled those seen in cases of nail moniliasis of man. Hyphae were found in the nail scrapings and on culture C. albicans was found.

The results obtained by Ehrmann and Wiedmann are hard to evaluate and, for several reasons, not convincing.

The above examination of literature reveals a good deal of clinical evidence of the pathogenicity of C. albicans for mucous membranes and many reports of the occurrence of this yeast on keratinized tissues. However, it reveals very little, in fact practically no information about the chemical character of its possible pathogenic activity on skin and skin appendages.

The work, about to be presented, on the growth in vitro of C. albicans on keratin as sole source of nitrogen, it is hoped, will satisfy to some extent the need in this respect. Although the purely chemical investigation of this subject cannot provide a complete answer to the question of pathogenicity, it has been considered a sound basis for future work in this field.

II. METHODS.

1. Preparation of keratin.

Several workers have found that grinding in a ball mill produces changes in the chemical composition of keratin: the finer the powder the more pronounced the change. After 125 hours of grinding in a ball mill an appreciable loss of N and S occurred; the amount of tryptophan, tyrosine and cystine in keratin decreased. Powdered keratin was found accessible to some degree to the action of common proteolytic enzymes. Milling apparently has an oxidizing effect, since water extract of powdered keratin was found to contain inorganic sulphates (Routh and Lewis,1938; Routh, 1940; Olcott, 1943; and Cohen,1944). Therefore keratin used in the experimental work was ground for a few hours only.

Selected cattle hooves, fresh from the abbatoir were carefully trimmed, washed with water and scraped with a scalpel, or if necessary, with bench grinder to remove any impurities. Hooves cleaned in this manner were wrapped individually and stored in the refrigerator until required for filing. Filing was performed by hand with a rasp, from the hard outer layer only (unguis). The softer keratin (subunguis) was not used since it consists of a different type of keratin with a lower S-content (Olcott). Only a relatively thin layer of the hard keratin was filed down thus making sure that no unkeratinized layers were included in the sample. The filings, partly air dried in the laboratory, were then ground in a ball mill, with smooth stones, for two hours only. The keratin was then sifted. The fraction which passed through a 100 mesh sieve was rejected being too fine, whereas the coarser material collected on a 40 mesh sieve was ground for another two hours and fractionation was repeated. Most of the ground keratin obtained was in the 40 to 100 grade mesh. The keratin was then washed with water, and the first liter used for 150 gms. of keratin became murky; when filtered, it gave a

positive ninhydrin test. Washing was repeated using one liter of fresh water at a time. This produced a homogeneous fraction of granular keratin which after a final rinsing with distilled water did not give any reaction with ninhydrin. The keratin was then collected on a Buchner funnel, spread on a tray, dried in an oven at 50°C. for a week and stored in closed jars at room temperature.

The removal of any watersoluble compound giving a positive ninhydrin test is of utmost importance because such substances with α-amino nitrogen can well serve as source of nitrogen for the fungus. Their presence would render any results inconclusive.

Cuttings of human nails were washed thoroughly with soap and water and dried at room temperature. Since 2 hour grinding had no effect on the size of the nail cuttings they were comminuted with scissors, and subsequently washed with 0.1%/sodium taurocholate and extracted with ether and acetone.

2. Media.

The first tests as to the availability of keratin as sole source of nitrogen for C. albicans were performed on a solid medium (I) consisting of -

glucose	2%
$MgSO_4 \cdot 7H_2O$	0.05%
KH_2PO_4	0.1%
Washed agar	2%
keratin	0.5%

The keratin powder was prepared from cattle hooves and ground in a ball mill for 12 hours. The keratin was not washed.

The composition of the medium in the preliminary experiment was as follows (medium II) -

glucose	2%
$MgSO_4 \cdot 7H_2O$	0.05%
KH_2PO_4	0.1%
hoof keratin	1%

To secure a good growth of the fungus vitamins as described below were added:

biotin	2 µg.
thiamine	400 µg.
inositol	2000 µg.
water	10 ml.

According to the procedure of Lodder and Kreger-van Rij, 1 ml. of this concentrate was added to each 100 ml. of medium.

The keratin used in the preliminary experiment was obtained from both white and streaked hooves. Preliminary washing of the keratin with water was not as thorough as it was in the main experiment.

In the main experiment, 0.4% of keratin obtained from white hooves and 0.3% of nail keratin were used (medium III).

The experiments were set up in 1 liter Erlenmeyer flasks, containing 500 ml. of medium in the preliminary, and 300 ml. in the main experiment.

Experiments with nail cuttings were made with 250 ml. of medium.

In the first experiment, the Erlenmeyer flasks were plugged with cotton wool. A considerable reduction of the volume occurred through evaporation. Therefore, in the main experiment rubber stoppers, each fitted with a glass tube of 1 cm. in diameter and plugged at one end with cotton wool, were used. In this way evaporation was lessened markedly and only negligible loss of water was observed. Only 10 ml. of water had to be added towards the end of four months to restore the original volume.

Sterilization of keratin presented a major technical problem. It

was most desirable to avoid heat sterilization, however the use of chloroform was not successful. Keratin treated with ethylene oxide resulted in sterile material, but thus treated keratin was found to be chemically altered (Phillips). This finding was corroborated in the present experiments when such keratin, used as a source of nitrogen, did not support the growth of C. albicans.

In the preliminary experiment keratin was sterilized by autoclaving in distilled water. Since the water showed a positive, but very weak ninhydrin test after sterilization, the keratin was washed twice under sterile conditions. The last sample of water did not give a positive ninhydrin reaction. The separately sterilized remaining constituents of the medium were then added to the washed keratin.

Unfortunately, contamination was rather high. In the main experiment, preliminary washing of keratin with water was more often repeated, as described before. As a result, the ninhydrin test of the water in which the keratin was sterilized was negative after autoclaving. Furthermore, glucose was sterilized separately. Due to these precautions contamination was very low in the main experiment.

3. Inoculation and incubation.

C. albicans, strain # 544, isolated from infected nails was used in the experiments. The inoculum was prepared from a 48 hour old culture on solid yeast medium (1% peptone, 2% cerelose). The growth was washed off the slope with distilled water, centrifuged, resuspended in water and centrifuged again. A suspension with a light transmission of 13.5% (Filter # 660, Evelyn Photoelectric Colorimeter) was prepared: 1 ml. of the suspension was used as inoculum per 100 ml. of medium.

All flasks were incubated at 37°C.

The inoculum used in the preliminary experiments was derived from

the freshly isolated strain. To preserve its presumed keratinolytic properties the strain was subcultured repeatedly on a medium containing 1% keratin, 2% cerelose and 2% agar. 13 transfers were made in the course of a year and each time the yeast produced good growth on this medium. After the strain had been grown on keratin medium for eighteen months it was used as a source of inoculum for the main experiment. Due to unavoidable circumstances the inoculum was not as heavy as in the foregoing experiment, its light transmission being 54%.

4. Investigative methods.

The cultures were observed closely and any changes noted.

As will be reported later C. albicans, in its mycelial phase, became closely entangled with the keratin particles under the conditions of these experiments. It was found difficult to separate the fungus growth from the keratin. Therefore it was decided to investigate the culture filtrate and determine, if any, the nitrogen containing degradation products of keratin.

In the preliminary experiment sampling of the culture fluid was performed every week. 20 ml. of the liquid were withdrawn and replaced by fresh medium. The sample was Seitz filtered and kept in the refrigerator.

In the main experiment every two weeks the entire contents of an Erlenmeyer flask were filtered in the same way and stored in the cold.

Two to three days before a sampling was performed subcultures of flasks to be sampled were made in order to determine the purity of growth or the sterility of uninoculated controls.

The preliminary experiment consisted of the pH determination, ninhydrin reaction and determination of total soluble nitrogen in the filtrate. The main experiment further included determination of α-amino nitrogen, and glucose, and paper chromatography of amino acids in the hydrolized and

unhydrolized filtrate.

A Beckman Glass Electrode pH Meter H2 was used for the measurement of the hydrogen ion concentration.

The ninhydrin test was performed with 0.2% ninhydrin (Hawk, Osler and Summerson).

Glucose was measured by the method described by Benedict in 1911 in samples taken from flasks with media containing glucose.

