ANTIGENIC ANALYSIS OF YEAST CELL-WALLS AS AN AID TO CLASSIFICATION

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Presented for the degree of Doctor of Philosophy
in the Faculty of Science, University of Glasgow

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March, 1974

To Jan

"What's the use of their having names," the Gnat said, "if they wo'n't answer to them?"

"No use to them," said Alice, "but it's useful to the people that name them, I suppose. If not, why do things have names at all?"

Lewis Carroll.

ACKNOWLEDGMENTS

I would like to express my gratitude to Dr. D. E. S. Stewart-Tull for his supervision of this research and for his advice, helpful suggestions and discussions. I wish to thank Dr. J. C. Gentles, Department of Medical Mycology, University of Glasgow, for his patience and understanding throughout this work. I would also like to thank Professor A. C. Wardlaw and Dr. D. E. S. Stewart-Tull for reading this manuscript and for their valuable criticisms and suggestions.

To my uncle, Mr. A. D. Govan, I express my gratitude for kindly binding the manuscripts. I record my appreciation to Mrs. J. Tait for capable typing services and to the technical staff of the Department of Microbiology for assistance on many occasions.

OBJECT OF THE RESEARCH

The inherent heterogeneity of yeasts is reflected in the controversial literature on yeast taxonomy. Present classification schemes are based mainly on physiological properties and the number of characteristics is constantly increasing. Serological reactions of yeasts have been investigated by many workers (Hasenclever and Mitchell, 1960, 1964; Tsuchiya, Fukazawa and Kawakita, 1965; Murray and Buckley, 1966; Campbell, 1968; Richards, 1972). However, serological characteristics have not been included in standard classification schemes because of anomalous results and poor differentiation. These drawbacks may have arisen because of whole yeast cells being used for preparation of antisera, with consequent interference by antibodies against common cytoplasmic components.

This thesis is concerned with the antigenic properties of yeast cell-walls as a possible aid to classification. Investigation of bacterial cell-walls has provided information on chemical and serological properties which is of value in bacterial differentiation (Cummins and Harris, 1956, 1958; Cheeseman and Silva, 1959). Yeast cell-walls were shown by Stewart-Tull, Timperley and Horne (1966) to possess antigenic and chemical properties which might prove useful in yeast taxonomy. To extend this study the present work was undertaken. It was considered that extensive information concerning the antigenic relationships of cell-wall components was required before it would be possible to decide whether this approach would have significance in the study of yeasts. Consequently, the type species of twenty of the genera recognised by Lodder and Kreger-van Rij (1952) were investigated. In addition, several other Saccharomyces and Candida species were included because of the importance of these genera.

This investigation involved the large-scale production of cell-walls

of each species and preparation of specific anti-yeast cell-wall sera by immunisation of rabbits. The sugar composition of the cell-walls was determined to assess its importance as a criterion in yeast classification. The main purpose of the study was to conduct an extensive serological investigation by agglutination and complement fixation tests to reveal the antigenic relationships of the type species.

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LIST OF ABBREVIATIONS

Agglutin	=	agglutination
^{o}C	=	degrees centigrade
CBS	=	Centraalbureau voor Schimmelcultures
CF	=	complement fixation or complement fixing
CFT	=	complement fixation test
d^2	=	average squared Euclidean distance
Δ^2	=	squared Euclidean distance
DNA	=	deoxyribonucleic acid
E	=	extinction
ESS	=	error sum of squares
g	=	gram or acceleration due to gravity
G + C	=	guanine plus cytosine
hr	=	hour
HU_{50}	=	50% haemolytic unit
l	=	litre
M	=	molar
μ	=	microns
μg	=	microgram
mg	=	milligram
ml	=	millilitre
N	=	normal
nm	=	nanometre
PCV	=	packed cell volume
pH	=	negative logarithm of hydrogen ion concentration
PMR	=	proton magnetic resonance
RNA	=	ribonucleic acid
r.p.m.	=	revolutions per minute

S_{sm}	=	simple matching coefficient
spp.	=	species
v/v	=	volume for volume
WHO	=	World Health Organisation
w/v	=	weight for volume

GENERA

Bullera	B.
Candida	C.
Debaromyces	D.
Endomycopsis	E.
Hanseniaspora	H'spora
Hansenula	H.
Kloeckera	K.
Lipomyces	L.
Nadsonia	N.
Nematospora	Nem.
Pichia	P.
Rhodotorula	R.
Saccharomyces	S.
Schizosaccharomyces	Schiz.
Schwanniomyces	Schw.
Sporobolomyces	Sp.
Torulopsis	T.
Trichosporon	Tr.
Trigonopsis	Trig.
Wickerhamia	Wick.
Wingea	W.

REVIEW OF THE LITERATURE

CLASSIFICATION OF YEASTS

The first microscopic observation of "yeasts" was made by Antonie van Leeuwenhoek (1680), who described spherical bodies in the sediment of beer. In 1837, Cagnard-Latour discovered that these spherical bodies which belonged to the plant kingdom were responsible for the fermentation. Schwann supported these observations and termed the organisms "Zuckerpilz" or "sugar fungus" from which the name "Saccharomyces" was derived. When other organisms, which appeared morphologically similar to those found in the beer sediment, were isolated, they were included in this group although they did not possess identical properties. For example, some reproduced by fission rather than budding; others did not have the ability to ferment carbohydrates.

The term "yeasts", as described by Lodder and Kreger-van Rij (1952), represents "a group of micro-organisms which is neither well-defined nor homogeneous". They added that "the boundaries of the yeast domain are vague, and subject to arbitrary decisions". Alexopoulos (1962) described yeasts as those members of the Ascomycetes, subclass Hemiascomycetidae, order Endomycetales, family Saccharomycetaceae, which (i) possess a predominantly unicellular thallus, (ii) reproduce asexually by budding or fission and (iii) produce ascospores in a naked ascus originating either from a zygote or parthenogenetically from a single cell. Non-ascospore forming yeasts, which conform in other respects to the above definition, are also included provided that they are not obviously related to another group of fungi.

This definition of the yeasts does not include members of the family Sporobolomycetaceae which form ballistospores and which Alexopoulos classified as members of the Basidiomycetes. However, Lodder and Kreger-van Rij (1952) and Phaff, Miller and Mrak (1966) considered that the Sporobolomycetaceae should be included in the yeasts.

Criteria of Classification

The classification of yeasts is based on a number of morphological and physiological characteristics. In general, morphological features have been used to classify yeasts to the level of a genus, whereas physiological properties differentiate the species.

Morphology

Reess in 1870 attempted to classify the known yeasts by cell morphology and asexual reproductive characteristics. The shape of the vegetative cell can be variable; spheroidal, ovoid, subglobose, cylindrical or apiculate. For example, the triangular cells of Trigonopsis and the apiculate cells of Nadsonia, Hanseniaspora and Saccharomycodes are important taxonomic criteria. However, as the majority of yeasts have no distinctive or constant cell shape, it is doubtful whether the morphology of the vegetative cell as a taxonomic criterion is significant.

The ability of an organism to form pseudomycelium or true mycelium is a useful characteristic in differentiating between genera such as Torulopsis and Candida. Pseudomycelium consists of a filamentous structure in which cells are formed by budding. True mycelium occurs in those yeasts which divide by fission with the formation of a filament. The ability of an organism to form filamentous structures is dependent upon the constitution of the medium (Nickerson, 1963; Romano, 1966) and therefore suitable cultural conditions must be maintained if mycelial formation is to be regarded as a valid taxonomic characteristic.

Asexual Reproduction

Vegetative reproduction occurs in yeasts either by means of fission, as in the genus Schizosaccharomyces, or by budding as in the majority of yeasts, or by "bud-fission" which occurs in species of the

family Nadsonoideae. Fission involves the replication of a vegetative cell by the ingrowth of a transverse septum from the cell-wall which bisects the longitudinal axis of the cell. Budding occurs as an evagination at different points in the surface of the cell which enlarges to form a new cell, after which it separates from the mother cell. Bipolar budding or bud-fission takes place at the poles of the cell, as in the genus Hanseniaspora. Sterigmatomyces species do not bud, but form conidia or sterigmata; this property distinguishes the genus from all other genera of yeasts. In addition to budding, members of the Sporobolomycetaceae form ballistospores by a mechanism considered to be similar to that of the basidiospore discharge mechanism occurring in the Basidiomycetes. Exceptions to the classical multipolar bud formation have considerable significance in taxonomy of yeasts.

Sexual Reproduction

Ascosporogenous yeasts can be homothallic or heterothallic and can occur in the vegetative state either in haplophase or in diplophase, or a mixture of both. The mode of ascus formation, as well as the shape of spores and colour of spores, can be used to differentiate yeasts. Distinctive types of ascospore are 'hat-shaped' or 'Saturn-shaped' as in the genus Hansenula (Wickerham and Burton, 1954); 'cap-shaped' as in the genus Wickerhamia (Soneda, 1960); brown, warty and spherical as occurs in the genus Nadsonia (Nadson and Konokotina, 1911 a, b, 1912, 1926).

Formation of an ascus in homothallic yeasts can occur either by the conjugation of two haploid cells or by fusion of nuclear material of a bud and mother cell, or by reduction and division within a vegetative diploid cell. Heterothallic yeasts in haplophase undergo conjugation when cells of the opposite mating strains are in contact with one another as in the genus Hansenula. The diplophase is normally heterozygous for the mating type and individual cells are usually bisexual, although

Wickerham (1958) observed unisexual diploid cells. Considerable importance has been attached to the characteristics of ascospores and of ascospore formation when considering the taxonomic position of a yeast. Although a useful asset in taxonomy, sporulation is of less value than physiological properties (Barnett, 1960, 1961).

Physiological Criteria

Physiological properties which are of taxonomic significance for the yeasts include the ability to ferment carbohydrates, the pattern of utilisation or assimilation of carbon and nitrogen containing compounds, and the production of extracellular polysaccharides. To a lesser extent, vitamin requirements, growth under high osmotic pressure, and the range of temperature at which growth will occur should be taken into consideration. The differentiation of genera is facilitated only in certain instances by considering physiological characteristics. Physiological properties are better used for classification at species level (Kreger-van Rij, 1969).

a) Fermentation of sugars

The genus Hansenula consists of species some of which ferment sugars strongly, whereas others are non-fermentative. The genera Lipomyces and Rhodotorula comprise species which do not ferment carbohydrates and this characteristic is a useful taxonomic criterion. The emphasis placed on fermentation properties of yeasts is not as great as that found in bacteriology, for example, in the Enterobacteriaceae the ability to ferment lactose is a major diagnostic test. Fermentation properties of a species can be variable particularly if the culture is maintained for a prolonged period on a medium containing particular carbohydrates (Scheda and Yarrow, 1966), and this may cause confusion when constructing a classification scheme.

b) Assimilation of organic compounds

Assimilation of carbon-containing compounds is mainly used for the differentiation of species rather than genera. Wickerham and Burton (1948) selected thirty compounds as being most useful for classification. Barnett (1960, 1966) discussed the biochemical reactions concerned in assimilation and derived a scheme of taxonomy based thereon. As with fermentation, prolonged cultivation of yeasts results in adaptation to utilisation of compounds not assimilated by the original isolate; a disadvantage during classification.

Nitrate and other nitrogen-containing compounds can be assimilated by some yeasts, and this property is not only important at a species level but also for differentiation of genera. For example, Saccharomyces, Pichia and Debaromyces species do not utilise nitrate, whereas all Hansenula species can utilise it. Utilisation of amino acids, purines and pyrimidines was found by LaRue and Spencer (1967 a, b, 1968) to have little application to yeast taxonomy.

c) Production of extracellular polysaccharides

Production of extracellular polysaccharides is primarily useful in differentiating pairs of two similar genera; for example, the formation of a starch-like polysaccharide was used to separate the genus Cryptococcus from the genus Torulopsis (Lodder and Kreger-van Rij, 1952). Some species in the genera Hansenula and Pachysolen produce phosphomannans (Jeanes, Pittsley, Watson and Dimler, 1961) which were characterised by Slodki, Wickerham and Cadmus (1961) and Slodki (1963). These workers considered that the chemical properties of extracellular polysaccharides were a distinctive feature of each species.

d) Pigment and enzyme production

Lipase and urease activities and pigment production are employed to a limited extent as taxonomic criteria, usually as confirmatory tests. Lipase activity occurs in Candida lipolytica and Trichosporon pullulans and hydrolysis of urea is associated with the asporogenous yeasts. Pigment production is useful in the classification of species of Rhodotorula and Sporobolomyces which have carotenoid pigments.

Deoxyribonucleic acid analyses

The analysis of the base composition of deoxyribonucleic acid (DNA) has been used in bacterial taxonomy to elucidate relationships in bacteria (Marmur, Falkow and Mandel, 1963). Comparison of the DNA guanine plus cytosine (G + C) contents of bacterial species can indicate possible relationships. However, this procedure has limited value because of poor resolution (Hill, 1966). Storck (1966) established DNA base ratios of some fungi, and referring to results obtained by Vanyushin, Belozersky and Bogdanova (1961) showed that the G + C content of Ascomycetes was in the region of 50%, and that of Basidiomycetes greater than 50%; the lowest G + C ratios were found in Phycomycetes. Stenderup and Leth Bak (1968), Nakase and Komáyata (1968), and Storck, Alexopoulos and Phaff (1969) published DNA base ratios of a variety of yeasts. Their results showed a significant difference between the ascosporogenous yeasts and those species belonging to the genera Cryptococcus and Rhodotorula. It has been suggested that the latter are related to the Basidiomycetes. Also, in Candida and Torulopsis, there are species with G + C values similar to those of Ascomycetes and species whose values are similar to those of Cryptococcus and Rhodotorula species.

Leth Bak and Stenderup (1969), using the DNA-RNA hybrid technique

of Gillespie and Spiegelman (1965) to assess DNA homology in species of Candida, found that their results reflected the relationships determined by G + C content. The contribution of DNA analyses to yeast taxonomy could provide an indication of the phylogenetic relationships of yeasts, and to the direction in which to search for the sexual state of asporogenous yeasts. However, it is unlikely that this technique could be used as a practical aid in identification.

COMPOSITION OF YEAST CELL-WALLS

Cummins and Harris (1956, 1958) showed that genera of Gram positive bacteria could be differentiated by the amino acid and sugar composition of their cell-walls. Cheeseman (1959), Cheeseman and Silva (1959) and Silva and Cheeseman (1959) examined cell-wall extracts of lactobacilli by chromatography and found there was usually a similarity in the major cell-wall components of species within the genus. Since then many bacterial cell-walls have been examined and it has been shown that cell-wall taxonomy indicates whether a species has been correctly classified.

Investigation of yeast cell-wall components started when Salkowski (1894 a, b) demonstrated the presence of "yeast cellulose" and "yeast gum". The principal components of yeast cell-walls are "yeast cellulose" or glucan , investigated by Salkowski (1894 a) and later by Zechmeister and Toth (1934, 1936); "yeast gum" or mannan (Salkowski, 1894 b; Haworth, Hirst and Isherwood, 1937; Haworth, Heath and Peat, 1941); chitin (Schmidt, 1936; Nabel, 1939); protein (Northcote and Horne, 1952; Roelofsen, 1953); and lipids (Eddy, 1958; Masschelein, 1959). In a preliminary investigation Stewart-Tull, Timperley and Horne (1966) showed that, although there were qualitative differences in the components of various yeast cell-walls, these were of limited value in classification.