Total soluble nitrogen was determined in 5 ml. filtrate by the Micro-Kjeldahl method, using the Parnass-Wagner apparatus.

Estimation of α-amino nitrogen and paper chromatography of amino acids were performed on samples giving a positive ninhydrin test. For these determinations it was found necessary to concentrate the filtrate five times.

The Van Slyke Blood Gas Apparatus (Fisher Scientific Company) was used for the assay of α-amino nitrogen (Van Slyke, Dillon, MacFadyen and Hamilton, 1941).

The identification of amino acids by paper chromatography was largely based on methods described by Block (1950) and by Block, Durrum and Zweig (1955).

5 ml. of the concentrated filtrate were decolourized with activated charcoal and desalted by adsorption on a column of an artificial resin, Amberlite IR-120 (H form) made by Rohm and Haas (Philadelphia). The elution was performed with 3% NH_4OH. The eluate was evaporated to dryness in vacuo and the residue dissolved in 0.5 ml. of 10% isopropanol.

Another sample, 10 ml. of the concentrated filtrate, was evaporated in vacuo and refluxed with 6N hydrochloric acid for 20 hours. The hydrochloric acid was driven off by evaporation in vacuo. The sample was decolourized and desalted in the same way with artificial resin as described above.

30 ul. of the so prepared sample, now concentrated 50 times in relation to the original filtrate, were applied to large sheets (18¼" x 22½") of filter paper Whatman No. 1. Descending procedure in Shandon type chromatographic chambers was used. 80% phenol was used as the first solvent in an atmosphere of ammonia in the presence of NaCN. The second solvent consisted of:

70 parts absolute tertiary butanol

15 " 90% formic acid

15 " water (double distilled)

as recommended for paper chromatography of sulphur containing amino acids by Block.

All chemicals used were of Analar grade as supplied by the British Drug House.

Both the first and the second solvents required at least 42 - 45 hours to migrate to the bottom edge of the sheets.

The papers were developed with 0.25% ninhydrin in acetone.

The identification of amino acids was established by comparison with a map prepared with standard amino acids (Cave and Co. Ltd., Toronto) as found in hydrolysates of keratin. The map had been established previously by using the same technique. The identification was confirmed by repeating the chromatography of the sample to which the tentatively identified amino acids had been added in pure form.

III. RESULTS.

1. Growth of C. albicans on solid medium (I) with keratin as sole source of nitrogen.

On this medium an abundant growth of C. albicans was obtained within three to five days. Whereas a growth of the yeast-like phase was observed on the surface of the medium, growth of the mycelial phase occurred in the agar, below the yeast-like colony. However, the filaments did not seem to be directed towards the keratin particles.

When keratin scrapings were scattered on the surface of the same medium growth of C. albicans did not differ. In this experiment, too, the presence of keratin did not influence the direction of filamentous growth.

These cultures, kept at 37°C, were observed up to four weeks at which time the media dried out. Since no visible change of the keratin particles could be observed by this method, it was decided to investigate the character of growth of C. albicans and its influence on keratin as sole source of nitrogen in liquid media.

When C. albicans was cultured on medium I without agar an abundance of both growth forms was obtained. Microscopic examination revealed pronounced filament formation around keratin particles. When first tested after seven days, the ninhydrin test was negative, but became positive after six to seven weeks.

2. Preliminary experiments.

Preliminary experiments were arranged in the following way (Table I):

TABLE I.

Composition of media and arrangement of the preliminary experiment.

Culture 1	keratin	salts	glucose	C. albicans
" 2	keratin	salts	glucose	-
" 3	keratin	-	-	C. albicans
" 4	keratin	-	-	-
" 5	-	salts	glucose	C. albicans
" 6	-	salts	glucose	-
" 7	-	-	-	C. albicans

Submerged filamentous growth in culture No. 1 started within two to three days. Gradually, this growth form gave way to the unicellular yeast phase. The culture turned yellow after five weeks, indicating the formation of flavins as already observed by Levine et al in cultures of C. guilliermondii and C. flaveri. The ninhydrin test turned positive after four weeks' culturing, being weak at first and deepening gradually. The pH dropped slowly from its initial level of 6.1 to 4.5 within five weeks, and rose again to a value of 5.0 - 6.0.

Weekly determinations of the total nitrogen in the filtered culture liquid showed an increase from 0.002 mg. N/ml. to 0.12 mg. N/ml. within three months. Then the rate of increase of the total soluble nitrogen diminished. At the beginning of the fourth month glucose was added, raising the concentration in this flask back to 2%. After the addition of glucose the amount of total soluble nitrogen in the medium fell by 20% and gradually increased within the two following weeks at twice the rate as compared to the parallel period just prior to the addition of glucose. Although the rate of increase finally slowed down, 0.1956 mg. N/ml. were found in the filtered culture medium after seven months.

Cultures No. 3 and 7 did not show any visible growth, however, the organism was found viable on subculturing.Some growth of C. albicans (yeast phase) was observed in culture No. 5 which lead to a thorough supplementary experiment, to be discussed later. When glucose was added to culture No. 3 after three months filamentous growth started within a week.

The ninhydrin test in culture No. 2 became and remained slightly positive after three months. Culture No. 3 began to show a positive reaction three weeks after the addition of glucose. The test remained negative in cultures No. 4 - 7.

Total soluble nitrogen was not found in measurable quantities in cultures No. 2, 4, 5, 6, 7. The same holds true for culture No. 3 until the addition of glucose. Seven weeks after the addition of glucose 0.03 mg. N/ml. were found in the filtered culture medium.

The supplementary experiment mentioned earlier was devised to examine the growth of C. albicans in a nitrogen-free medium II. Six different experiments were run. The growth of C. albicans was measured photometrically and the results obtained are presented in Figure 1.

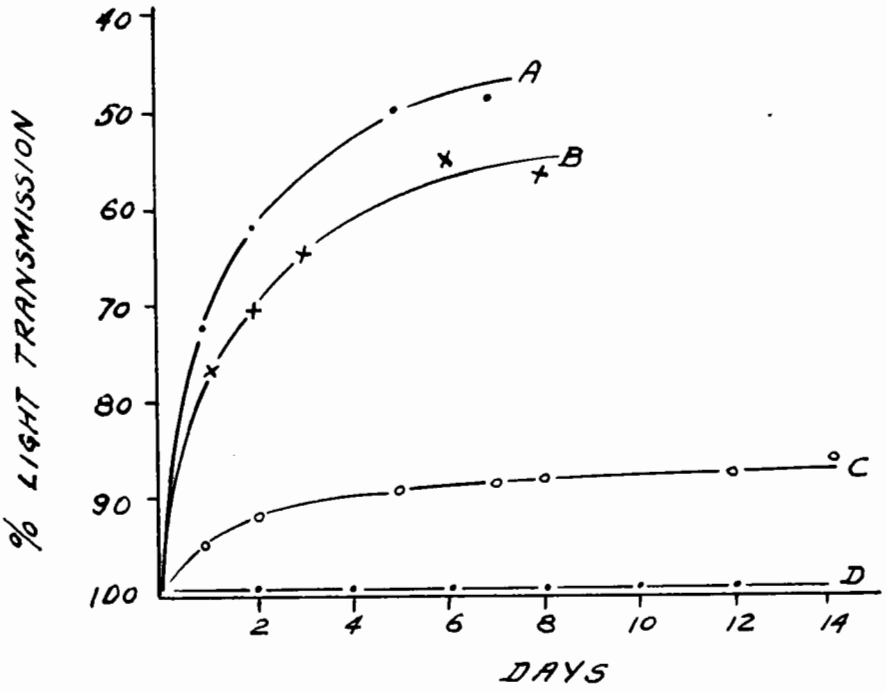


Fig. 1. Growth of C. albicans in four different media.

A. salts, glucose, and 0.3% ammonium sulphate as source of nitrogen,

B. salts, glucose, and filtrate obtained by autoclaving 1% keratin in dist. water,

C. salts and glucose (no nitrogen),

D. salts, with organic and inorganic nitrogen (no glucose).

The results of the experiments showed that C. albicans - excluding a negligible initial development - cannot grow on a nitrogen deficient medium.