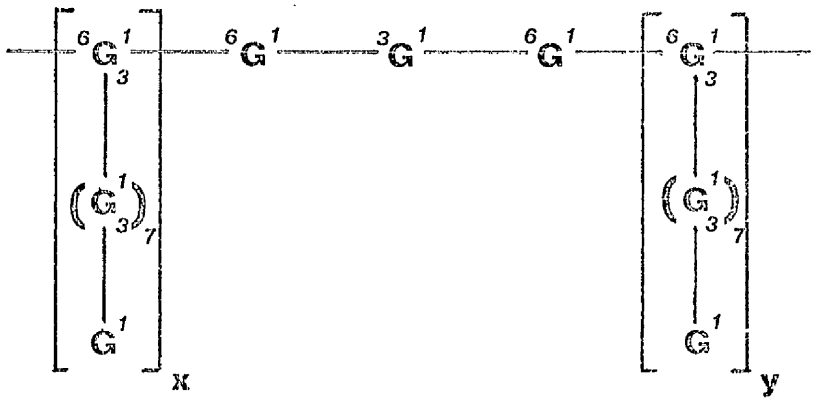
TABLE 1 :

Characteristics of glucans in the cell-walls of yeasts

YEAST	Glycosidic linkage	Characteristics	Reference
S. cerevisiae	β1-3 β1-6	Highly branched polymer	a) Hassid, Joslyn and McCready (1941) b) Bell and Northcote (1950) c) Misaki, Johnson, Kirkwood, Scaletti and Smith (1961)
	β1-3 β1-6	Unbranched linear polymer	d) Peat, Whelan and Edwards (1958)
	β1-6 β1-3 (few)	i) Water-soluble linear polymer	e) Manners and Masson (1969)
	β1-3 β1-6	ii) Insoluble highly branched polymer	
Schizosaccharomyces spp and *Cryptococcus* spp	α1-3	i) alkali-soluble polymer	a) Houwink and Kreger (1953) b) Kreger (1954)
	β1-3 β1-6	ii) alkali-insoluble polymer	c) Bacon, Jones, Fermer and Webley (1968) d) Hasegawa, Nordin and Kirkwood (1969)

FIGURE 1 :

Structure proposed for the cell-wall glucan of S. cerevisiae



$x + y = 40$

Misaki, Johnson, Kirkwood, Scaletti and Smith (1968)

Characteristics of cell-wall glucan

Chemical studies of glucans, mainly from baker's yeast, have demonstrated the types of glycosidic linkages and the degree of branching and polymerisation of the glucose residues. The majority of reports are chemical studies, each of a small number of yeast species. As shown in Table 1 there are few instances of comparative investigations on a wide range of yeasts.

The glucan from S. cerevisiae was shown to possess a highly branched structure with β 1-3 and β 1-6 linkages (Hassid et al., 1941; Bell and Northcote, 1950; Misaki et al., 1968). By contrast, Peat et al., (1958) considered that the glucan polymer was linear. Bacon and Farmer (1968) and Bacon, Farmer, Jones and Taylor (1969) obtained water-soluble and insoluble glucans from S. cerevisiae. The water-soluble glucan was subsequently shown to be linear, with β 1-6 linkages and a small proportion of β 1-3 linkages (Manners and Masson, 1969). This water-soluble glucan seems to be similar to that described by Peat et al., (1958). The residual insoluble glucan was similar to that described by Misaki et al., (1968), as in Figure 1, although it was not as highly branched and possessed longer β 1-3 chains.

Comparative studies of yeast glucans by Houwink and Kreger (1953) and Kreger (1954) revealed that in Schizosaccharomyces cell-walls there was 30-35% of an atypical alkali-soluble glucan which had an X-ray diffraction pattern markedly different from the "hydro-glucan" of baker's yeast. The latter, after boiling with hydrochloric acid, was alkali-soluble. The proportion of glucan resembling that of S. cerevisiae was about 10% in Schizosaccharomyces species. Bacon et al., (1968) showed that the atypical glucan of Schizosaccharomyces was also found in Cryptococcus terreus and Cryptococcus albidus and contained α 1-3 linked units. The α 1-3 content of the glucan was shown by Jones, Bacon, Farmer and Webley

TABLE 2 :

Action of endo-β glucanases on yeast cell-wall glucans.

SPECIES	Hydrolysis by : Endo-β 1-3 glucanase			Endo-β 1-6 glucanase		
	Complete	Incomplete or weak	None	Complete	Incomplete or weak	None
Saccharomyces spp	+++				+	
Hansenula anomala						
H. ciferri	+++			+++		
Nadsonia elongata						
Schizosaccharomyces spp		+			+	
Lodderomyces elongisporus						
Rhodotorula rubra			-			-
Debaromyces hansenii	+++*	+		+++*	+	

* strongly hydrolysed by combined enzymes

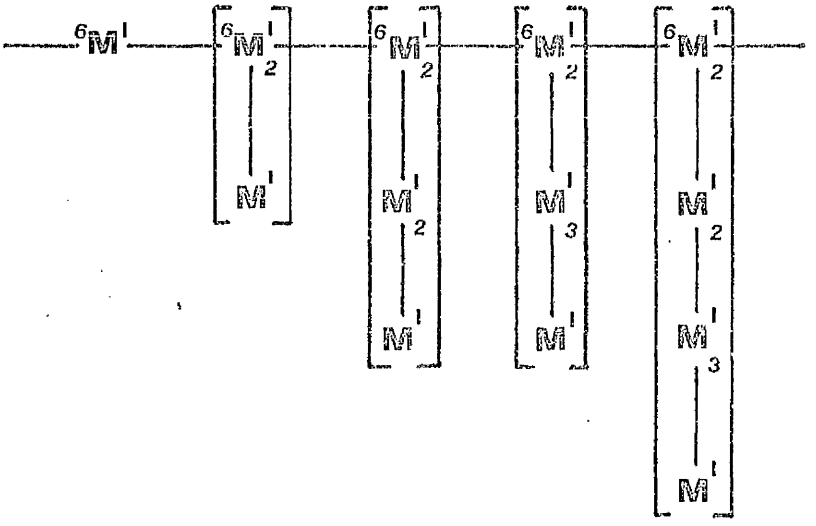
TABLE 3 :

Characteristics of mannans in the cell-walls of yeasts

YEAST	Glysodic linkage	Characteristics	References
S. cerevisiae	α 1-6 α 1-6 backbone α 1-2 side α 1-3 chain	Highly branched mannose polymer	a) Haworth, Hirst and Isherwood (1937) b) Haworth, Heath and Peat (1941) c) Cifonelli and Smith (1955) d) Peat, Whelan and Edwards (1961) e) Peat, Turvey Doyle (1961) f) Lee and Ballou (1965) g) Stewart, Mendershausen and Ballou (1968) h) Stewart and Ballou (1968)
S. rouxii	α 1-6 α 1-3 (few)	Highly branched polymer	a) Gorin and Perlin (1956, 1957)
C. albicans C. stellatoidea C. tropicalis C. parapsilosis	α 1-6 α 1-2 α 1-6 α 1-2	Highly branched polymer	a) Bishop, Blank and Gardner (1960) b) Šikl, Masler and Bauer (1964, 1967) c) Yu, Bishop, Cooper, Hasenclever and Blank (1967)
C. albicans C. stellatoidea	α 1-6 α 1-2 α 1-3 (few)	Highly branched polymer	a) Stewart and Ballou (1968)
Pichia pastoris Citeromyces matritensis	α 1-6 backbone β 1-2 side α 1-2 chain	Highly branched polymer	a) Gorin, Spencer and Bhattacharjee (1969)

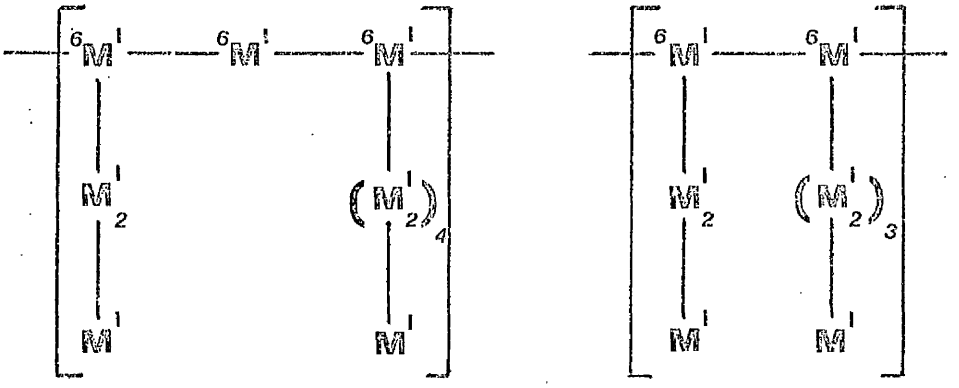
FIGURE 2 :

Structures proposed for cell-wall mannan



S. cerevisiae

(Stewart and Ballou, 1968)



4 strains 1 strain

M : α-D-mannopyranose

C. albicans

(Šikl, Masler and Bauer, 1967)

(1969) to be affected by the composition of the growth medium. This α 1-3 linked glucan has been found in the cell-walls of the Basidiomycetes and Ascomycetes (Kreger, 1954; Bacon et al., 1968), in Endomyces decipiens and in species of Cryptococcus and Schizosaccharomyces (Hasegawa et al., 1969).

Enzymic studies of Tanaka and Phaff (1965) using endo-β-1-3 and endo-β-1-6 glucanases indicated that the former was more effective than the latter in causing lysis of baker's yeast cells. The ratio of β 1-6 : β 1-3 bonds susceptible to the respective enzyme is 1 : 2 in baker's yeast compared to 1.3 : 1 in Hansenula anomala. The comparative action of endo-β glucanases on different species of yeasts was investigated by Tanaka, Phaff and Higgins (1965), who divided species into five groups based on enzyme susceptibility (Table 2).

Characteristics of cell-wall mannan

The presence of mannan in baker's yeast was first reported by Salkowski (1894 b), who termed his preparation "yeast gum". Garzuly-Janke (1940) demonstrated mannan in a wide variety of yeasts, with only a few exceptions. Subsequently, the preponderence of α-linkages in the mannans was shown although the positions of these linkages were not completely resolved (Table 3). Haworth et al., (1937, 1941) found that the mannan was a highly branched polymer with α-linkages. This was confirmed by Cifonelli and Smith (1955). Peat et al., (1961 a, b) showed that α 1-6 linked mannose residues in the backbone of the mannan were not all substituted. Lee and Ballou (1965), Stewart et al., (1968) and Stewart and Ballou (1968) found by means of controlled acetolysis of the mannan that the α1-2 and α 1-3 bonds present in the side-chains were attached to the backbone of the polymer by α 1-2 bonds (Figure 2). Mannans from different strains of S. cerevisiae were similar, but there were differences in the ratios of oligosaccharides in the side-chains

(Stewart and Ballou, 1968). No alteration in the composition of the mannan was found by altering growth conditions, although the ratio of glucan to mannan could be affected by nutritional factors (Dunwell, Ahmad and Rose, 1961). S. carlsbergensis mannan is similar to that of S. cerevisiae except that it has a smaller number of trisaccharide chains and a larger number of unsubstituted α 1-6 linked mannose units in the backbone. Gorin and Perlin (1956, 1957) studied a S. rouxii mannan which was excreted into the medium and found side-chains of one or two mannose residues and a deficiency of α 1-3 linkages.

Candida albicans was shown to be a highly branched α 1-6 linked polymer with α 1-2 linked short side-chains (Bishop et al., 1960). C. stellatoidea, C. parapsilosis and C. tropicalis possessed similar mannans with only slight variations (Yu et al., 1967). Sikl et al., (1964, 1967) investigated mannans from the culture filtrates and washings of five strains of C. albicans and confirmed the absence of α 1-3 linkages. Four strains had identical mannans, (Figure 2), with side-chains containing up to five mannose residues, and an unsubstituted mannose between substituted residues in the backbone structure. One strain had a maximum of four sugar residues in the side-chain and no unsubstituted residues in the backbone. These results were contradicted by Stewart and Ballou (1968), who found a small number of α 1-3 linkages in the four and five unit side-chains of the mannan of C. albicans and a higher proportion of α 1-3 linkages in C. stellatoidea mannan.

Recently, Gorin et al., (1969) examined yeast mannans using the technique of proton magnetic resonance (PMR), and found an indication of β -linkages in the spectra. Chemical analyses of the mannans of Pichia pastoris and Citeromyces matritensis confirmed the typical α 1-6 linkages in the backbone structure with α 1-2 - and β 1-2 linked side-chains. This type of analysis was first used as a taxonomic criterion by Gorin, Mazurek and Spencer (1968). The PMR spectra of mannans from different

yeasts were successfully used as "finger-prints" to indicate the similarity of the mannans. PMR spectroscopy as an aid in identification and chemotaxonomy of yeasts was extensively reviewed by Gorin and Spencer (1970). Unfortunately, PMR spectroscopy of mannans could not be used as a routine procedure in a diagnostic laboratory.

Phosphomannans

Northcote and Horne (1952), Lindquist (1953) and Eddy (1958) found 0.2 to 1% phosphate in mannan. Mill (1966) obtained mannose-6-phosphate as a hydrolysis product of S. cerevisiae mannan, and found that the amount of phosphate varied with strain and with cultural conditions. The phosphate groups in Kloeckera apiculata mannan occurred at the C3 or C4 positions in the mannose residues of the backbone (Stewart and Ballou, 1968). On the other hand, Cawley and Letters (1968) showed that the phosphate in S. cerevisiae mannan occurred in the phospho-diester link between C6 and C1 positions of two mannose residues. It has been suggested that the presence of phosphomannans might have taxonomic significance, but it is obvious that more extensive studies must be pursued using a wide range of yeasts before the taxonomic value can be considered.

Galactomannans

Low yields of galactomannans were isolated from Nadsonia spp. and Trichosporon spp. (Spencer and Gorin, 1968; Gorin and Spencer, 1968), and from Schizosaccharomyces spp., Torulopsis spp. and Candida spp. (Gorin, Spencer and Eveleigh, 1969; Gorin, Spencer and Magus, 1969). The occurrence of galactomannans does not appear to be specific at either species or genus levels.

Studies with exo- α-mannosidase

Studies using an exo- α-mannosidase from an Arthrobacter species gave direct evidence of the α 1-6 linked mannan backbone. The enzyme hydrolyses α 1-6, α1-3 and α 1-2 linkages in side-chains leaving the main chain intact (Jones and Ballou, 1968). However, the enzyme cannot hydrolyse β-linkage or side-chains containing galactose. The use of this enzyme is important in the determination of mannan structures and might become useful in the differentiation of yeasts.

Immunochemistry of mannans

Hasenclever and Mitchell (1964 a), Summers, Grollman and Hasenclever (1964) demonstrated that cell-wall mannan was the major antigen in Candida species. Inhibition of precipitin reactions by oligosaccharides from the mannan side-chains of S. cerevisiae and C. albicans revealed that the main antigenic determinants were the α 1-2 and α 1-3 linked residues of which the α 1-3 linked terminal residues were more inhibitory (Suzuki, Sunayama and Saito, 1968; Suzuki and Sunayama, 1968). Further work is necessary to determine whether the chemical properties of the antigenic determinants of a wide variety of yeasts will assist the understanding of phylogenetic relationships amongst species of yeasts.

Chitin content of cell-walls

Schmidt (1936) first detected chitin in filamentous yeasts. Kreger (1954) demonstrated that chitin was present in greater quantities in filamentous than in non-filamentous yeasts, and that Schizosaccharomyces spp. possessed no chitin. Eddy (1958) concluded that there was 0.8 to 0.9% glucosamine in the cell-walls of baker's yeast, but Korn and Northcote (1960) considered that only 9% of the glucosamine present was

in the form of typical crustacean chitin. Bacon, Davidson, Jones and Taylor (1966) agreed with Korn and Northcote (1960) in finding only 20% of the total chitin in bud-scar preparations.

Protein content of cell-walls

In baker's yeast the protein content has been shown to be between 5 and 7% by Roelofsen (1953), Falcone and Nickerson (1956) and Miller and Phaff (1958). Among the yeasts there is a wide range of protein content in the cell-walls; for example, Hanseniaspora uvarum has 7% (Miller and Phaff, 1958), and the triangular form of Trigonopsis variabilis has 14.6% (SentheShanmuganathan and Nickerson, 1962). Glucomannan-protein complexes have been extracted from cell-walls by a number of investigators (Northcote and Horne, 1952; Cifonelli and Smith, 1955; Kessler and Nickerson, 1959, and Sentandreu and Northcote, 1968). Korn and Northcote (1960) suggested that glucosamine may be the link between protein and mannan. The later results of Sentandreu and Northcote (1968) suggested similar circumstantial evidence of N-acetylglucosamine being linked to mannose residues in mannan and aspartic acid of the protein.

Enzymes associated with cell-walls

Several enzymes have been reported from the yeast cell-wall, e.g., invertase (Friis and Ottolenghi, 1959) and catalase (Kaplan, 1965). Neumann and Lampen (1967) regarded invertase as a mannan-protein, as did Boer and Steyn-Parve (1966) for acid phosphatase. However, invertase may exist in more than one active form, one type being located between the plasma membrane and cell-wall according to Lampen (1968) and is not attached to the mannan. The secretion of extracellular enzymes could well result in their loose association with the cell-wall and may contribute to the apparent enzyme activity of cell-walls. Using techniques such as the sensitivity of a hydrolase e.g., invertase to low

voltage electron beams of increasing penetrating power, the enzyme was located just below the outer layer of the wall (Preiss, 1958).

Lipid content of cell-walls

Masschelein (1959) found as much as 13.5% lipid in cell-walls of beer yeasts. Eddy (1958), however, reported that the lipid content was less than 2% in cell-walls of S. cerevisiae. As a result of the difficulty in preparing cell-walls completely free of membranes, there is doubt about whether the lipid is in the cytoplasmic membrane or; truly cell-wall lipid. Stodola, Deinema and Spencer (1967) investigated species of Torulopsis, Cryptococcus, Candida and Rhodotorula which excrete glycolipids into the culture medium. Staining with lipophilic dyes showed that the excreted glycolipids were trapped in the cell-wall but were not integral components of the wall.

In conclusion, the investigation of the chemical composition of yeast cell-walls has involved detailed chemical studies of the glucan and mannan components in a small number of yeasts (Phaff, 1971). The PMR spectra of mannans has been extensively used to differentiate a large number of yeasts (Gorin and Spencer, 1970), but the interpretation of the similarity of the spectra is difficult to assess without specialised knowledge.

SEROLOGICAL STUDIES OF YEASTS.

There are many papers which report the antigenic relationships among species within a single genus of yeasts. However, there appear to be no systematic studies of the inter-relationships of different groups of yeasts. For this reason, and also because a variety of unrelated serological procedures were used, it is difficult to correlate the various published results.

The following sections review the individual serological tests which have been applied to yeast antigens, namely, complement fixation, agglutination, precipitin and immunofluorescence.

Serological relationships determined by complement fixation

Widal, Abrami, Joltrain, Brissaud and Weill (1910) determined the interaction of *Candida*, *Actinomyces* and *Sporothrix* species with antisera to these organisms from infected patients and observed cross-reactions between the three organisms and the three antisera. Epstein (1924) separated species of *Candida* from other yeasts by significant differences in the cross-reaction titres. Strains within a species showed different degrees of reaction to each antiserum. These early experiments suggested that, although the *Candida* species were serologically distinct from other yeasts, there was also heterogeneity within the genus. Stone (1930) differentiated various yeasts isolated from clinical cases into seven serological groups, one of which was *C. albicans*. Stone and Garrod (1931) demonstrated that *S. cerevisiae* did not react with rabbit antisera to *Candida* spp. and that *C. tropicalis* and *C. albicans* were identical in their reactions.

An antigenic scheme was proposed by Martin (1942) for *C. albicans*, *C. stellatoidea*, *C. tropicalis* and *C. parapsilosis* consisting of three antigens occurring in different proportions, one being common to all four

species. Tomcsik (1930) prepared an antigenic extract from Saccharomyces spp. and determined the titre against rabbit whole cell antisera. Two groups of species were distinguished and it was noticed that the complement fixation test gave a better differentiation of the groups than a precipitin test.

Seeliger and Schröter (1963) compared various serological techniques in a study of the antigenic relationships of the genus Trichosporon. Three groups of species were proposed, although the groups were not distinct and overlapped antigenically. Complement fixation results reflected more closely the results of precipitin tests than agglutination tests. Müller (1966) considered haemagglutination to be more sensitive than complement fixation in producing greater differentiation in serological reactions and concluded that C. albicans had one more antigen than C. tropicalis. This antigen was located both in the cytoplasm and in the cell-wall.

Serological relationships determined by agglutination methods

Agglutination methods have been used extensively in the investigation of the immunological properties of yeasts. Hines (1924) failed to differentiate C. albicans from other yeasts by the technique of agglutinin absorption. Hopkins and Benham (1929) demonstrated serological differences amongst species of Candida but absorption results were not conclusive. However, Benham (1951) differentiated Candida spp. from S. cerevisiae and H. anomala. C. albicans, C. parapsilosis and C. krusei were differentiated from each other by absorption tests. C. albicans was serologically homogeneous, all strains of C. albicans being antigenically identical. Almon and Stovall (1934) found higher agglutination titres for S. cerevisiae and H. anomala with antisera to Candida species; C. albicans and C. tropicalis were identical. The Candida species formed a distinct group which could be identified by

C. albicans or C. tropicalis antisera absorbed with S. cerevisiae, which removed cross-reactivity for other yeasts.

Benham (1935) divided strains of cryptococci into four groups. The first group showed cross-reactivity with C. albicans, S. cerevisiae and other strains of cryptococci but absorption tests revealed the existence of three distinct types or species. The second and third groups contained strains which belonged to one distinct species for each group. The fourth group contained strains showing different serological characteristics which indicated the existence of several species within the group.

Jonsen, Thjotta and Rasch (1953) compared the serological properties of C. albicans and C. stellatoidea using quantitative agglutination tests. It was found that C. pseudotropicalis was agglutinated at a low titre by antisera to both C. albicans and C. stellatoidea. Absorption of C. albicans antiserum with C. stellatoidea cells removed the majority of agglutinating activity to C. albicans. A similar result was obtained when C. stellatoidea antiserum was absorbed with C. albicans; the majority of the antibody to C. stellatoidea was removed. C. pseudotropicalis showed little antigenic similarity with C. albicans and C. stellatoidea, the latter two being almost identical in their antigenic composition, but each possessed some individual specificity.

Martin, Jones, Yao and Lee (1937) regarded serology as a useful aid in the classification of Candida spp., although the degree of cross-reaction prevented the use of agglutination tests in routine identification. However, Keiper and Prescott (1938) regarded agglutination and precipitin methods as being more reliable than fermentation tests. Yeast isolates from pulmonary infections showed greater variation in fermentation characteristics than in serological properties.

Slide agglutination was studied by Rosenthal and Furnari (1958) as a quick method for the identification of C. albicans. Unlike Jonsen

TABLE 4 :

Agglutination reactions of absorbed sera to Candida species and Torulopsis glabrata

(reprinted by courtesy of the publishers of the Journal of Bacteriology)

Agglutination reactions of Torulopsis glabrata with adsorbed antisera to Candida species

Antisera to:	Antisera Adsorbed with:							
	C. tropicalis	*C. parapsilosis*	*C. stellatoidea*	*C. guilliermondii*	*C. albicans*	*C. krusei*	*C. pseudotropicalis*	*T. glabrata*
C. tropicalis	--	2+ to 3+	2+ to 3+	+ to 2+	− to +	2+ to 3+	2+ to 3+	−
C. parapsilosis	+	−	2+	+	+	2+ to 3+	2+ to 3+	--
C. stellatoidea	−	−	−	−	− to +	+ to 2+	2+ to 3+	--
C. guilliermondii	−	2+ to 3+	2+ to 3+	−	+ to 2+	2+ to 3+	2+ to 3+	−
C. albicans	--	2+ to 3+	2+	+	−	2+ to 3+	3+	−
C. krusei	+ to 2+	+ to 2+	+ to 2+	+ to 2+	+ to 2+	−	2+	−
C. pseudotropicalis	+	+	+	+	+ to 2+	+ to 2+	−	--

− = No agglutination at 1:30 serum dilution; + = agglutination at 1:30 serum dilution; 2+ = agglutination at 1:60 or 1:120 serum dilution; and 3+ = agglutination at 1:240 or 1:480 serum dilution.

Agglutination reactions of Candida species and Torulopsis glabrata with adsorbed T. glabrata antiserum

Antigen:	Antiserum Adsorbed with:							
	C. tropicalis	*C. parapsilosis*	*C. stellatoidea*	*C. guilliermondii*	*C. albicans*	*C. krusei*	*C. pseudotropicalis*	*T. glabrata*
C. tropicalis	−	3+	3+	+ to 2+	+ to 2+	2+ to 3+	2+ to 3+	--
C. parapsilosis	−	−	−	−	− to +	2+	2+	--
C. stellatoidea	− to +	−	−	−	−	− to +	− to +	--
C. guilliermondii	−	3+	3+	−	+ to 2+	3+	3+	−
C. albicans	−	3+	2+	− to +	−	3+	3+	−
C. krusei	−	−	− to +	−	−	−	−	−
C. pseudotropicalis	− to +	−	− to +	2+	− to +	−	−	−
T. glabrata	2+ to 3+	3+	3+	2+ to 3+	3+	3+	3+	−

− = No agglutination at 1:30 serum dilution; + = agglutination at 1:30 serum dilution, 2+ = agglutination at 1:60 at 1:120 serum dilution; and 3+ = agglutination at 1:240 or 1:480 serum dilution.

Agglutination reactions of Candida species with Candida antisera adsorbed with Torulopsis glabrata

Antisera to:	Antigen							
	C. tropicalis	*C. parapsilosis*	*C. stellatoidea*	*C. guilliermondii*	*C. albicans*	*C. krusei*	*C. pseudotropicalis*	*T. glabrata*
C. tropicalis	2+ to 3+	+ to 2+	− to +	+ to 2+	+ to 2+	− to +	− to +	−
C. parapsilosis	+ to 2+	3+	2+ to 3+	− to +	− to +	− to +	− to +	−
C. stellatoidea	+ to 2+	3+	3+	+ to 2+	+ to 2+	2+	+ to 2+	−
C. guilliermondii	2+ to 3+	− to +	− to +	2+ to 3+	2+ to 3+	−	−	−
C. albicans	2+	2+	+ to 2+	+ to 2+	2+	− to +	− to +	−
C. krusei	--	− to +	− to +	−	−	2+	− to +	−
C. pseudotropicalis	−	− to +	+ to 2+	−	−	+ to 2+	2+ to 3+	−

− = No reaction at 1:30 serum dilution; + = agglutination at 1:30 serum dilution; 2+ = agglutination at 1:60 − 1:120 serum dilution; and 3+ = agglutination at 1:240 or 1:480 serum dilution.

Hasenclever and Mitchell (1960)

et al., (1953) no reaction was found when C. pseudotropicalis or C. krusei was tested against C. albicans antiserum. Cells of C. albicans, C. tropicalis, C. guilliermondii and C. stellatoidea were agglutinated by antiserum to C. albicans. Absorption of C. albicans antiserum with either C. stellatoidea or with C. stellatoidea and C. guilliermondii together removed the specificity for these species. However, when C. tropicalis, C. stellatoidea and C. guilliermondii cells were used together to absorb C. albicans antiserum all the specificity against C. albicans cells was lost. Although these authors could not obtain a specific antiserum to C. albicans they considered that C. albicans antiserum absorbed with C. stellatoidea and C. guilliermondii was useful in the identification of isolates of C. albicans. Hasenclever and Mitchell (1960) studied the antigenic relationships of T. glabrata and seven species of Candida. Direct tube agglutination of all species with all antisera revealed extensive cross-reactions. Antisera to the Candida spp., after absorption with the other species of Candida, were subsequently tested against cells of T. glabrata and a close relationship was demonstrated in C. tropicalis, C. guilliermondii and C. albicans (Table 4). There were some discrepancies in the results as the cells of T. glabrata were agglutinated by antisera to C. parapsilosis, C. krusei and C. pseudotropicalis absorbed with C. tropicalis, C. guilliermondii or C. albicans. The authors considered that T. glabrata had surface antigens in common with deep antigens to C. parapsilosis, C. krusei and C. pseudotropicalis.

Hasenclever and Mitchell (1961) observed two serological groups of C. albicans designated A and B. The discovery of two serotypes of C. albicans could account for the discrepancies encountered in the antigenic analyses of different workers. C. albicans A was indistinguishable from C. tropicalis and C. albicans B strains were identical to C. stellatoidea (Hasenclever, Mitchell and Loewe 1961).

Of particular interest was the fact that absorption of antiserum of C. stellatoidea with whole cells of C. stellatoidea did not remove all the agglutinins to C. tropicalis and C. albicans type A. It was necessary to use disintegrated cells of C. stellatoidea to absorb the homologous antiserum in order to remove all the agglutinins. It is possible that the antigens common to C. tropicalis and C. stellatoidea are situated on the surface of the former and located within the cells in the latter as is thought to be the case with T. glabrata.

The surface antigens of C. albicans A and B, C. stellatoidea, C. tropicalis and S. cerevisiae were investigated by agglutinin inhibition with mannans from these species (Hasenclever and Mitchell, 1964 b). The amount of mannan of each species which caused a four-fold inhibition in an homologous agglutination was determined. Anomalous results were obtained since C. albicans A antiserum required 5 µg of C. albicans group A mannan for inhibition of the homologous reaction but required greater than 500 µg of C. albicans mannan to inhibit agglutination of S. cerevisiae or C. stellatoidea cells. The authors offered no explanation for these results and suggested that precipitin inhibition and quantitative precipitin tests were more precise although not so sensitive. It is apparent that investigation of antigens in situ by agglutination inhibition methods does not reveal the entire antigenic structure. The extraction of mannan could destroy antigenic determinants formed by the arrangement and configuration of molecular structures on the whole cell surface.