3. Main experiment.

Based on the experience gathered from the preliminary investigations and to enlarge and improve the scope of these studies, the main experiment was arranged as follows (Table II):

TABLE II.

Composition of media and arrangement of the main experiment.

Series 1	keratin (hoof)	salts	glucose 2%	C. albicans
2	keratin (hoof)	salts	glucose 4%	C. albicans
3	keratin (hoof)	salts	glucose 8%	C. albicans
4	keratin (hoof)	-	glucose 2%	C. albicans
5	keratin (hoof)	salts	-	C. albicans
6	keratin (hoof)	-	-	C. albicans
7	keratin (hoof)	-	-	-
8	keratin (hoof)	salts	glucose 2%	-
9	keratin (nail cuttings)	salts	glucose 2%	C. albicans
10	-	salts	glucose 2%	C. albicans
11	-	-	-	C. albicans
12	ammonium sulphate, 0.3%	salts	glucose 2%	C. albicans

Each series of this experiment consisted of twelve flasks. Observations of the growth of C. albicans and the chemical alterations of the filtered culture liquid were made every two or three weeks during a period of four months. The results will be presented in the following order:

1. Visual and microscopic observations on growth.
2. Study of pH changes.
3. Ninhydrin reaction.
4. Determination of total nitrogen.
5. Determination of residual glucose.
6. Determination of α-amino nitrogen.
7. Identification of amino acids by paper chromatography.

Visual and microscopic observations on growth.

Series No. 1 produced growth of unicellular structures and numerous delicate mycelial tufts clinging to the surface of keratin particles towards the end of the first week. Similar tufts were seen under the surface. Fermentation produced bubbles of gas which could be observed at the surface.

On the 26th day of the experiment tufts were numerous both along the walls of the flask and at the surface. Most of the growth appeared to be associated with the keratin particles. This phenomenon became even more conspicuous on the 40th day. At this time growth at the surface formed a dense mat which, either under its own weight or when disturbed, fell to the bottom, only to be replaced by a new mat mainly consisting of filamentous growth in the beginning. After four months the keratin was covered with a uniform blanket of white tufted growth and some yeast colonies.

The liquid, colourless and clear at first, showed by the 40th day a slight yellowish tint which turned distinctly yellow on the 54th day deepening with time to a strong greenish yellow colour.

In series No. 2 growth was characterized by fewer tufts and the formation of finer growth covering the keratin particles. On the 26th day growth covering the keratin was more abundant and consisted of both growth forms. Floating tufts made only an insignificant increase in size and number and did not form a mat. Around the 110th day growth in these flasks

started to change in appearance: billowing masses of filaments started to develop in the liquid and increased in size.

Growth in the series No. 3 started predominantly as the yeast-phase producing a light, uniform layer covering the keratin particles. Only isolated tufts were seen in some flasks. The growth covering the keratin became thicker than in series No. 2 but no change was observed between the 110th and 144th day of the experiment.

Growth in series No. 4 started slowly as the yeast phase. Later clumps of filaments were seen on the walls. At the 26th day luxuriant filamentous growth developed in the liquid. The light billowing masses, apparently having no contact with keratin, increased in volume. On the 68th day three quarters of the liquid were occupied by this growth and on the 110th day it had spread through the whole liquid. Growth at the surface was never observed.

Series No. 5 and 6 produced no visible growth.

Series No. 7 and 8 remained sterile. The colour of the keratin in No. 8 became a light tan while it remained unchanged in No. 7.

Growth in series No. 9 started as yeast phase mainly. No growth at the surface nor the formation of gas bubbles were observed in the beginning. Large loose filamentous masses similar to those seen in series No. 4 appeared in the liquid on the 40th day. While the floating filamentous growth increased, the thin layer covering the keratin particles did not thicken conspicuously. On the 68th day the appearance of floating tufts at the surface was observed; these had formed a dense mat by the 134th day of the experiment. The loose billowing mycelium then filled half of the liquid. A pale yellow colour developed at the end of the fourth month.

In series No. 10 a perceptible growth was observed on the bottom of the flasks. This bottom growth did not increase during the time of the

experiment. No other forms of growth were observed.

No change from an initial slight opacity became visible in series No. 11.

The most vigorous growth started in series No. 12. The yeast-phase grew on the bottom. Abundant filamentous tufts were formed along the walls and at the surface where they formed a ring. When the ring fell to the bottom another one formed in its place. Profuse new filamentous growth appeared on the folds of the fallen ring, a feature not observed in series No. 1. The bottom growth changed slowly into the filamentous phase and appeared as small granules, while delicate filamentous masses billowed in the liquid. The yellow colour appeared at the same time as in series No. 1.

Microscopic examinations of samples of growth were made on five successive occasions and, although a loopfull or one entanglement on a needle can hardly be considered representative of the whole culture, nevertheless a consistency of certain features was observed: samples of growth from series No. 1, 2 and 3 were predominantly in the form of a mixture of both yeast cells and short filaments, all elements being distinctly big and stout, indicating favourable ecological conditions. Series No. 3 was characterized by the predominance of the yeast phase. In series No. 4 and 9 every mount showed an entanglement of long, thin filaments accompanied by very few yeast cells. Series No. 12 presented a different picture: masses of long and short thin filaments interspersed densely with abundant yeast phase cells, the latter having all the appearances of poorly nourished, fragile elements. Chlamydospores were seen in most of the mounts.

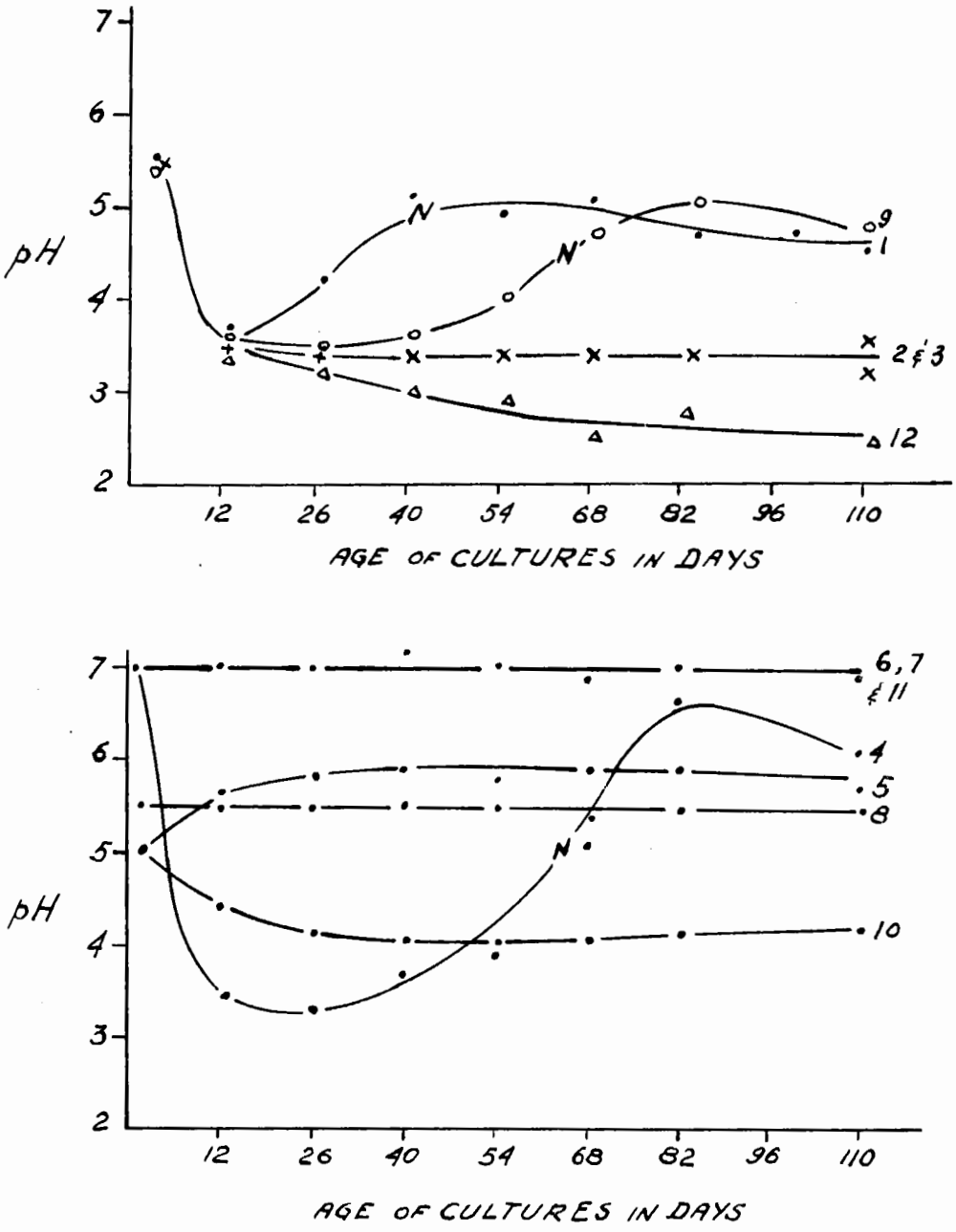
Sterility and viability tests were repeatedly made. No other growth than that of C. albicans was ever obtained. Series No. 7 and 8 remained sterile. The inoculum of series No. 11 remained viable although no active growth took place.