An antigenic analysis of fourteen Candida species was carried out by Murray and Buckley (1966) using agglutination and agglutinin absorption. Antigenic differences between C. albicans group A and C. tropicalis were found and between C. albicans group B and C. stellatoidea which were not discovered by Hasenclever et al., (1961). Disintegrated cells were used for absorption of antisera as whole cells did

not remove all the reactivity to the absorbing strain.

Campbell and Allan (1964) proposed a tentative antigenic structure for S. cerevisiae and S. ellipsoideus. Nine other Saccharomyces species were tested against S. cerevisiae antiserum and were divided into two groups. One antigen was common to all the species examined, another was common to all except S. ellipsoideus, one antigen was specific to S. cerevisiae and one to S. ellipsoideus. As S. ellipsoideus was antigenically more distinct than the rest of the species investigated, the authors considered that S. ellipsoideus should be retained as a distinct species.

The serological investigation of Saccharomyces species by slide agglutination was continued by Campbell and Brudzynski (1966). Certain antigens were common to S. cerevisiae, S. carlsbergensis and S. ellipsoideus. S. rouxii was very similar to, but not identical with, S. carlsbergensis. Some strains of S. carlsbergensis, S. ellipsoideus and S. diastaticus were serologically identical. Campbell (1968) divided seventeen species of Saccharomyces into four main serogroups with four absorbed sera and the members of the first three groups were further differentiated using five more absorbed sera. The serological properties were compared with physiological properties of Saccharomyces species (Campbell, 1970), and it was discovered that if two yeasts were serologically identical they were also more than 85% physiologically similar.

Extending the serological investigation to forty-three yeast species Campbell (1971 a) distinguished six serogroups using the absorbed sera previously used. However, the degree of differentiation was poor as species in the same genus could belong to different serogroups, for example, C. guilliermondii and C. krusei were in groups D and F respectively; or all species in a genus could belong to a serogroup in common with species of another genus, such as all the species of

Brettanomyces and Debaromyces which were in group D. Numerical taxonomy was used by Campbell (1971 b), who proposed an identification scheme for forty-seven species of thirteen genera which were characterised by both serological and selected physiological properties. This scheme was in general agreement with the classification of Lodder (1970). However, the resolution of antigenic differences is poor in Campbell's analyses as Richards (1972) stated "his (Campbell's) analyses were insufficiently precise for the detailed definition of serotypes", and concluded that "these conclusions (Campbell, 1971) cannot necessarily be accepted as a measure of comparison between definitive antigenic analysis and conventional yeast taxa".

Stewart-Tull et al., (1966) studied the serological and chemical properties of cell-walls of yeasts of eleven genera. Sugar constituents of the cell-walls were mannose and glucose except for R. mucilaginosa and Sp. salmonicolor which contained galactose and fucose, and H'spora valbyensis which contained galactose in addition to mannose and glucose. The cell-walls of species which contained no additional sugars reacted with antiserum to C. albicans, whereas the cell-walls of species with sugars additional to glucose and mannose did not agglutinate with antiserum to C. albicans. The presence of sugars other than mannose and glucose conferred a completely different serological specificity compared to the majority of yeasts which possess only glucose and mannose and cross-react serologically.

An extensive serological investigation of yeast whole cells was done by Tsuchiya and his colleagues using slide agglutination and agglutinin absorption. An antigenic scheme constructed from slide agglutination tests is, however, suspect because of the low degree of antigenic differentiation which can be achieved as stated by Richards (1972): "the studies of Tsuchiya et al., (1965) were designed for the finite definition of antigen composition, and the poorer precision of

their analysis can only be explained by their use of a slide agglutination test method". The antigens recognised were divided into two groups, those which were heat-labile and those which were heat-stable. Tsuchiya, Fukazawa, Miyasaki and Kawakita (1955) examined the antigenic structure of seven Candida species in which nine heat-stable and three heat-labile antigens were proposed; each species possessed a particular combination of antigens. C. albicans, C. pseudotropicalis and C. guilliermondii were the only species which possessed an antigen specific to themselves. Over eighty strains of Candida were shown to have identical antigenic properties to the seven species investigated indicating there was no strain variation (Tsuchiya, Kamijo, Fukazawa, Miyasaki and Kawakita, (1956). Sixteen more Candida species were investigated serologically using the antigenic scheme previously determined (Tsuchiya, Kamijo, Fukazawa, Kawakita and Nishikawa, 1956; Tsuchiya, Kawakita, Hayashi, Sato and Takahashi, 1957; Tsuchiya, Kawakita, Hayashi and Kobayashi, 1958), which resulted in the recognition of twenty-one heat-stable antigens and eight heat-labile antigens in the twenty-three species. The twenty-three species were divided into six serogroups depending on the possession of particular antigens.

The antigenic structures of asporogenous and ascosporogenous yeasts were compared (Tsuchiya, Fukazawa, Hayashi, Hayashi and Doi, 1957; Tsuchiya, Fukazawa, Hayashi, Amemiya and Sano, 1957), and it was demonstrated that C. robusta and S. cerevisiae were identical antigenically as were Kluyveromyces fragilis, K. marxianus, C. macedoniensis and C. pseudotropicalis. The genus Rhodotorula was examined serologically (Tsuchiya, Fukazawa, Amemiya, Yonezawa and Suzuki, 1957) and no reactions occurred with antiserum to Candida species. Consequently, an antigenic scheme was proposed with no relation to the one for Candida species. Seven heat-stable antigens were found for the six species investigated but no heat-labile antigens were found. R. glutinis and R. aurantiaca

were found to have identical antigens. R. minuta and R. pallida were completely distinct from the other species and showed no cross-reactivity.

The antigenic analysis of the genus Hansenula was done using the same antigen scheme proposed for Candida species (Tsuchiya, Fukazawa, Hayashi, Nishikawa and Doi, 1957; Tsuchiya, Fukazawa, Sato, Amemiya and Murata, 1958; Tsuchiya, Kawakita and Yamase, 1964; Tsuchiya, Yamase and Udagawa, 1964). Nine heat-stable antigens and one heat-labile antigen were designated for the species, the heat-labile antigen was found only in H. jadinii. Species with different physiological properties were found to be antigenically indistinguishable - for example, H. beijerinckii and H. californica, and H. silvicola and H. subpelliculosa. All the strains of a particular species were found to be serologically identical and the authors suggested that antigenic characteristics were a useful guide to phylogenetic relationships as originally proposed by Wickerham and Burton (1962).

Monospecific antisera prepared by absorption for every antigen attributed to the genera Hansenula and Candida were used in the serological study of Saccharomyces species (Tsuchiya, Fukazawa, Sato, Kawakita, Yonezawa and Yamase, 1958; Tsuchiya, Fukazawa and Yamase, 1961; Tsuchiya, Fukazawa, Kawakita, Imai and Shinoda, 1965). Further heat-stable antigens were recognised giving a total of forty antigens present in the three genera; some existing antigens were shown to be composed of more than one antigen. Physiological properties of all the strains tested were compared with the antigenic properties and all species were placed in five groups typified by Kluyveromyces marxianus, S. rouxii, S. cerevisiae and S. chevalieri, S. bisporus and S. delbrueckii. There was variation in both physiological and antigenic characteristics of strains of a particular species and the authors emphasised that both serology and physiology were essential, neither criterion being sufficient in itself.

In the genus Debaromyces, D. hansenii was found to be antigenically similar to C. guilliermondii, except that it possessed an additional antigen. Schwanniomyces occidentalis had identical heat-stable antigens to D. hansenii, but differed in the possession of a heat-labile one. D. globosus was antigenically quite distinct from the other species (Tsuchiya, Fukazawa, Sano, Shimura and Murata, 1960) and is classified as S. kloeckerianus at present.

A study of the genus Torulopsis (Tsuchiya, Fukazawa and Kawakita, 1961 a, b) revealed that T. glabrata and T. holmii were serologically related and were members of the C. albicans group. T. colliculosa was antigenically identical to S. rosei and S. fermentati. T. holmii was identical to S. exiguus, which is regarded as being its ascosporogenous counterpart. T. inconspicua was antigenically similar to Pichia membranaefaciens and T. ernobii similar to C. parapsilosis.

Three serogroups were demonstrated in the study of the genera Kloeckera and Hanseniaspora (Tsuchiya, Kawakita, Imai and Miyagawa, 1966). The first group was comprised of K. apiculata and its perfect form H'spora valbyensis, which were antigenically identical, the second group consisted of K. africana, K. javanica var javanica and K. corticis, which are all antigenically identical, and the third group consisted of K. javanica.

Species of the genus Sporobolomyces were found to have an antigenic scheme related to that of Rhodotorula species, but not to that of Candida species (Tsuchiya, Fukazawa and Suzuki, 1969). Sp. salmonicolor, Sp. odorus and Sp. roseus were shown to be antigenically identical and were members of the same group as R. glutinis. Sp. pararoseus was serologically related to these species and was placed in the same group, unlike Sp. gracilis which had no antigens in common with the other Sporobolomyces species and was related to R. minuta and R. pallida.

TABLE 5 :

Antigenic and physiological properties of yeasts (Tsuchiya et al, 1965)

(reprinted by courtesy of the publishers of Mycopathologia et Mycologia Applicata)

Fermentative, assimilative and serological characteristics of several strains of various species of yeasts

Group	Species	No. of strains	Fermentation	Assimilation	Antigenic structure	
	P. fermentans	5	D – – – –	D – – – – –	1,5,11	2
	P. krusei	1	D – – – –	D – – – – –	1,5,11 b	2
	C. krusei	138	D – – – –	D – – – – –	1,5(11) b	2
	C. catenulata	3	D g – – –	D G – – – –	1,5,11, b	2
	P. membranaefaciens	6	⊥ – – – –	D – – – – –	1,5,11,12	2
	C. mycoderma	8	⊥ – – – –	D – – – – –	1,5,11,12	2
	T. inconspicua	3	⊥ – – – –	D – – – – –	1,5(11)(12)	2
	C. reukaufii	2	d – – – –	D g ⊥ ⊥ – –	1(5)(11)(12)b, f	2
II	*D. fluxorum*	2	– – – – –	D – – – – –	1,5,11, m	2
	C. rugosa	12	– – – – –	D G – – – –	1, 11,19, g	2,3,4
	C. brumptii	4	d – – – –	D G – M – –	1, 19	2
	P. farinosa	2	D ⊥ – – –	D G – – – –	1, 29	2,3,4,
	S. pastori	1	D – – – –	D – – – – –	1, 25	3,4, 17,24
	C. melinii	4	⊥ – ⊥ ⊥ –	D – S M – K	1,5, 25	2,3,10,17
	H. angusta	4	D – – – –	D – S M – K	1(5)11	2, (17)
	C. solani	3	D – s – –	D g S M – –	1,5,11,12,37	2, 20,21
	H. californica	2	d – – – –	D – S – – K	1,16,20,21,22	2,14(15)(17)
	H. beigerinckii	3	D – S – –	D G S M – K	1,16,20,21,22	2,14,15,(17)
	H. mrakii	5	D – – – –	D – – – – K	1,16,20,21	2,14,15,(17)
	H. saturnus	9	D – S – –	D – S – – K	1,16,20,21	2(14)(15)(17)
	H. suaveolens	4	D – S – –	D – S – – K	1,16,20,21	2,14,15,(17)
VI	*H. silvicola*	2	D g – – –	D G S M – K	1(16)20	2,14(15)
	H. subpelliculosa	1	D – S m –	D ⊥ S M – K	1(16)20	2,14,15
	H. schneggii	3	D g s M –	D G S M – K	1,16,20	2,14(15)(17)
	H. anomala	42	D g S M –	D g S M – K	1,16,20	2,14,15,(17)
	C. pelliculosa	3	D G S M –	D G S M – K	1,16,20	2,14,15
	H. jadinii	1	D – S – –	D – S M – K	1(16) (c)	(2)14, 17
	C. utilis	6	D – S – –	D – S M – K	1(16) c	(2)(14) 17
	T. utilis	1	D – S – –	D – S M – K	1(16) (c)	(2)14, 17
	T. candida	3	⊥ – ⊥ – –	D G S M 1 –	1,9,(14),33	2,3,4
	D. subglobosus	1	d ⊥ s ⊥ –	D G S M 1 –	1,9, 14, 33	2,3,4
	T. famata	5	⊥ – – – –	D G S M – –	1(9)(14)	2,3 4
	D. kloeckeri	12	d – ⊥ ⊥ –	D G S M – –	1(9)(14)	2,3,4
	D. nicolianae	4	d g s m –	D G S M – –	1(9)(14)	2,3,4
	D. hansenii	12	d ⊥ s ⊥ –	D G S M ⊥ –	1(9)14	2,3,4
	T. globosa	2	D – S m –	D – S M – K	1,9,14	2,3,4
	H. matritensis	2	D – S m –	D – S m – K	1,9(14)	2,3,4
	Schw. occidentalis	2	D – S – –	D G S M – –	1,9,14 j	2,3,4
	C. guilliermondii	68	D g s ⊥ –	D G S M – –	1,9	2,3,4
	C. melibiosi	2	D g S m –	D G S M – –	1,9	2,3,4
	D. globosus	2	D – S – –	D – S – – –	1, 27, i	3, 10,24
	C. zeylanoides	2	– – – – –	D – – – – –	1,13,(a),c,h	2,3,4,17
	H. holstii	2	D – – – –	D – S M – K	1(13)	2,3,(14)(15)
V	*T. arnobii*	1	d – – – –	D – S M – –	1,13, c	2,3,5(14)(15)
	C. parapsilosis	84	D g ⊥ – –	D G S M – –	1,13, c	2,3,5(14)(15)
	C. pelliculima	4	D ⊥ – – –	D g S M – –	1,13, d	2,3,5,14,(15)
	S. rosei	12	D ⊥ S – –	D ⊥ S – – –	1,4,24	3
VII	*T. stellata*	2	D – S – –	D – S – – –	1,4,24	3
	S. fermentati	1	D – S M –	D – S M – –	1,4.24	3
	T. colliculosa	5	D – S M –	D – S M – –	1,4,24	3

Group	Species	No. of strains	Fermentation	Assimilation	Antigenic structure	
	T. glabrata	162	d – – – –	D – – – – –	1, 6,34, k	3, 10
	S. delbrueckii	17	D G – – –	D G – – – –	1,4,6,23	3,5(10)
	S. bisporus	4	D – – – –	D G – – – –	1,4, 26	3, 10
	S. acidifaciens	5	D – – – –	D G – – – –	1,4, 26	3, (10)
	S. exiguus	7	D G s – –	D G s – – –	1,4,6,26	3(5)10,32
I	*T. holmii*	5	D G S – –	D G S – – –	1(4)6,26	3(5)10,32
	C. stellatoidea	6	D – – M –	D G – M – –	1,4	2,3,5,10,32
	T. saka	1	D g S m –	D G S M – –	1,4,6	2,3,5
	C. tropicalis	201	D G S M –	D G S M – –	1,4,6	2,3,5
	C. claussenii	3	D G – M –	D G S M – –	1,4,6,7	2,3,5
	C. albicans	1368	D g ⊥ M –	D G S M – –	1,4,6,7	2,3,5
	K. apiculata	2	D – – – –	D – – – – –	1,10,28	8
	Hs. valbyensis	1	D – – – –	D – – – – –	1,10,28	8
	S. marxianus	19	D g S – –	D G S – 1 –	1,10,28, a	8, 31
	C. macedoniensis	4	D G S – –	D G S – L –	1,10,28, (a)	8, 31
	S. fragilis	2	D G S – L	D G S – L –	1,10,28, a	8, 31
	C. pseudotropicalis	18	D g S – L	D G S – L –	1(10)28, a	8, 31
	S. rouxii	11	D – – m –	D G – M – –	1(10)28	(8) 32
	S. carlsbergensis	7	D g S M –	D G S M – –	1(10)28	(8) 32
II	*S. italicus*	1	D G s M –	D G S M – –	1,10,28(18)	(2)(3)8,14,31
	S. chevalieri	3	D g S M –	D G S M – –	1(10), 18	2,3(8)(14)31
	S. oviformis	1	D – S M –	D G S M – –	1,10, 18	2,3, 14,31
	S. uvarum	3	D g S m –	D G S M – –	1,10, 18	2,3, 14,31
	S. cerevisiae	11	D g S M –	D g S M – –	1,10, 18,a,e	2,3, (14)31
	C. robusta	10	D G S M –	D G S M – –	1,10, 18,a,e	2,3, (14)31
	S. willianus	6	D g S M –	D G S M – –	1,10, 18(a)(e)	2,3, (14)31
	S. steineri	1	D G S M –	D G S M – –	1,10, 18, e	2,3 14,31
Rhodotor. Group	*Sp. salmonicolor*	1	– – – – –	D g S – – K	1, 9	8
	Sp. odorus	1	– – – – –	D ⊥ S – – K	1, 9	8
	R. glutinis	3	– – – – –	D G S M – K	1,2,5	8
	R. aurantiaca	1	– – – – –	D G S M – K	1,2,5	8
	R. mucilaginosa	44	– – – – –	D g S M – –	1, 3	8
	Sp. pararoseus	1	– – – – –	D g S M – –	1, 11	
	R. rubra	18	– – – – –	D G S M – –	1,2,(3)	8
	Sp. gracilis	1	– – – – –	D ⊥ – – – –	(4) (6)	
	R. pallida	1	– – – – –	D g – – – –	4, 6,10	
	R. minuta	9	– – – – –	D G S – – –	4, 7	
Cryptococc. Gr.	*Cr. neoformans*	66	– – – – –	D g S M – –	1, 6,8	2,4
	C. humicola	3	– – – – –	D G S M L –	1, (6)7	2(4)
	C. curvata	4	– – – – –	D G S M L –	1, 9	(2)(4)
	Cr. diffluens	3	– – – – –	D g S M – K	1,3	4
	Cr. albidus	3	– – – – –	D g S M – K	1,3, 7	(4)
	Cr. laurentii	2	– – – – –	D g S M L –	1, 5	
	T. aeria	2	– – – – –	D G S M I K	1, 11	
Sch. Gr.	*Schiz. pombe*	19	D – S M –	D – S M – –	1,3	
	C. lipolytica	3	– – – – –	D – – – – –	1,2...	
	T. bacillaris	2	D – S – –	D – S – – –	1,2...	

D, G, S, M, I. and K mean positive reactions in fermentation or assimilation of glucose, galactose sucrose, maltose, lactose and potassium nitrate, respectively. The small letter indicates weakly positive or positive reactions only in a few strains of the species. ⊥ means seldom positive or very weakly positive.

The importance of antigenic analysis to classification of yeasts was stressed repeatedly by Tsuchiya and his colleagues. A classification scheme was proposed (Tsuchiya, Fukazawa and Kawakita, 1965) which was based on serological and physiological properties. The initial emphasis was placed upon serology from which seven groups were distinguished with designated antigens based on the system described for Candida species (Table 5). Three other groups existed, Rhodotorula, Cryptococcus and Schizosaccharomyces, which were antigenically distinct. The authors concluded that in order to improve the natural classification other criteria should be taken into consideration, especially the serology of yeasts which, up to the present, has not been generally accepted in standard classification schemes.

Discrepancies occur in the work of Tsuchiya whereby an organism (A) is shown to possess an antigen because of its agglutination by an absorbed monospecific antiserum. However, another absorbed monospecific antiserum to the same antigen, but derived from an antiserum to a different yeast species, often failed to agglutinate the original organism A. The authors regarded this as being due to a difference in agglutinability and the ability to produce antibody. However, it is also possible that this is due to the erroneous designation of numbered antigens to each species. From the serological results presented above it is apparent that as yet no satisfactory complete yeast classification has been achieved using antigenic analyses of whole cells.

Serological relationships determined by precipitin methods with soluble yeast antigens

Mueller and Tomcsik (1924) prepared two antigenic extracts from S. cerevisiae which formed a precipitin with rabbit antisera to heat-killed whole yeast cells; the antigen dilution titre being 1/400,000. Both extracts failed to stimulate an antibody response when injected into

rabbits. Balls (1925) used an antigenic preparation from yeast autolysate. Considerable cross-reactions were observed among the ten species investigated, indicating a lack of specificity which was confirmed by Yukawa and Ohta (1929). Tomcsik (1930) distinguished two serogroups in the genus Saccharomyces using the precipitin test combined with complement fixation. Kesten, Cook, Mott and Jobling (1930) distinguished between two Candida isolates, C. albicans, Hansenula species and S. cerevisiae, using precipitin tests with absorbed antisera. Stone and Garrod (1931) prepared antigenic extracts of Candida isolates and found they were identical by precipitin tests. Lamb and Lamb (1935) demonstrated three Candida species, C. albicans, C. parapsilosis and C. krusei which were serologically distinct. Trimble (1957) absorbed C. albicans antiserum with C. stellatoidea and found that the absorbed serum reacted with C. albicans and C. tropicalis antigen preparations but not with C. parapsilosis, C. krusei, C. pseudotropicalis, C. guilliermondii or S. cerevisiae. Elinov and Zaikina (1959) continued the investigation of serological relationships within the genus Candida, and showed that C. pseudotropicalis and C. krusei antigens did not react with C. albicans antiserum to the same extent as C. tropicalis and C. albicans. C. krusei antiserum when absorbed with other Candida species still retained activity to C. krusei. Extracts of C. albicans, C. tropicalis and C. pseudotropicalis produced one precipitin line against homologous antisera, whereas C. krusei extract produced two precipitin lines - this suggested that serologically C. krusei was not closely related to the other species. However, few definite conclusions can be drawn from these two studies.

Campbell and Gilmour (1969) compared the precipitin test results with those previously obtained by agglutination and fluorescent antibody technique, and found that they were identical. This indicated that a cell-wall mannan was responsible for the antigenic differences of these

groups. The characterisation of yeast cell-wall mannans and polysaccharides by means of the precipitin test has contributed greatly to the understanding of the antigenic determinants present in the yeast cell-wall. Sakaguchi, Suzuki, Suzuki and Sanayama (1967) prepared mannan fractions from C. albicans and S. cerevisiae. Rabbit antisera to whole cells of both species were used to investigate the antigenic properties of the mannan. S. cerevisiae mannan reacted to the same degree as C. albicans mannan against C. albicans antiserum. On the other hand, the titre of the C. albicans mannan was 25% of that of S. cerevisiae mannan against S. cerevisiae antiserum which implies that C. albicans mannan contained all the major antigenic determinants of S. cerevisiae mannan in addition to those specific to itself. C. albicans antiserum absorbed with S. cerevisiae mannan retained activity to C. albicans mannan, whereas C. albicans mannan removed a significant amount of the antibody from S. cerevisiae antiserum reacting to S. cerevisiae mannan. The authors concluded that the antigenic differences of the mannans could not be explained solely by the chemical structure and linkages of the mannans, and they considered that macromolecular structure of the polysaccharides contributed to the antigenicity.

Hasenclever and Mitchell (1964 a) studied the reactions of polysaccharides extracted from Candida, Hansenula, Cryptococcus, Torulopsis and Saccharomyces species to antisera to C. albicans, C. stellatoidea and C. tropicalis by means of quantitative precipitin experiments. The results indicated close antigenic relationship among C. albicans serotype A, C. albicans serotype B, and C. tropicalis. C. stellatoidea would appear to be closely related to C. albicans serotype B but not to C. albicans serotype A or to C. tropicalis.

Precipitin inhibition of C. stellatoidea antiserum with oligosaccharides prepared from the cell-wall mannan was most marked with

pentasaccharides and hexasaccharides; the α 1-2 and α 1-3 linkages were considered important (Mitchell and Hasenclever, 1970). Suzuki, Sunayama and Saito (1968) and Suzuki and Sunayama (1968) demonstrated that the α 1-3 linked terminal mannose residue of the side-chains of mannans of S. cerevisiae and C. albicans contributed greatly to the antigenicity of the mannan. Side-chains ending in α 1-3 linked mannose units caused more inhibition of precipitin reactions than side-chains of the same length ending in α 1-2 linked mannose units. The advantage of the precipitin test is that a chemically defined substance can be shown to have antigenic properties and by fractionating a complex structure, such as a cell-wall, one can elucidate the individual components that possess antigenicity. With regard to serological interactions amongst whole cells of various species or genera, extraction and comparison of antigenic components will not necessarily reveal the true relationship, as the antibody response is directed not only against the primary configuration but also against the tertiary configuration of a molecule. With extracted antigens an antiserum may be produced which does not react with all components of the cell surface and thus the complete antigenic mosaic cannot be determined.

Biguet, Havez, Tran Van Ky and Degaey (1961) investigated the serological properties of the cytoplasmic contents of species of C. albicans by means of immunoelectrophoresis. C. albicans extract produced nine precipitin arcs against C. albicans antiserum; two antigens appeared to be specific for C. albicans and another was specific for C. albicans and C. stellatoidea. A common antigen against C. albicans antiserum was revealed in C. albicans, C. stellatoidea, C. tropicalis, C. zeylanoides, C. macedoniensis and C. pseudotropicalis which was not present in C. brumptii or C. krusei. The precipitin arcs of C. tropicalis and C. zeylanoides were indistinguishable. Later work (Biguet, Tran Van Ky and Andrieu, 1962) involved the cross-testing of

every antigen against antisera to every species. Forty-eight distinct antigens were detected; the majority were shared antigens except for a few which were specific to a particular species.

Tran Van Ky, Biguet and Andrieu (1963) prepared an antigenic extract from culture supernates of Candida species. After extensive dialysis to remove mannan components, immuno-electrophoresis showed seven antigenic components in C. albicans when tested against C. albicans antiserum. Five of these antigens were identical to antigens revealed on cytoplasmic material. The two antigens specific to the culture supernate were also specific for C. albicans and were not present in culture supernates of C. stellatoidea, C. tropicalis, C. zeylanoides, C. krusei, C. macedoniensis and C. pseudotropicalis.

Hasenclever and Mitchell (1964 a) determined the antigenic relationship of yeast polysaccharides by precipitin-inhibition and immuno-diffusion. Double diffusion tests showed that all Candida mannans produced reactions of identity with C. stellatoidea and C. albicans serotype B antisera. C. tropicalis antiserum reacted with mannans of C. tropicalis, C. stellatoidea and C. albicans serotype B. The precipitin lines of the latter two showed spur formation with the precipitin line of C. tropicalis indicating partial identity.

Immunodiffusion has been used predominantly in the diagnosis of systemic yeast infection. Taschdjian, Kozinn, Okas and Caroline, (1967) investigated the specificity of various antigenic preparations of C. albicans in gel diffusion tests against sera from patients with systemic conditions; a cytoplasmic antigen preparation gave the most reliable results. This preparation was also shown by Taschdjian, Kozinn, Fink, Cuesta, Caroline and Kantrowitz (1969) to give a higher percentage of positive results than were obtainable by the agglutination test. Stickle, Kaufman, Blumer and McLaughlin (1972) found that sera from patients

suffering from a wide range of systemic mycoses did not produce precipitin lines against C. albicans cytoplasmic antigen preparation. Double diffusion tests using suitable cytoplasmic antigens and antisera are reliable and specific and therefore of significant importance in the diagnosis of candidiasis.

Serological relationships determined by immunofluorescence

The first use of the fluorescent antibody technique in yeast differentiation was by Gordon (1958 a, b), who separated C. albicans from other Candida and yeast species. Kaplan and Kaufman (1961) described the identification of two serotypes of C. albicans by immunofluorescence. Gordon (1962) absorbed C. albicans antiserum with C. parapsilosis cells and subsequently conjugated it with fluorescein. This conjugated serum reacted with C. albicans and C. tropicalis but did not react with C. parapsilosis, C. stellatoidea and T. glabrata. Similarly T. glabrata antiserum when absorbed with C. albicans reacted with C. tropicalis but not with C. albicans or C. stellatoidea. By the use of two absorbed antisera, Gordon, Elliott and Hawkins (1964) distinguished C. albicans type A, C. albicans type B, C. tropicalis, C. stellatoidea and T. glabrata. Rimbaud, Bastide and Nakam (1966) studied fifteen species of Candida and demonstrated a close antigenic relationship between C. albicans, C. brumptii, C. parapsilosis, C. tropicalis, and C. pelliculosa, but considered that C. stellatoidea, C. lipolytica and C. zeylanoides were distinct antigenically.

Within the genus Saccharomyces, four serogroups were demonstrated by Campbell (1968) but fluorescent antibody techniques did not differentiate between members of the same group. In further studies, Campbell (1971) divided species of twelve genera of yeasts into six serogroups; species belonging to the same genus did not necessarily belong to the same serogroup. Richards (1972) proposed an antigenic

scheme for eight Saccharomyces species in which there were no natural serogroups as previously proposed by Campbell, (1968). Richards emphasized the reduction in the intensity of fluorescence after absorption of an antiserum, which indicated that antibody reacting with the antigens present on the yeast cells was partially absorbed. As the assessment of fluorescence was arbitrary and subjective (-, ±, ++, +++), and as there was probably more than one antigen involved in the absorption, it is doubtful whether one can allocate different antigens to species, let alone differentiate serogroups. Campbell (1968) found that agglutination methods produced a finer degree of resolution than immunofluorescent methods. Immunofluorescent techniques are more suitable for the identification of yeast cells in tissues for the rapid diagnosis of infection, e.g., cryptococcosis (Eveland, Marshall, Silverstein, Johnson, Iverson and Winslow, 1957).