Studies of pH changes.

The pH values of the media prior to inoculation were as follows:

Series No. 1, 2, 3, 8 and 9	4.45 - 5.50 (Keratin-salts-glucose)
Series No. 4, 6, 7 and 11	7.0 - 7.35 (media without salts)
Series No. 5, 10 and 12	5.0 - 5.1

Figure 2 shows the changes of pH occurring in the media.



(N = ninhydrin reaction becomes positive)

Fig. 2. pH changes in the 12 series of the main experiment.

A drop of the pH was observed in all media on which fungus growth took place including series No. 10 where only an initial and limited growth developed. All these media contained glucose. The pH fell to an approximately low value of 3.4 - 3.7 within the firstten days irrespective of glucose concentration. In series No. 10 alone the pH ceased to drop at 4.4. A comparison of the notes on visual observations will reveal that this early phase was characterized chiefly by the development of the yeast-like growth form.

The initial drop of pH was followed by

1. An immediate rise in series No. 1.
2. A delayed rise in series No. 4 and 9.
3. A stabilization on the same level in series No. 2 and 3.
4. A further drop to pH 2.5 in series No. 12.

Series No. 1 and 9 reached the same maximum at pH 5.0; in series No. 4 the pH rose to 6.2.

In series No. 5 pH changes were observed although no growth took place.

Ninhydrin reaction.

Positive ninhydrin reactions indicative of the presence of α-amino nitrogen in the culture liquids were obtained in series No. 1, 4, 9 and 12. The first appearance of the positive reaction and its subsequent intensity are listed in Table III.

TABLE III.

Appearance and approximate intensity of ninhydrin reaction.

	Days after inoculation								
	0	13	26	40	54	68	84	110	144
Series 1				x	x	xx	xxx	xxx	xxx
2									
3									
4						x	x	x	x
5					(x)	(x)			
6									
7									
8									
9						x	x	x	x
10									
11									
12				x	x	xx	xxx	xxxx	xxxx

Series No. 1, 4 and 12 gave a blue-purple colour whereas in series No. 9 a red-purple shade appeared. The remaining series did not give a positive ninhydrin test except in series No. 5 where a faint positive reaction was observed on two occasions only.

Determination of total nitrogen.

The determination of total nitrogen in the filtered culture medium by the Micro-Kjeldahl method gave the following results (Table IV).

TABLE IV.

Total nitrogen in µg. per ml. in the filtered culture media.

	Age of cultures									
	0	13	26	40	54	68	84	99	110	134
Series 1	6.2		0.0	7.8	26.6	22.6	27.4	38.2	46.1	54.8
2	7.8	3.7	0.0	5.4	15.4	4.6	3.4		9.7	5.4
3	10.6	6.1	0.2	3.8	11.8	5.0	12.2		10.1	5.4
4	7.0	6.1	0.0	7.4	7.0	3.4	19.8		12.5	10.2
5	6.0	8.1	0.0	11.0	11.8	2.6	3.4		5.3	6.4
6	6.2	9.3	9.4	19.2	9.8	6.6	6.2		8.9	6.4
7	3.4	7.3	0.0	6.2	7.8		3.8		5.7	3.2
8	1.4	8.9	1.8	0.6	7.8	1.4	6.2		4.5	6.8
9	0.8	8.9	0.0	1.8	1.4	0.2	4.6		4.9	
10	0.0	0.2	5.4	0.0	0.0	0.0	0.0		0.2	6.6
11	4.4	0.0	0.2	0.0	0.0	0.0	0.0		0.0	3.4
12	635.3	636.9	609.4	598.6	586.6	587.0	598.6		542.5	550.0

The increase in total nitrogen in series No. 1 started about the 54th day of incubation. It will be recalled that the ninhydrin reaction became positive around the 40th day. In series No. 4 the increase in nitrogen also lags behind the first positive ninhydrin reaction, while in series No. 9, in spite of a positive ninhydrin reaction there was no measurable increase in total nitrogen. Values obtained in series No. 2 - 9 fell within the limits of experimental variations.

Results obtained in series No. 12 in which ammonium sulphate was the source of nitrogen for C. albicans differed from the other ones. A decrease in total nitrogen took place indicating the uptake of nitrogen by the fungus.

Assuming that the soluble N was derived directly or indirectly from

keratin, it is interesting to consider the values of soluble nitrogen as a percentage of the nitrogen initially present in the medium in the form of keratin. Series No. 1 - 8 contained initially 192 mg. N; series 9 contained 120 mg. N, while series 12 contained 191 mg. N (Table V).

TABLE V.

Total nitrogen in filtered culture media as percentage of the initial keratin nitrogen

	0	13	26	40	54	68	84	99	110	134
Series 1	0.97		0.0	1.22	4.16	3.53	4.28	5.91	7.18	8.54
2	1.22	0.57	0.0	0.84	2.41	0.72	0.53		1.51	0.83
3	1.65	0.95	0.03	0.59	1.85	0.78	1.91		1.56	0.83
4	1.09	0.95	0.0	1.16	1.09	0.53	3.09		1.92	1.59
5	0.93	1.30	0.0	1.72	1.85	0.40	0.53		0.83	0.99
6	0.97	1.45	1.45	3.09	1.53	1.03	0.97		1.39	0.99
7	0.53	1.14	0.0	0.97	1.22		0.59		0.88	0.50
8	0.22	1.41	0.28	0.09	1.22	0.22	0.97		0.70	1.06
9	0.16	1.83	0.0	0.37	0.29	0.04	0.96		0.99	
10	)									
11	) no keratin in medium									
(12	99.78	100.04	95.69	94.01	92.13	92.19	94.01		85.17	86.38)

On the 99th day, glucose was added to two flasks of series No. 1 raising its concentration to 2%. Subsequently , these two flasks were sampled by removing 11 ml. of liquid at weekly intervals. Both cultures produced a new abundant growth of the yeast-phase after the addition of glucose. There was no deepening of the yellow colour of the liquid.

The changes occurring in these flasks after the addition of glucose (pH; presence of α-amino nitrogen; quantity of total soluble nitrogen) were studied and are presented in Table VI.

TABLE VI

Changes occurring in cultures of series No. 1 after addition of glucose.

	pH	Intensity of ninhydrin reaction	µg. total N/ml.	µg. N/ml. in series No. 1
Prior to addition of glucose	4.6	+++	38.2	38.2
Glucose added				No glucose added
After 1 week	2.9	-	44.2	46.1
" 2 "	3.3	(+)	60.0	
" 3 "	3.9	+	41.0	
" 4 "	4.3	++	32.5	
" 5 "	4.3	++	37.5	
" 6 "	4.2	+++	40.6	54.8
" 7 "	4.3	+++	45.0	
" 9 "	4.2	+++	50.0	

The pH first dropped to pH 2.9 and then rose gradually to 4.3 after the fourth week. The pH remained at this level during the following five weeks, failing to resume the original level of series No. 1 again.

The ninhydrin reaction became negative in the first week and then

slowly began gaining in intensity. After six weeks it reached its original intensity.