Problems associated with the serological investigation of yeasts

The reasons for the poor serological classifications may be due to a number of factors:

a) The poor immunogenicity of yeasts

Early work to obtain antibody against yeast cells was unsuccessful. Malovez(1901) injected rabbits with live cells of Saccharomyces species and obtained maximum agglutination titres of between 1/30 and 1/90. Autoclaving the cells enhanced the response, but Defalle (1902) did not obtain a greater response when autolysed cells were inoculated. Bissérié (1901) immunised rabbits with yeasts and obtained higher agglutination titres, in the region of 1/200. Lichtenstein (1914) found that intravenous injection of S. cerevisiae cells into rabbits stimulated antibody which had an agglutination titre between 1/2000 and 1/5000. By contrast, Fineman (1921) had difficulty in stimulating antibody

formation in rabbits after injection of five Candida isolates; two antisera were produced, one with a titre of 1/20 against both strains and the other with a homologous titre of 1/50. Fineman concluded that "agglutinins are not produced by the thrush parasite in sufficient quantities to be of diagnostic or differential value". Throughout the studies of Tsuchiya and co-workers anomalous results occurred in which a yeast was demonstrated to possess a particular antigen by use of heterologous absorbed antisera, although homologous sera did not contain this specificity. The authors considered such an antigen to be a hapten which is capable of agglutination with corresponding antisera but which will not induce an antibody response.

b) Occurrence of natural antibody

The occurrence of natural antibody to yeast in normal sera of rabbits and human beings causes difficulties in serological procedures. Drake (1945) found by slide agglutination that antibody to C. albicans was present in 89% of human and 63% of rabbit sera; antibody to C. krusei in 22% and 61% respectively; and antibodies to S. cerevisiae in 53% and 67% respectively. The majority of the titres was less than 1/10. Winner (1955) found a much lower incidence of antibody to C. albicans in human sera, 31.6%, and those sera which had higher titres (greater than 1/16) were not always associated with infection. Norris and Rawson (1947) demonstrated that 64% of the sera of hospital patients contained antibodies against C. albicans and S. cerevisiae. Rawson and Norris (1947) found by absorption experiments that H. anomala, C. albicans and S. cerevisiae had an antigen or antigens in common which would account for the presence of agglutinins to both S. cerevisiae and C. albicans in the sera of patients.

c) Presence of deeply-located antigens

It is possible that the phenomenon proposed by Hasenclever and Mitchell (1960) is relevant, that is, the antigens are too deeply located in the cell to take part in agglutination, but can induce an antibody response. This would be analogous to the Rh antigen of human red blood cells. Hasenclever et al., (1961) and Murray and Buckley (1966) found that in certain instances absorption must be performed with disintegrated cells to achieve complete removal of antibody to the species used for absorption. This fact could also be responsible for the anomalous results of Tschuiya and his colleagues.

d) Autoagglutination of whole cells

Agglutination endpoints were difficult to determine in some instances because of autoagglutination. Seeliger and Schröter (1963) encountered this problem in a study of the genus Trichosporon and concluded that agglutination methods were inferior to complement fixation and precipitin methods.

e) Comparison of results of different serological methods

Negroni (1969) found that the agglutination test was of little diagnostic value, whereas complement fixation separated the systemic from the superficial infection in seventy candidiasis patients. In addition, Andersen (1968) found that 46% of the sera from eight hundred normal persons contained agglutinins to C. albicans, but only 2% contained complement fixing antibodies to C. albicans. The presence of such a high proportion of agglutinins in normal sera confirms that the agglutination test is an unreliable diagnostic procedure.

Slide agglutination has been criticised by Hasenclever et al., (1961) and Richards(1972) as being less reliable than tube agglutination or immunofluorescence. Certain antigenic differences could not be

demonstrated by slide agglutination, such as the distinction between C. albicans group A and group B strains which Tsuchiya, Fukazawa and Kawakita (1965) considered to be identical. Hasenclever et al., (1961) suggested that the 1/30 dilution of serum which they used might miss subtle antigenic differences. Since Tsuchiya and co-workers employed a serum dilution of 1/128 the major antigenic differences would be delineated but not the minor ones. A more sensitive method such as tube agglutination or complement fixation would, therefore, give better definition of antigenic structures.

During the course of the work presented in this thesis these problems were considered and where possible controlled by modification of existing techniques.

MATERIALS AND METHODS

CULTIVATION OF YEASTS AND PREPARATION OF CELL-WALLS

Strains of yeasts

Pure cultures of 26 species of yeasts were obtained from the Centraalbureau voor Schimmelcultures, Delft, Netherlands. The type strains of species and the type species of genera are listed in Table 6. For purposes of this thesis the inter-relationships of the genera were taken as described by Lodder (1970). Figure 3 shows the 20 genera grouped into families and sub-families within the three classes, Ascomycetes, Basidiomycetes and Fungi Imperfecti. Group I yeasts are members of the Ascomycetes, Group II yeasts are members of the Basidiomycetes and Group III yeasts are members of the Fungi Imperfecti.

Cultivation of yeasts

Yeasts were grown on 4% (w/v) malt extract agar consisting of 4% (w/v) malt extract (Boots Pure Drug Co. Ltd., Nottingham) and 2% (w/v) agar (Bacto-agar, Difco Laboratories Inc., Detroit) in distilled water. Agar slopes were inoculated and incubated at room temperature (approximately 20°C) or at 30°C depending upon the temperature sensitivity of each yeast. R. glutinis, K. apiculata, N. fulvescens, B. alba and Sp. roseus were incubated at room temperature for 72 hours, the remainder at 30°C for 48 hours. Roux bottles (1 litre) containing 200 ml. of malt extract agar were autoclaved at 121°C for 15 minutes and then laid flat to ensure the maximum surface area of the agar. The yeasts in slope cultures (48 or 72 hours old) were suspended in 5 ml of sterile distilled water and used to inoculate the agar layer in the Roux bottles. The entire agar surface was covered with the inoculum by tilting the Roux bottles before incubating at the appropriate temperature for 48 or 72 hours.

TABLE 6 :

List of species of yeasts

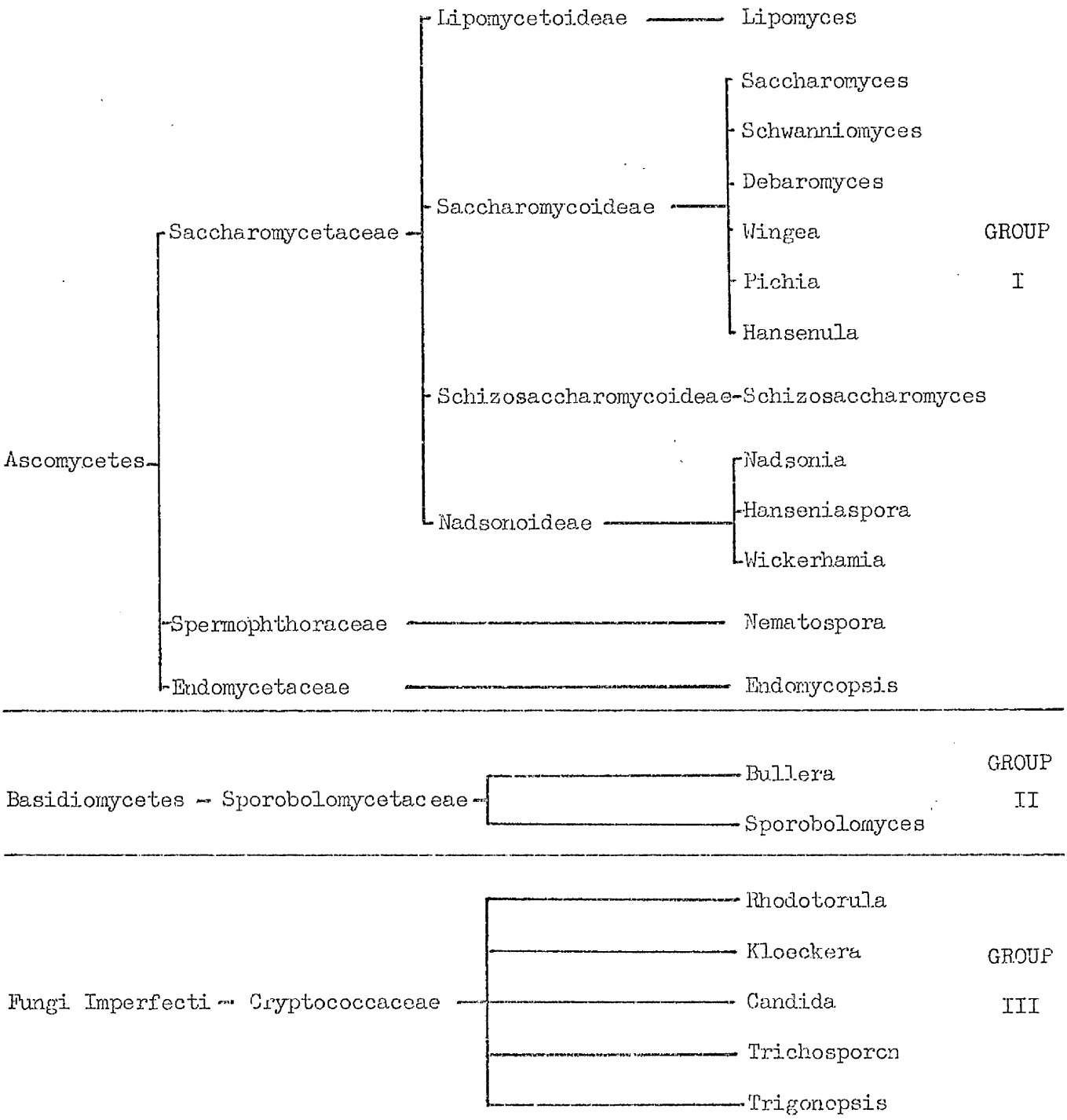
Lipomyces starkeyi	Lodder et van Rij	CBS 1807
Saccharomyces cerevisiae	Hansen	CBS 1171
Saccharomyces bayanus	Saccardo	CBS 380
Saccharomyces rouxii	Boutroux	CBS 732
Saccharomyces uvarum	Beijerinck	CBS 395
Schwanniomyces occidentalis	Kloecker	CBS 819
Debaromyces hansenii	(Zopf) Lodder et van Rij	CBS 767
Wingea robertsii	van der Walt	CBS 2934
Pichia membranaefaciens	Hansen	CBS 107
Hansenula anomala	(Hansen) M. et P. Sydow	CBS 5759
Schizosaccharomyces pombe	Linder	CBS 356
Nadsonia fulvescens	(Nadson et Konokotina) Sydow	CBS 2596
Hanseniaspora valbyensis	Kloecker	CBS 479
Wickerhamia fluorescens	Soneda	CBS 4565
Nematospora coryli	Peglion	CBS 2608
Endomycopsis capsularis	(Schionning) Dekker	CBS 2519
Bullera alba	(Hanna) Derx	CBS 501
Sporobolomyces roseus	Kluyver et van Niel	CBS 486
Rhodotorula glutinis	(Frensenius) Harrison	CBS 20
Kloeckera apiculata	(Rees emend. Kloecker) Janke	CBS 104
Candida albicans	(Robin) Berkhout	CBS 562

TABLE 6 : (continued)

Candida guilliermondii	(Castellani)	
	Langeron et Guerra	CBS 566
Candida krusei	(Castellani.) Berkhout	CBS 573
Candida stellatoidea	(Jones et Martin)	
	Langeron et Guerra	CBS 1905
Trichosporon cutaneum	(de Beurmann, Gougerot, et Vaucher)	
	Ota	CBS 2466
Trigonopsis variabilis	Schachner	CBS 1040

FIGURE 3 :

Classification of yeasts (Lodder, 1970)



Harvesting of cells

After incubation, the organisms were harvested by emulsifying the growth in distilled water with the aid of a glass rod. As yields varied considerably from one species to another, between 10 and 30 Roux bottles were required per species to obtain a sufficient quantity of cells. The suspension of cells was centrifuged in an MSE 18 centrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex), and washed six times in distilled water to remove any contaminating culture material. The cells were stored at -20°C or used immediately for preparation of cell-walls.

Preparation of cell-walls

Several methods were examined for the disintegration of yeast cells: ultrasonic disintegration, grinding cells with "Zeolite 4A" (sodium aluminium silicate), and mechanical disruption in the Braun disintegrator (Braun model MSK homogeniser, supplied by Shandon Southern Instruments, Camberley, Surrey). The Braun disintegrator was the most efficient machine for disintegration of cells with maximum recovery of cell-walls. Packed yeast cells were resuspended in distilled water to give a thick paste which was placed together with an equal volume of No. 12 Ballotini beads in a 100 ml steel bottle and cooled to 4°C. Disruption of the cells was achieved by shaking for successive periods of 1-minute at speed setting 2 (approximately 4000 r.p.m.) in a constant stream of vapourising CO_2 to avoid over-heating. The degree of disruption was assessed by taking samples at 2-minute intervals and staining with Gram stain to detect remaining whole cells. Disintegration was regarded as complete when no intact cells were observed in 20 fields of the light microscope at 1000 times magnification. This was usually achieved after 6 to 8 minutes of treatment.

Cell disintegrates and Ballotini beads were decanted into a 1 litre beaker and the beads allowed to settle. The supernatant fluid was decanted, the beads were washed with distilled water at 4°C, and the supernatant fluids were pooled. The combined supernatant fluids were transferred to 250 ml centrifuge bottles and centrifuged at 500 g for 10 minutes in an MSE 18 centrifuge at 4°C. The pellet was collected and the supernatant fluid was centrifuged at 1500 g for 10 minutes and a second pellet collected. Further centrifugation of the supernatant fluid at 10,000 g for 30 minutes did not sediment any further cell-wall material. Both pellets were examined microscopically to ensure that no whole cells were present, washed at least 6 times with distilled water and freeze-dried.

ESTIMATION OF MONOSACCHARIDE COMPONENTS OF CELL-WALLS

Hydrolysis of cell-walls

Freeze-dried cell-walls (10 mg) were weighed and suspended in 2 ml of 2N H_2SO_4 (Analar grade, BDH Chemicals Ltd., Poole, Dorset) in a large freeze-drying ampoule previously chrome-cleaned. The sealed tube was kept at 105°C for 2 hours. The hydrolysates were neutralised with solid $BaCO_3$ (Analar grade, BDH) and the $BaSO_4$ precipitate removed by filtration through Whatman No. 1 filter paper (W. and R. Balston Ltd., London). The filtrates were evaporated to dryness over a boiling water-bath and redissolved in 2 ml of distilled water. Controls of glucose, arabinose, galactose and glucosamine were treated in parallel to estimate the loss occurring during hydrolysis and subsequent preparation. For hexasomine determination the cell-walls were hydrolysed in 6N HCl (Analar, BDH) for 18 hours at 105°C. The hydrolysates were filtered and evaporated to dryness over a boiling water-bath and dissolved in 2 ml of distilled water.

Chromatography of yeast cell-wall hydrolysates

The H_2SO_4 yeast cell-wall hydrolysates were applied to Whatman No. 4 paper as discrete spots. Glucose, galactose, mannose, rhamnose, fucose and arabinose standard solutions were similarly applied as reference sugars as was a mixture containing all these sugars. One-dimensional descending chromatography was done using ethyl acetate + pyridine + water (160 + 40 + 20 by volume) as the solvent (Jermyn and Isherwood, 1949). To differentiate between glucose, galactose and mannose, chromatography was carried out for 18 hours; and to differentiate between rhamnose, fucose and arabinose, and the 3 previously mentioned sugars chromatography was done for 6 hours. The sugars were revealed by spraying with aniline hydrogen phthalate.

Total hexose estimation by the anthrone method

Estimations of the hexose content of cell-wall hydrolysates were carried out by an anthrone test (Scott and Melvin, 1953). Standard solutions containing 10, 20, 40, 60, 80 and 100 µg of glucose in 3 ml of distilled water, plus a reagent blank of 3 ml of distilled water, were included in the assay. Each hydrolysate (0.04 ml) was made up to 3 ml with distilled water, with the exception of the glucose control where 0.01 ml was used. An "Agla" micrometer syringe (Burroughs Wellcome and Co., London) was used to measure the volumes of hydrolysates for assay as volumes of 0.01 ml could be measured accurately. Tubes containing the hydrolysates in distilled water were placed in an ice-bath and 6 ml of anthrone reagent (Appendix) was added. After mixing thoroughly the tubes were transferred to a boiling water-bath for 3 minutes and subsequently cooled to room temperature. Extinction values (E) were determined at 625 nm in a Pye Unicam SP 600 spectrophotometer against the reagent blank. A standard curve was drawn of E 625 nm plotted

against the amount of hexose in each standard and unknown values calculated by simple proportion from a curve.

Total pentose estimation by the orcinol method

Estimations of the pentose content of cell-wall hydrolysates were made by a modification of the orcinol method (Dische and Schwartz, 1937). Standard solutions containing 10, 20, 40, 60, 80 and 100 µg of arabinose (Analar, BDH) in 3 ml of distilled water, and a reagent blank of 3 ml of distilled water were set up. Each hydrolysate (0.2 ml) was made up to 3 ml with distilled water with the exception of the arabinose control of which 0.01 ml was taken. Volumes of samples were measured using the Agla micrometer syringe. Ferric alum reagent (3 ml) (Appendix) was added to each sample plus 0.3 ml of the orcinol reagent (Appendix). The mixtures were placed in a boiling water-bath for 20 minutes, cooled to room temperature, and the E 660 nm of each sample determined in a spectrophotometer against the reagent blank. A standard curve was drawn of the E 660 nm values against the amount of pentose in each standard solution, and unknown values calculated by simple proportion.

Total hexosamine estimation by the Cessi and Piliego method

A modification of the Cessi and Piliego (1969) method was used to estimate the hexosamine content of cell-wall hydrolysates. Standard solutions containing 10, 20, 40, 60, 80 and 100 µg of glucosamine (Analar, BDH) in 2 ml of distilled water and a reagent blank of 2 ml of distilled water were prepared. Each hydrolysate (0.2 ml) was made up to 2 ml with distilled water with the exception of the glucosamine control of which 0.01 ml was taken. To each sample 5.5 ml of acetylacetone reagent was added (Appendix) the sample tubes were stoppered and sealed with tape before being placed in a boiling water-bath for 20 minutes. After

cooling to room temperature, the mixtures were transferred to 100 ml distillation flasks which were attached to a small water-cooled condenser. Distillation was done at a rate of 1 ml per minute and 4 ml of distillate was collected in a 10 ml measuring cylinder containing 6 ml of aldehyde reagent (Appendix). The solutions were left for 30 minutes to allow the colour to develop and the E 545 nm measured against the reagent blank. A standard curve was drawn of E 545 nm values plotted against the corresponding amount of hexosamine in each standard, and the unknown values calculated by simple proportion.

Glucose estimation by the glucose-oxidase technique

The glucose content of cell-wall hydrolysates was estimated by a linked enzyme system of glucose oxidase, peroxidase and a chromogen (Saifer and Gerstenfeld, 1958). The reaction can be represented by:

$$\text{Glucose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Glucose}} H_2O_2 + \text{Glucuronic Acid}$$

$$H_2O_2 + \text{Reduced chromogen} \xrightarrow{\text{peroxidase}} \text{Oxidised Chromogen} + H_2O$$

The enzyme reagent (Appendix) was dissolved in 0.1 M phosphate buffer pH 7.0 (Appendix). Standard solutions contained 10, 20, 40, 60, 80 and 100 µg of glucose (Analar, BDH) in 2 ml of distilled water and a reagent blank of 2 ml distilled water was included. Each hydrolysate (0.1 ml.) was made up 2 ml with distilled water, with the exception of the glucose control of which 0.01 ml was taken. The micrometer syringe was used to measure hydrolysate volumes as before. Two millilitres of the reagent solution were added to each sample at timed intervals and the reaction allowed to proceed for 30 minutes in a 37°C water-bath before being stopped by the addition of 0.2 ml of 4N HCl

(Analar, BDH) which stabilised the colour. The E 425 nm of each sample was measured spectrophotometrically against the reagent blank. A standard curve was drawn of E 425 nm of each standard solution plotted against the amount of glucose in the standard solution and unknown values calculated by simple proportion.

Galactose estimation by the galactose-oxidase technique

A coupled enzyme system of galactose oxidase, peroxidase and chromogen was used to estimate the galactose content of the cell-wall hydrolysates. The reaction can be represented by:

$$\text{Galactose} + O_2 \xrightarrow[\text{Oxidase}]{\text{Galactose}} \text{Galacto-hexodialdose} + H_2O_2$$

$$H_2O_2 + \text{reduced chromogen} \xrightarrow{\text{peroxidase}} \text{oxidised chromogen} + H_2O$$

The enzyme reagent (Appendix) was dissolved in 0.1 M phosphate buffer pH 7.0. Standard solutions contained 10, 20, 40, 60, 80 and 100 μg of galactose (Analar, BDH) in 2 ml of distilled water and a reagent blank of distilled water was included. Each hydrolysate (0.2 ml) was made up to 2 ml with distilled water with the exception of the galactose control of which 0.02 ml was taken. Two millilitres of the reagent solution was added to each sample at timed intervals and reacted for 60 minutes in a 37°C water-bath before being stopped by the addition of 6 ml of glycine buffer pH 9.7 (Appendix) which stabilised the colour. The E 425 nm of each sample was measured in a spectrophotometer against the reagent blank. A standard curve was drawn of the E 425 nm of each standard solution plotted against the amount of galactose present in the standard, and unknown values calculated by simple proportion.

Loss during hydrolysis

The percentage recovery of each sugar after hydrolysis was calculated and a correction factor was applied to each sugar estimation to give a true indication of the amount of sugar present in the cell-walls hydrolysates.

SEROLOGICAL INVESTIGATION OF YEAST CELL-WALLS

Preparation of antisera

Lyophilised cell-walls in 5 mg amounts were suspended in 1 ml of normal saline and thoroughly mixed with 0.5 ml. of Arlacel A (Honeywell-Atlas Ltd., Carshalton, Surrey) and 1 ml of Marcol 52 (Esso, Abingdon, Berkshire). Six-month old female Dutch rabbits (Hyline Commercial Rabbits, Hartford, Cheshire) were given an intramuscular injection of the water-in-oil emulsion containing yeast cell-walls into the inner aspect of the hind limbs, and subsequently 3 further injections in the same site at 2-week intervals. The animals were bled prior to injection and 10 days after each injection. Serum samples were stored at -20°C without preservative.

Absorption of antisera

To 0.25 ml of inactivated antiserum (56°C for 30 minutes) diluted 1/2 with complement fixation test (CFT) diluent, 10 mg of cell-walls in 0.25 ml of CFT diluent were added. The reaction was allowed to proceed at 4°C for 18 hours after which the mixture was centrifuged to sediment antigen-antibody complexes and excess cell-walls. To the supernatant fluid a further 10 mg of cell-walls were added in 0.5 ml of CFT diluent and left at 4°C as before. The supernatant fluid was tested for residual antibody activity against the absorbing cell-walls,

and if any remained the absorption was repeated.

Cell-wall agglutination test

A fine suspension (0.1 mg/ml) of cell-walls in normal saline was prepared by ultrasonic agitation. The test was set up in Dreyer tubes with two-fold dilutions of the antiserum in 0.2 ml volumes in normal saline and an equal volume of the cell-wall suspension. Saline controls were included containing 0.2 ml of the cell-wall suspension plus 0.2 ml of saline. The contents of the tubes were thoroughly mixed and incubated at 56°C for 2 hours after which the titres were read. Longer periods of incubation or overnight incubation at 4°C resulted in sedimentation of the cell-walls which masked the agglutination endpoint. Pre-injection rabbit sera were similarly titrated to ensure that no antibody was present in the rabbits used for immunisation. The endpoint of the titration was taken as the highest dilution of antiserum which produced definite agglutination of the cell-wall suspension; approximately equivalent to a 50% endpoint.

Complement fixation test (CFT)

Standardisation of erythrocyte suspension

A suspension of sheep erythrocytes in Alsever's solution (Oxoid, London) was centrifuged and the plasma removed by aspiration. The packed cells were resuspended in barbitone-buffered complement fixation test diluent (Oxoid) and washed three times in the same diluent. A capillary tube was filled with the packed cells and centrifuged in a haematocrit head (MSE Minor Centrifuge and MSE Haematocrit Head). The packed cell volume (PCV) was read directly on an MSE Micro Haematocrit Reader and the packed erythrocytes were diluted accordingly in the diluent to give a 4% (v/v) suspension.

Preparation of CFT haemolytic indicator system

Rabbit anti-sheep erythrocyte haemolytic antibody (Wellcome Reagents Ltd., Beckenham Kent) of haemolytic titre 1/2000 to 1/3000 was diluted to 1/500 in a volume equal to that of the erythrocyte suspension. The haemolytic antibody solution was carefully added with mixing to the erythrocyte suspension and incubated at 37°C for 15 minutes for sensitisation. The final suspension contained 2% (v/v) of sensitised erythrocytes.

Titration of complement

Lyophilised guinea-pig serum (Wellcome Reagents, Ltd.) was reconstituted with distilled water and dilutions prepared in CFT diluent from 1/60 to 1/288 in succeeding dilution increases of 20%. Each complement dilution (0.1 ml) was added to wells in a standard WHO haemagglutination tray containing 0.2 ml of CFT diluent. Two controls were included, one of which contained 0.3 ml of CFT diluent and no complement, and one which contained 0.1 ml of the 1/60 dilution of complement in 0.2 ml of CFT diluent. The tray was placed on filter paper soaked with CFT diluent in a plastic box fitted with a tight lid and incubated for 18 hours at 4°C on a shaking table. After incubation, 0.1 ml of sensitised erythrocyte suspension was added to all the wells except for the duplicate 1/60 dilution of complement to which 0.1 ml of unsensitised erythrocyte suspension was added. The tray was incubated at 37°C for 1 hour, the erythrocytes were kept in suspension by swirling the tray every 10 minutes, and at the end of the incubation period the test was left at room temperature until the erythrocytes sedimented. After ensuring that no lysis occurred in the controls, the complement dilution at which there was 50% haemolysis was noted. This complement dilution was taken as containing one 50% haemolytic unit (HU_{50}). For

subsequent complement fixation tests, 4 HU_{50} of complement were used.

Anticomplementary activity of rabbit anti-yeast cell-wall sera

Antisera to yeast cell-walls were heated at 56°C for 30 minutes to inactivate their own complement. Two-fold dilutions of antisera in 0.1 ml amounts were made in CFT diluent in WHO trays starting at a 1/5 dilution. Complement controls were included consisting of 0, 1, 2 and 4 HU_{50} of complement in CFT diluent and also controls containing the 1/5 dilution of antisera in CFT diluent to which no complement was added. After the addition of 4 HU_{50} of complement and 0.1 ml of diluent to the antisera dilutions, the tests were incubated for 18 hours at 4°C. Sensitised erythrocytes were added to all the wells, and the complement fixing titres recorded after incubation at 37°C and sedimentation of the erythrocytes.

Anticomplementary activity of cell-walls

Suspensions of cell-walls containing 1 mg/ml in CFT diluent were ultrasonicated and two-fold dilutions were made. Complement controls were included and controls containing the lowest dilution of the cell-wall suspension. Complement (4 HU_{50}) was added to the dilutions with the exception of the duplicate cell-wall controls. Incubation was carried out at 4°C and 37°C and the titres read as described.

Complement fixation test

Two-fold dilutions of antiserum in CFT diluent were made in 0.1 ml volumes with an initial dilution of 1/20. Volumes of complement (0.1 ml) containing 4 HU_{50} and cell-wall suspensions containing 0.1 mg cell-walls/ml were added. The tests were held at 4°C for 18 hours. Subsequently 0.1 ml of a standardised suspension of sensitised erythrocytes (2% v/v) was added and the tests were held for a further period of 1 hour at 37°C

after which the titres were read. Controls included in the test were:

a) Antiserum + Complement + sensitised erythrocytes

b) Antiserum + Antigen + sensitised erythrocytes

c) Complement + Antigen + sensitised erythrocytes

d) 4 HU_{50}, 2 HU_{50}, 1 HU_{50}, 0 HU_{50} } Complement + sensitised erythrocytes

In controls lacking one or more reagents, the equivalent volume was made up with diluent.

NUMERICAL ANALYSES

Coding of characters

For purposes of numerical taxonomy, the chemical properties of the cell-wall were divided into multistate characters; four characters were assigned to each of the hexose, glucose and mannose content of the cell-wall, two characters to hexosamine content, and one character to galactose content. Additive scoring was used for the multistate characters. The serological reactions of cell-walls were coded 0 or 1 depending upon the reactivity against each of the antisera in agglutination and complement fixation tests. A titre of less than 25% of the homologous titre was scored as 0. A titre of 25%, or greater, of the homologous titre was scored as 1.

Evaluation of similarity

The similarities amongst the yeasts were assessed by the Simple Matching Coefficient (Sokal and Michener, 1958), and the Average Squared Euclidean

Distance (Sokal, 1961). Both values had to be used because not all clustering methods were valid with the former value but all were valid with the latter.

a) Simple Matching Coefficient (S_{sm})

Both negative and positive matches are included in the expression:

$$S_{sm} = \frac{m}{(m + u)}$$

where m is the number of positive and negative matches, and u is the number of non-matches.

b) Average Squared Euclidean Distance (d^2)

For each character an object may be + or -, or 1 and 0 in binary form. This value determines the position of the object on an axis on which it can occupy either of the 2 points 0 and 1. Each character has an axis at right angles to the previous ones of which there can be any number in the theoretical Euclidean hyperspace. The distance between 2 objects can be calculated from the expression:

$$\Delta^2 = (a_1 - a_2)^2 + (b_1 - b_2)^2 + ... + (n_1 - n_2)^2$$

where Δ^2 is the square of the distance, a_1 is the value (0 or 1) for the first character of the first object, a_2 is the value for the first character of the second object, b_1 and b_2 are the values of the second character for both objects, and n_1 and n_2 represent the values of the last character for both objects. The Average Squared Distance is obtained by dividing the squared distance by the number of characters (n). The Average Squared Distance (d^2) is related to the Simple Matching Coefficient by the expression:

$$d^2 = 1 - S_{sm}$$

Clustering Methods

Single linkage (Sneath, 1957)

The two objects with the highest similarity (or smallest distance) in the group under consideration form a cluster. At the next highest similarity value, the next two objects form a cluster or if one is already in a cluster the other is added to the cluster. This process continues with clusters fusing together when one object of one has the next highest similarity value with one in the other cluster. Thus, while two clusters may be linked by this method on the basis of a single bond, many of the objects of the two clusters may be quite distant from each other.