The initial high rate of increase in total nitrogen was followed by a sharp decrease after two weeks, and a rise again after four weeks (Fig. 3).

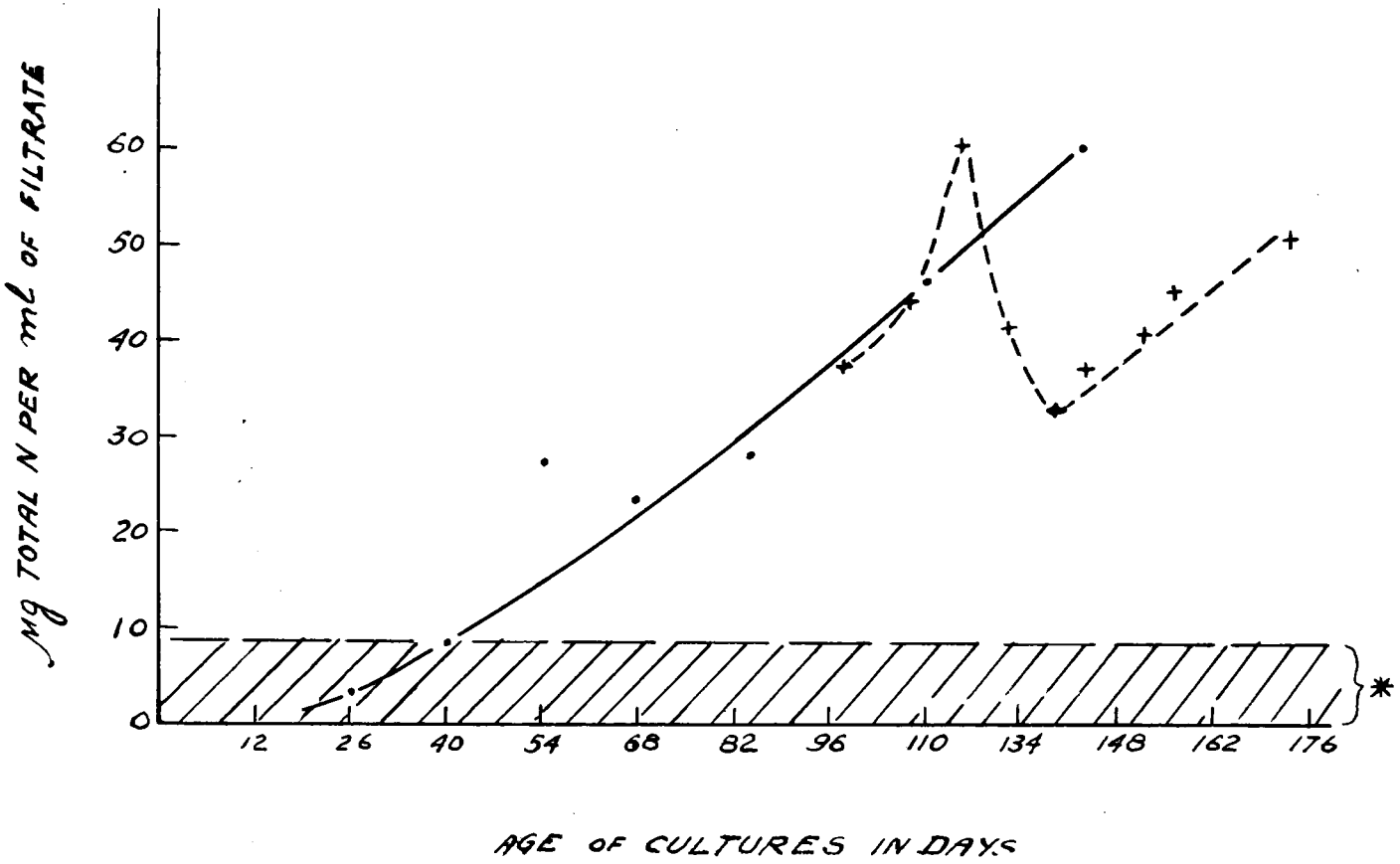


Fig. 3. Changes in total N in culture liquid in series No. 1.
(glucose added on the 99th day; - - -)

* Total N in culture liquid in series 2 - 9 incl.

Determination of α - amino nitrogen.

Determinations of α-amino nitrogen were done on concentrated filtered culture media which gave a positive ninhydrin test. The values found are listed in Table VII which also contains the values of total nitrogen. The amounts of α-amino nitrogen in percentage of the total nitrogen were calculated for series No. 1 and 12 only, since the very low values obtained in the remaining series would tend to be misleading.

TABLE VII.

∝-amino nitrogen in µg/ml. in culture media.

	Age of cultures in days	∝-amino-nitrogen in µg/ml.	Total nitrogen in µg/ml.	∝-amino nitrogen in % of total nitrogen
Series 1	40	2.9	7.8	38.4
	54	4.0	26.6	15.0
	68	4.6	22.6	15.8
	84	2.3	27.4	8.4
	110	2.7	46.1	5.8
	134	6.2	54.8	11.3
Series 12	40	2.7	598.6	0.45
	54	2.2	586.6	0.37
	68	2.2	587.0	0.37
	84	3.4	598.6	0.56
	110	3.0	542.5	0.57
	134	4.4	550.0	0.80
Series 4	68	1.7	3.4	
	84	0.5	19.8	
	110	1.6	12.6	
	134	1.7	15.2	
Series 9	68	0.3	0.2	
	84	0.9	4.6	
	110	0.9	5.0	
	134	1.3		

Paper chromatography of amino acids.

Amino acids were identified by paper chromatography in unhydrolized and hydrolized filtrates which gave a positive ninhydrin reaction. In addition, one sample of series No. 8 was chromatographed.

The spots obtained on chromatograms prepared from hydrolized samples were always distinct and dark, as compared with pale spots from unhydrolized filtrates. Since five to twenty µg. of single amino acids are required to produce distinct spots these findings are not surprising in view of the low values found for α-amino nitrogen in the unhydrolized filtrates, as shown in Table VII.

TABLE VIII.

Identification of amino acids in unhydrolized and hydrolized filtered culture media by paper chromatography.

Series No.	1				4				5				8				9				12			
Age of cultures in days.	A	B	C	D	A	B	C	D	B	C	D		B	C	D		B	C	D		A	B	C	D	A	B	C	D
	unhydrol				hydrol.				unhyd			hydrol	unhyd			hydrol	unhyd			hydrol	unhydrol				hydrol.			
									*				*				* *				*							
Alanine	x	x	x		x	x	x	x	x			x	x			x				x		x	x		x	x	x	x
Aspartic acid	x	x	x		x	x	x	x				x	x			x				x		x	x		x	x	x	x
Cystine							x	x				x											x				x	x
Glutamic acid	x	x	x		x	x	x	x				x	x			x				x					x	x	x	x
Glycine	x	x	x		x	x	x	x	x			x	x			x				x		x	x		x	x	x	x
Histidine	x		x			x	x	x				x	x												x	x	x	x
Leucine	x	x	x	x	x	x	x	x	x			x	x			x				x	x				x	x	x	x
Methionine sulfox			x				x	x																				x
Serine	x	x	x	x	x	x	x	x	x			x	x			x				x	x		x	x	x	x	x	x
Lysine								x																				x
Threonine	x	x	x		x	x	x	x				x	x			x				x					x	x	x	x
Tyrosine																						x						
Valine	x	x	x	x	x	x	x	x	x			x	x			x				x					x	x	x	x

A: 40 days
B: 54 "
C: 68 "
D: 84 "

* no spots obtained on chromatogram.

All chromatograms obtained were characterized by a similar pattern (see Table VIII). There was a consistent appearance of the following amino acids: alanine, aspartic acid, glutamic acid, glycine, leucine, serine, threonine, and valine. These recurrent spots are shown in Fig. 4 and Fig. 5. Other amino acids, as arranged in their frequency of occurrence, were histidine, cystine, methionine sulphoxide, lysine, and tyrosine.

Leucine, serine, and valine appeared on the first chromatogram of the unhydrolized sample of series No. 1 after 40 days of culturing; in addition, alanine, aspartic acid, glutamic acid, glycine and threonine appeared on chromatogram from the same sample after hydrolysis. It is interesting to see that upon second sampling, 14 days later, these five amino acids and hystidine appeared free in the culture liquid of this series.

Chromatograms obtained from unhydrolized samples of series No. 4, 5, 8, and 9 were either negative or showed pale spots of alanine, glycine, leucine, serine, and valine.

The chromatograms prepared from unhydrolized samples of series No. 12 were characterized by the consistent absence of valine.

Cystine was found on only one chromatogram prepared from an unhydrolized sample (series No. 12).

Determination of glucose.

The fate of glucose in the filtered culture media is presented in Table IX.

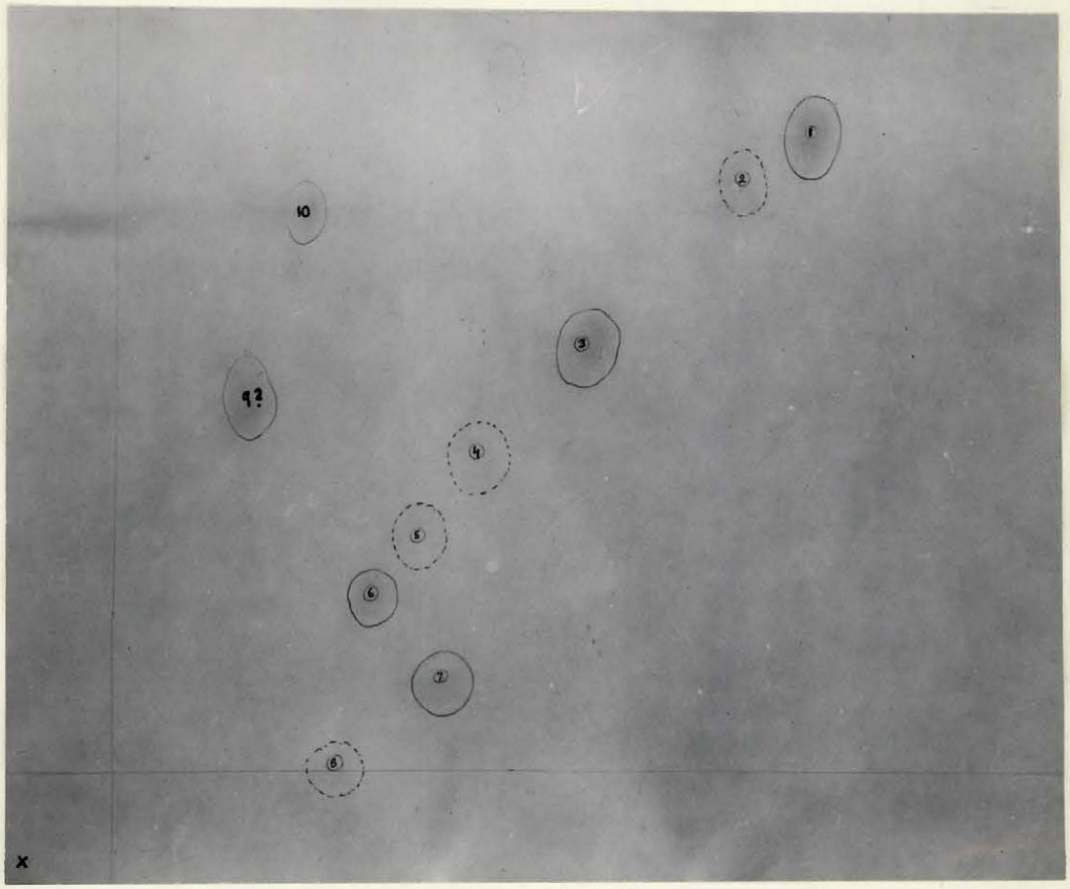


Fig. 4. Paper chromatogram of amino acids in the culture liquid of series No. 1 (84 days, unhydrolized).

1. Leucine
2. Valine
3. Alanine
4. Threonine
5. Glycine
6. Serine
7. Glutamic acid
8. Aspartic acid
9. Histidine
10. Methionine sulfoxide

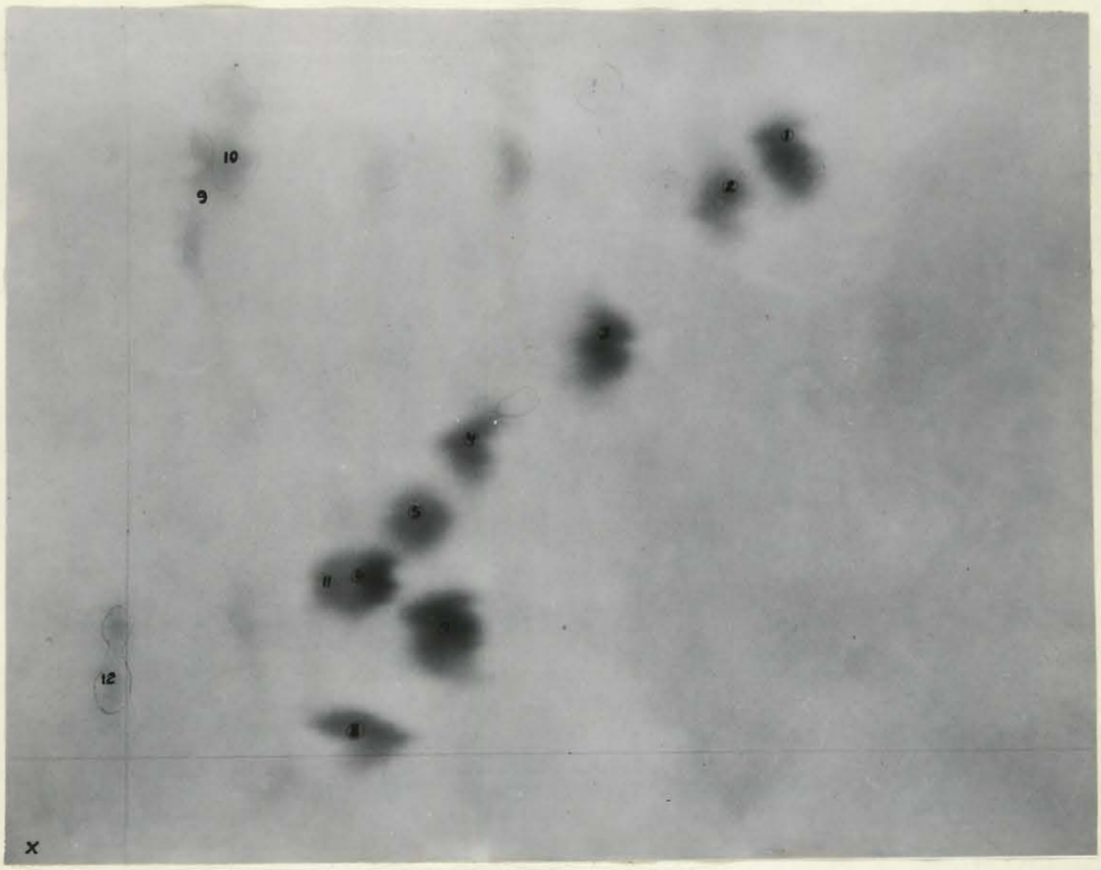


Fig. 5. Paper chromatogram of amino acids in the culture liquid of series No. 1 (84 days, hydrolized).

1. Leucine	5. Glycine	9. Histidine
2. Valine	6. Serine	10. Methionine sulfoxide
3. Alanine	7. Glutamic acid	11. Lysine
4. Threonine	8. Aspartic acid	12. Cystine

TABLE IX.

Glucose (%) in the filtered culture media.

	Initial value(%)	Age of cultures in days						
		26	40	54	68	84	110	134
Series No. 1	2	*	**	**	**	**	**	**
" 2	4	1.10	1.03	**	**	**	**	**
" 3	8	3.87	2.73	2.74	2.79	3.01	2.34	1.95
" 4	2	*	**	**	**	**	**	**
" 9	2	*	**	**	**	**	**	**
" 10	2	1.95	1.91	1.76		1.60		1.51
" 12	2	0.84	**	**	**	**	**	**

* Less than 0.62%

** Less than 0.31%

IV. DISCUSSION.

1. Evidence for the keratinolytic activities of C. albicans.

The purpose of this work was to obtain as much information as possible on the keratinolytic activities of C. albicans. The luxuriant growth of C. albicans in media containing keratin as sole source of nitrogen might be considered to prove these keratinolytic activities beyond any doubt. A possible objection to such an assumption is that C. albicans might grow on a nitrogen-free medium due to fixation of atmospheric nitrogen. A thorough review of the literature revealed only two investigations of fixation of atmospheric N by yeasts (Frei; Kossowicz). Both authors claim that some yeasts are capable of utilizing nitrogen from that source; however, C. albicans is not referred to in their papers. Since these experiments were performed without using labelled N the results must be considered inconclusive by modern standards (Wilson, 1952).

Observations of experiments described in Part III clearly indicate that under the conditions of these experiments C. albicans did not develop more than a negligible initial growth in a medium to which no nitrogen was added. These facts were observed in series No. 10 of the main experiment, in culture No. 5 of the preliminary experiment, and confirmed later in quantitative experiments in which growth was measured photometrically (Fig. 1). Therefore, it is to be concluded that growth of C. albicans in cultures results from nitrogenous compounds included purposely in the media.

However, it was observed that growth of C. albicans took place on agar plates containing powdered keratin and keratin scrapings, although the filamentous growth did not seem to be influenced by the presence of keratin particles. These early observations indicated that the growth of the fungus might be due to the water soluble nitrogen derived from keratin during the autoclaving. That such was indeed the case was proven by the appearance of

a positive ninhydrin test with filtrates from powdered keratin before autoclaving and a more pronounced one after autoclaving.

In both the preliminary and main experiments therefore, the greatest care was taken in the preparation of granular keratin to remove these fractions. Although the ninhydrin test was negative, small quantities of soluble nitrogen were detected in all media containing keratin, which amounted to an average of 8 µg/ml. This amount represents only 0.005% of hydrolysed protein and its role in a medium would be to support only a limited initial growth.

Therefore, it is to be concluded that good growth observed in these experiments was conditioned by the presence of keratin in series No. 1, 2, 3, 4, and 9 and ammonium sulphate in series No. 12.

Proteins may be hydrolysed by the action of hydrogen ions. The scleroproteins, such as keratin are more resistant to the action of acids than the other proteins. The pH in all keratin containing media which supported growth of C. albicans was on the acid side. Therefore the question might be raised whether the breakdown of keratin was due to enzymatic activities of the organism or to acid hydrolysis. In series No. 2 and 3, the pH was the lowest in keratin containing media and remained at that level for 4 months. It might be argued that in these two series acid hydrolysis might have supplied the organism with soluble forms of N, which were completely utilized for growth, leaving none to react with ninhydrin or to be detected by micro-Kjeldahl method. However, in series No. 1 the pH rose very soon from its low level and most growth occurred (also positive ninhydrin reaction and accumulation of soluble N) after the pH reached the level of about 5.

It might be argued again that the initial acidity in series No. 1 (pH 3.7) resulted in sufficient amounts of solubilized N to account for all the subsequent growth; if this were so then this argument should hold true

even more so in the case of series No. 2 and 3 since the pH in these two series remained at the low level for four months. Yet, these two series showed not only less growth, but in series No. 3 obvious stoppage of growth occurred (for some reason, which will be discussed later) and no soluble N appeared in the medium and no ninhydrin reaction ever became positive. These observations rule out the possibility of the pH prevailing in these experiments causing hydrolysis of keratin. The development of growth in the absence of positive ninhydrin reaction in series No. 2 and 3 indicate that any small amounts of enzymatically solubilized nitrogen were at once utilized for growth and none accumulated in the medium. Rather, the plausible conclusion from the study of the pH changes in the three series to be drawn is that a prolonged acid pH of around 3 does not favour growth of C. albicans in media with keratin as sole source of N; in other words it does not favour enzymatic breakdown of keratin. Such a conclusion also would be in line with most data obtained with enzymatic breakdown of protein, in general where hydrolysis occurs at a pH around neutral (trypsin 8 - 8.5; papain 7 and 5). Existence of pepsin-like enzymes in C. albicans is improbable: it is contradicted by the growth pattern in series No. 1 and also by the fact that optimum pH for pepsin activity is 1.5 - 2.0, which is lower than the pH reached at any time in these experiments.

Positive ninhydrin reactions in the filtrates indicate measurable quantities of nitrogenous compounds with free amino groups in the α-position to a free carboxyl group. Since, outside of α-amino acids, only a small number of peptides would react with ninhydrin, the ninhydrin reaction almost exclusively specifies the presence of α-amino acids. The presence of amino acids - also measured by the Van Slyke method and paper chromatography in series No. 1, 4, and 9 - suggests the hydrolysis of keratin by direct action of the fungus. However, it is impossible to conclude from these experiments

whether the amino acids in the media were derived directly from the keratin or were the products of autolysis of aging yeast elements, as might be deduced from observations made in series No. 12 (Table VII).

This distinction could not be established in the experiments. The presence of amino acids five to six weeks after the beginning of the experiments, therefore, can only mean that either the steadily accumulating and non-assimilated products of keratin hydrolysis or of autolysis of the fungus, or both, reached a concentration high enough to give a positive test. However, in the writer's opinion, most probably the first process, direct keratin hydrolysis, was predominantly responsible in securing the positive test. In whatever way the ninhydrin test became positive this fact must be considered as another evidence of the keratinolytic powers of C. albicans.

Series No. 1 probably provided the best ecological conditions for the growth of C. albicans with keratin as sole source of nitrogen. The consistent accumulation of nitrogenous compounds in the culture media is the result of a vigorous metabolic activity in this series. Here too, significant amounts of nitrogenous compounds in addition to amino acids (Table VII), either directly or indirectly derived from keratin, provide further evidence for the keratinolytic activities of C. albicans.

2. Role of glucose in inducing growth of C. albicans in keratin containing media.

A medium containing glucose and salts and either ammonium sulphate or amino acids as source of nitrogen supported good growth of C. albicans. When glucose was omitted from such a medium, growth failed to develop (Fig. 1).

Growth occurred only in keratin containing media to which glucose was added (series No. 1, 2, 3, 4, and 9).

The experiment presented in Fig. 3 also stresses the importance of glucose for the growth of C. albicans under these experimental conditions. The glucose initially present in the medium soon became exhausted (Table IX). It may be assumed that glucose is dissimulated by C. albicans as described by van Niel and Cohen; or, in addition, it may be partly stored as observed

by Willstätter and Rhodewald in baker's yeasts, and reported by Norbring-Hertz for C. albicans. As soon as either form of carbohydrate is exhausted the fungus must derive its carbon and energy from other sources, in this case from breakdown products of keratin, most probably amino acids. This must lead to a more extensive and complete dissimilation of amino acids.

When glucose was added to cultures 99 days old an increased growth resulted (Fig. 3). The ninhydrin test became negative, and the pH fell from 4.6 to 3.0 during the first two weeks after addition of glucose. In the same period the total nitrogen content increased considerably as compared with the controls. These findings indicate that the addition of glucose results not only in an increased growth of the fungus but also in an increased breakdown of keratin. While the free amino acids disappeared from the media as a result of being consumed by the expanding yeast population, the raised amount of other nitrogenous compounds denotes the increased keratinolytic activities of C. albicans. After the exhaustion of the added glucose the sudden decrease of total nitrogen during the following two weeks indicates that the fungus returned to deriving its carbon and energy from the nitrogenous compounds already available in the media. This represents the reversion of the metabolism to its original pattern.

In the preliminary experiment (culture No. 1 in Table I), each 20 ml. aliquot of the medium removed for analysis was replaced by new, sterile solution of glucose and salts of the same concentration. The much higher amounts of total nitrogen found in this experiment may well be explained by the previously discussed effects of glucose on growth and its direct connection with keratin breakdown.

The facts discussed here demonstrate that glucose is necessary to induce growth and the keratinolytic action of C. albicans. Furthermore, the parallelism between growth of C. albicans and the increased appearance

of hydrolisation products of keratin after addition of glucose gives additional proof of the intimate connection between growth and keratinolytic activities of C. albicans.

3. Influence of glucose concentration.

The influence of the glucose concentration on the growth of C. albicans became apparent in series No. 1, 2, and 3. While series No. 1 (2% glucose) provided the best conditions for growth and keratinolytic activity of the fungus, series No. 3 (8% glucose) did not show any further development after a certain growth during the first few weeks. Series No. 2 (4% glucose) showed a growth similar to that in series No. 1 at first, then the morphological appearance of the cultures changed and the resulting bulky, filamentous growth resembled that produced in series No. 4.

The pH in series No. 1 (Fig. 2) after an initial drop to 3.7 soon rose to a constant level around 5. In series No. 2 and 3 it remained at pH 3.4.

Series No. 12 (2% glucose and ammonium sulphate) produced very good growth resembling series No. 1, although the pH dropped and remained at 2.7, the lowest level ever observed in the cultures.

Series No. 1 showed a steadily increasing amount of total nitrogen accompanied by a consistently positive ninhydrin reaction. A positive ninhydrin test was also observed in series No. 12. Neither series No. 2 nor No. 3 showed a positive ninhydrin test or the presence of nitrogenous compounds in the media.

Quantitative determinations of glucose revealed that in series No. 3 glucose was always present in a considerable amount, whereas in series No. 1 and 2 it had disappeared within two and six weeks respectively after the start of the experiment. In series No. 4 (no salts) glucose likewise disappeared early, but the rise of pH was slower than in series No. 1.

Van Niel and Cohen observed the formation of acetic acid as a normal intermediate in the breakdown of glucose by C. albicans. Although acetic acid determinations in the cultures have not been done yet the consistent low pH in series No. 3 may point to a heavy accumulation of acetic acid as the result of the breakdown of most of the original amount of glucose. Since a low pH does not affect the growth of C. albicans (series No. 12) this assumed high amount of acetic acid has to be regarded as responsible for cessation of growth in series No. 3.

In series No. 2 (4% glucose) similar conditions must have prevailed. However, the glucose determinations indicate that glucose was utilized more slowly thus avoiding an accumulation of comparable quantities of acetic acid. While the higher proportion of acetic acid might have been responsible for cessation of growth in series No. 3, the lower amount of acetic acid formed in series No. 2 induced a change in the gross appearance of the fungus cultures. Since similar but more accentuated morphological features were observed in series No. 4 (no salts, pH 6), it must be concluded that such an accumulation of acetic acid has the same detrimental effect on growth as deficient media. If this explanation is correct the cessation of growth in series No. 3 is due to a blocked metabolism.

These findings are not in accordance with those observed by Dion, 1950. The author found that proteolytic breakdown of KLIM by a number of fungi increased with increasing amounts of glucose. 8% glucose secured the greatest proteolytic activity. The present experiments with C. albicans have shown that glucose is not only necessary to induce the growth of C. albicans on keratin as sole source nitrogen, but that the concentration of glucose should not exceed 2%.

4. Quantitative utilization of keratin and the resultant amino acids.

Series No. 1 (keratin as source of N) and 12 (ammonium sulphate as

source of N) developed comparable amounts of growth and also showed similarity in the simultaneous appearance of the first ninhydrin reaction and of the yellowish colour. Abundant surface growth characterized both series. The foregoing points of similarity may be adduced as grounds for making further comparisons between these two series.

In series No. 12, 26 mg. of nitrogen were taken by the fungus from the medium within 134 days. The total nitrogen in culture media of series No. 1 after the same period of time was found to be 16.44 mg. nitrogen. If it be assumed that the fungus in series No. 1 absorbed approximately the same amount of nitrogen from the medium as in series 12, 42.44 mg. nitrogen (26 + 16.44), representing 22.1% of the original keratin, would have been split by C. albicans to water soluble nitrogenous compounds. Of this total 13.5% of the original keratin would be considered to be absorbed by the fungus while 8.6% is present in the medium.

Most of these soluble nitrogenous compounds derived from keratin by enzymatic processes were other than amino acids (Table VII). Comparison between the paper chromatograms of unhydrolized and hydrolized samples illustrates that most of the total nitrogen in the media was present in the form of peptides.

The constant low level of amino acids in the media points to their rapid utilization by C. albicans, from which source its growth is maintained even in the absence of glucose. The sudden disappearance of amino acids from the medium after addition of glucose (Table VI) shows that the amino acids could be, and were, completely utilized when an increased demand for nutrition arose as a result of the increasing yeast growth.

The assumption that the amino acids present in the medium are due to direct hydrolysis of keratin is borne out by the fact that the amino acids found correspond well to those identified by Blackburn among breakdown

products of wool digested with papain in sodium sulphite solution; they also correspond closely to those identified by Daniels as obtained in cultures of Microsporum canis with human hair as the sole source of nitrogen (Table X).

TABLE X.

Amino acids identified in enzymatic hydrolysates of keratin.

	Papain	Microsporum canis	Candida albicans
Alanine	+	+	+
Arginine		+	
Aspartic acid	+	+	+
Cystine			+
Glutamic acid	+	+	+
Glycine	+	+	+
Histidine		+	+
Leucine	+	+	+
Iso-leucine	+		+
Lysine		+	+
Methionine sulfoxide			+
Serine	+	+	+
Threonine	+	+	+
Tyrosine			+
Valine	+	+	+

Some of the spots on the chromatograms obtained by the action of C. albicans on keratin have not been identified yet. However, it is already evident that almost any amino acid so far detected in keratin hydrolysates also appears under the conditions of these experiments. The widest variety

in both unhydrolized and hydrolized samples was found in series No. 1.

Growth itself proves keratinolytic activity of C. albicans; whenever this growth was sufficiently abundant, positive ninhydrin reaction appeared. Further, only a luxuriant growth in keratin containing media resulted in accumulation of soluble forms of nitrogen in the culture liquid.

The foregoing evidence proves that C. albicans possesses keratinolytic capacities. However, this activity, under the presented experimental conditions, is not as pronounced as that encountered in other fungi that parasitize keratinized tissues, e.g. Microsporum canis. The experimental findings rather confirm clinical observations which agree that whenever a skin infection is claimed to have been caused by C. albicans it is a slowly developing condition.

SUMMARY.

Experiments in culturing Candida albicans on hoof and nail keratin as sole source of nitrogen were done in order to investigate the pathogenicity of this yeast for keratinized tissues.

It was found that:

1) C. albicans can grow on media containing either keratin, salts and glucose or keratin and glucose.
2) Glucose is necessary to initiate growth.
3) Out of three glucose concentrations tested (2, 4, and 8%) best results were obtained with 2% glucose.

The appearance of nitrogenous breakdown products of keratin in culture media was followed for four months qualitatively by ninhydrin tests and paper chromatography and quantitatively by determination of total nitrogen and α-amino nitrogen.

The first amino acids appeared in the culture liquid 40 days after inoculation. Fourteen amino acids were identified. The total nitrogen increased during the four months' course of the experiments and was found to contain only small proportions of free amino acids.

It was thereby proved that C. albicans can digest keratin.

Claim to originality and contribution to scientific knowledge.

The above study definitely established the presence of keratinolytic activity of Candida albicans, this species being the only yeast ever tested successfully in this respect. The significance of these findings extends into the medical field. Mycotic infections of skin and its appendages can be induced experimentally in humans and animals only with great difficulty; moreover, little or nothing is known about the way in which the parasitizing fungus acts. Therefore, in view of these considerations, greater attention must be given to the results of in vitro experiments. The presence of keratinolytic properties in C. albicans in vitro, which has been established in this work provides the first and sole experimental explanation and corroboration of clinical observations and strongly infers the pathogenicity of this yeast for keratinized tissues in vivo. Therefore, the presence of C. albicans in diseased skin or nail should no longer be regarded as incidental but as causative and accordingly antimycotic therapy should be considered.

Further, detailed consideration of the chemical changes observed in the course of this work has thrown some light on the metabolism of this yeast and on the enzymatic breakdown of keratin as conditioned and influenced by glucose and the products of its fermentative breakdown. It is strongly felt that further work in this particular direction may well lead to elucidation of the mechanism of keratinase activity.

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