Complete linkage (Sorensen, 1948)

Clustering by complete linkage does not appear to have been used in numerical taxonomy of microorganisms. It is similar in procedure to clustering by single linkage except that an object is permitted to join a cluster if it has the same or greater similarity values with all the members of the cluster. Clusters fuse when the similarity values of all the members of both clusters are equal to or greater than the value under consideration. Thus, a single similarity value with just one member of the cluster is insufficient.

Average linkage (Sokal and Michener, 1958)

The procedure of clustering in order of descending similarity values is followed as before. The condition for entry of any individual into a cluster is based on the average of the similarities of that individual with the members of the cluster. Clusters fuse on the basis of the average of the similarities of the members of both clusters. When a cluster is formed, or added to, the new similarity between it and all the other clusters or individuals is re-calculated.

Ward's Method (Ward, 1963)

Ward's method of clustering is based on the premise that the greatest amount of information is available when a collection of objects is ungrouped. If a group is formed it is recognised by the characteristics common to all members in the group instead of the total number of characteristics of all the members of the group. Ward's linkage method selects two objects to form a cluster which produces the least impairment of the available information. This is calculated by means of a value-reflecting number calculated from the "error sum of squares", (ESS):

$$ESS = \sum_{i=1}^{n} x_i^2 - \frac{1}{n}\left[\sum_{i=1}^{n} x_i\right]^2$$

where x_i is the score of the i th individual and is calculated from the average squared distance value. As the clusters are formed, the ESS increases to the level at which all members are contained in one cluster.

Numerical analyses

Numerical analyses were made on an IBM 370/155 at the Edinburgh Regional Computing Centre. The analyses were part of a program package "CLUSTAN 1A" (Wishart, 1969) with amendments by Middleton (1972). The printout produced the similarity matrix, the level and the composition of every cluster formed, and the correct sequence of the strains for a dendrogram.

RESULTS

MONOSACCHARIDES IN YEAST CELL-WALLS

Paper chromatography of cell-wall hydrolysates

Sulphuric acid hydrolysates of cell-walls were examined by descending paper chromatography in ethyl acetate/pyridine/water (160/40/20 v/v). After spraying sugar chromatograms with aniline phthalate, spots corresponding to glucose, mannose and galactose were revealed, Table 7. All strains contained detectable amounts of glucose and mannose; no pentose sugar was detected on the chromatograms. Two of the strains, L. starkeyi and Wick. fluorescens also contained galactose.

Quantitative analysis of monosaccharide components

Hexose content of cell-wall hydrolysates

The results of quantitative analysis for hexose using the anthrone test on neutralised H_2SO_4 hydrolysates of all the 26 species of yeast are shown in Table 8. Each tabulated result, expressed as a percentage of the cell-wall weight is the mean of at least two independent tests. The Table shows that the hexose contents of hydrolysates ranged from 7 to 69% of the cell-walls; the average value was 39%.

However, the results show that each group contains species with high and low hexose levels, summarised as follows:

Group I : mean 38% ; range 16-62%

Group II : mean 28% ; range 24-32%

Group III : mean 43% ; range 7-69%.

Even within a genus there was considerable variation between species. For example, in Saccharomyces the hexose contents of the 4 species varied from 27 to 62% and in Candida from 18 to 67%.

TABLE 7 :

Chromatographic determination of cell-wall monosaccharides

GROUP	SPECIES	Monosaccharides in cell-wall hydrolysates		
		Glucose	Mannose	Galactose
I	*L. starkeyi*	+	+	+
	S. cerevisiae	+	+	-
	S. bayanus	+	+	-
	Schw. occidentalis	+	+	-
	P. membranaefaciens	+	+	-
	W. robertsii	+	+	-
	H. anomala	+	+	-
	H'spora valbyensis	+	+	-
	Wick. fluorescens	+	+	+
III	*C. albicans*	+	+	-
	R. glutinis	+	+	-

TABLE 8 :

Monosaccharide content of yeast cell-wall hydrolysates

(expressed as percentage dry cell-wall weight)

GROUP	SPECIES	Percentage Hexose	Glucose	Galactose	Mannose
I	L. starkeyi	22	9	11	2
	S. cerevisiae	62	38	0	24
	S. bayanus	48	29	0	19
	S. rouxii	45	13	0	32
	S. uvarum	27	7	0	20
	Schw. occidentalis	27	12	0	15
	D. hansenii	34	19	0	15
	P. membranaefaciens	26	20	0	6
	W. robertsii	35	19	0	16
	H. anomala	50	24	0	26
	Schiz. pombe	58	45	12	1
	N. fulvescens	33	8	9	16
	H'spora valbyensis	16	12	0	4
	Wick. fluorescens	51	15	16	20
	Nem. coryli	38	13	0	25
	E. capsularis	38	12	13	13
II	B. alba	32	28	0	4
	Sp. roseus	24	6	0	18
III	R. glutinis	7	3	0	4
	K. apiculata	69	19	0	50
	C. albicans	42	25	0	17
	C. guilliermondii	40	4	0	36
	C. krusei	67	17	0	50
	C. stellatoidea	18	12	0	6
	Tr. cutaneum	42	4	23	15
	Trig. variabilis	62	10	38	14

Glucose content of cell-wall hydrolysates

Glucose analyses by the glucose oxidase method were made on the same H_2SO_4 hydrolysates. The results, based on duplicate determinations, showed a wide range of values in the cell-walls of the 26 type species, Table 8. The lowest glucose level was the 3% value found in R. glutinis and the highest value was the 45% in Schiz. pombe; the overall average value was 16%. The values for the mean and range in each Group were as follows:

Group I : mean 18% ; range 7-45%

Group II : mean 17% ; range 6-28%

Group III : mean 12% ; range 3-25%.

As with total hexose there was considerable variation between species within a single genus. In Saccharomyces the range was from 7 to 38% and in Candida from 4 to 25%.

Galactose content of cell-wall hydrolysates

Although galactose is known to be absent from the cell-walls of many yeasts (Phaff, 1971), all the H_2SO_4 hydrolysates of the 26 species studied were analysed for this sugar using galactose oxidase. The results of duplicate analyses showed that only 7 of the 26 strains in the collection contained galactose and these 7 were distributed in Groups I and III. Galactose was either completely absent or was detectable in amounts ranging from 9 to 38% of the cell-wall, Table 8.

Mannose content of cell-wall hydrolysates

Paper chromatography indicated that mannose was a significant component of the cell-wall in each of the strains examined. Since there was no satisfactory specific test for mannose, the content of this sugar in cell-wall hydrolysates was estimated by subtracting the glucose and galactose values from the total hexose. The calculated results in Table 8

TABLE 9 :

Ratio of glucose to mannose in cell-wall hydrolysates

GROUP	SPECIES	Glucose : Mannose
I	L. starkeyi	4.5 : 1
	S. cerevisiae	1.6 : 1
	S. bayanus	1.6 : 1
	S. rouxii	0.4 : 1
	S. uvarum	0.4 : 1
	Schw. occidentalis	0.8 : 1
	D. hansenii	1.3 : 1
	P. membranaefaciens	3.7 : 1
	W. robertsii	1.2 : 1
	H. anomala	0.9 : 1
	Schiz. pombe	45.0 : 1
	N. fulvescens	0.5 : 1
	H'spora valbyensis	3.0 : 1
	Wick. fluorescens	0.8 : 1
	Nem. coryli	0.5 : 1
	E. capsularis	0.9 : 1
II	B. alba	7.0 : 1
	Sp. roseus	0.3 : 1
III	R. glutinis	0.8 : 1
	K. apiculata	0.4 : 1
	C. albicans	1.5 : 1
	C. guilliermondii	0.1 : 1
	C. krusei	0.3 : 1
	C. stellatoidea	2.0 : 1
	Tr. cutaneum	0.3 : 1
	Trig. variabilis	0.7 : 1

TABLE 10 :

Hexosamine and pentose content of yeast cell-wall hydrolysates

GROUP	SPECIES	Percentage Pentose	Hexosamine
I	L. starkeyi	0.8	0.3
	S. cerevisiae	1.1	0.5
	S. bayanus	1.4	0.6
	S. rouxii	1.7	0.5
	S. uvarum	1.1	0.5
	Schw. occidentalis	1.4	1.3
	D. hansenii	1.7	0.8
	P. membranaefaciens	1.2	0.9
	W. robertsii	1.2	0.6
	H. anomala	1.2	0.7
	Schiz. pombe	2.9	0.8
	N. fulvescens	0.8	0.5
	H'spora valbyensis	1.4	0.1
	Wick. fluorescens	1.7	0.6
	Nem. coryli	1.8	0.1
	E. capsularis	0.8	1.5
II	B. alba	2.4	0.5
	Sp. roseus	1.1	0.4
III	R. glutinis	1.2	0.3
	K. apiculata	2.6	0.4
	C. albicans	2.0	0.4
	C. guilliermondii	1.7	0.4
	C. krusei	1.4	0.3
	C. stellatoidea	2.3	0.2
	Tr. cutaneum	2.4	1.1
	Trig. variabilis	3.9	0.6

show a range of 1 to 50% mannose. The mean values for mannose and the ranges are as follows:

Group I	:	mean 16%	;	range 1-32%
Group II	:	mean 11%	;	range 4-18%
Group III	:	mean 24%	;	range 4-50%.

Mannose levels in the 4 Saccharomyces species ranged from 19 to 32%, and in the 4 Candida species from 6 to 50%.

Since glucose and mannose were present in all of the cell-walls, and since the percentage of each varied widely between strains, glucose to mannose ratio was calculated from the results, Table 9. Schiz. pombe showed an exceptionally high ratio of 45:1. The mean values of glucose: mannose and the ranges were as follows:

Group I	:	mean 1.4	;	range 0.4-4.5	(excluding Schiz. pombe)
Group II	:	mean 3.7	;	range 0.3-7.0	
Group III	:	mean 0.8	;	range 0.1-2.0	

It was evident that there was a considerable range of glucose:mannose in each group.

Pentose content of cell-wall hydrolysates

Although pentose was not detected in any of the cell-wall preparations examined by chromatography, the 26 type strains were subjected to quantitative analysis for pentose using the orcinol test. The results of pentose determinations on cell-wall hydrolysates (Table 10) showed a range from 0.8% to 3.9% with a mean value of 1.8%. The results can be summarised as follows:

Group I	:	mean 1.4%	;	range 0.8-2.9%
Group II	:	mean 1.8%	;	range 1.1-2.4%
Group III	:	mean 2.2%	;	range 1.2-3.9%.

TABLE 11 :

Homologous Agglutination Titres

GROUP	SPECIES	Reciprocal of Homologous Agglutination Titres
I	L. starkeyi	16
	S. cerevisiae	32
	S. bayanus	16
	S. rouxii	8
	S. uvarum	64
	Schw. occidentalis	16
	D. hansenii	32
	P. membranaefaciens	16
	W. robertsii	32
	H. anomala	128
	Schiz. pombe	8
	N. fulvescens	32
	H'spora valbyensis	16
	Wick. fluorescens	4
	Nem.coryli	8
	E. capsularis	32
II	B. alba	16
	Sp. roseus	32
III	R. glutinis	16
	K. apiculata	32
	C. albicans	64
	C. guilliermondii	16
	C. krusei	64
	C. stellatoidea	64
	Tr. cutaneum	38
	Trig. variabilis	8

Hexosamine content of cell-wall hydrolysates

The hexosamine content of the cell-walls of the 26 type strains was estimated using a modification (Cessi and Piliego, 1960) of the Elson and Morgan method. Hydrochloric acid hydrolysates of cell-walls were assayed for hexosamine by the two-stage procedure using acetylacetone and p-dimethyl-aminobenzaldehyde. The hexosamine content of the cell-wall hydrolysates was low in all strains (Table 10); the mean content was 0.6% and the range from 0.1 to 1.5%. The results for each group were as follows:

Group I	:	mean 0.6%	;	range 0.1-1.5%
Group II	:	mean 0.5%	;	range 0.4-0.5%
Group III	:	mean 0.5%	;	range 0.2-1.1%.

Thus the majority of strains had hexosamine contents below 1%, the exceptions being Schw. occidentalis, E. capsularis and Tr. cutaneum which contained 1.3%, 1.5% and 1.1% respectively.

THE AGGLUTINATION OF YEAST CELL-WALLS WITH HOMOLOGOUS AND HETEROLOGOUS ANTISERA

Agglutination tests were carried out with dilutions of rabbit antisera and constant amounts of ultrasonically-dispersed suspensions of yeast cell-walls. Titres were recorded after 2 hr. incubation at 56°C, since longer incubation at this temperature or overnight exposure to room temperature of 4°C caused sedimentation of the cell-walls with resultant masking of the end point.

Agglutination titres obtained with homologous antisera

Table 11 shows the reciprocal of the titres of the 26 antisera against the corresponding cell-wall preparations. The majority of the

.	.	.	.	R	.	.	.	9
R	.	R	.	.	.	.	.	7
.	.	R	.	R	.	.	R	14
.	.	.	.	.	.	.	R	14
.	.	.	.	.	.	.	R	13
.	.	.	.	.	.	.	.	6
R	.	.	.	.	.	.	.	3
.	.	.	.	.	.	.	R	14
.	.	.	.	.	.	.	R	10
.	.	.	.	R	.	.	.	10
.	.	.	.	.	.	R	.	5
.	.	.	.	.	.	.	.	6
.	.	.	.	.	.	.	.	8
.	.	.	.	.	.	.	R	11
.	.	.	.	.	.	.	.	1
R	.	.	R	.	.	.	R	6
R	.	.	.	.	.	R	R	6
.	R	.	R	R	.	.	R	12
.	.	R	R	R	R	.	.	4
R	R	.	R	R	R	.	R	13
R	R	R	R	R	R	R	.	13
R	R	R	R	R	R	R	R	13
R	.	.	R	.	.	R	R	10
R	.	.	R	.	.	R	R	3
9	3	4	8	8	3	5	13	: mean value 9

1	2	0	0	2	4	2	16	2	1	0
1	1	0	4	0	8	2	2	4	2	1
64	2	1	2	2	8	2	32	4	2	16
8	0	2	0	2	2	2	4	0	1	4
4	0	0	2	0	4	2	8	2	4	16
0	0	0	0	2	4	2	8	2	2	1
0	0	2	4	0	4	2	0	2	2	0
32	2	4	1	1	4	2	2	4	2	16
4	0	0	4	2	4	2	8	2	4	4
16	0	1	0	1	4	1	64	1	1	0
1	2	4	1	0	2	2	0	0	8	0
1	0	0	0	2	2	1	2	2	2	0
1	0	1	0	2	4	2	0	2	0	1
32	2	2	2	2	4	4	2	2	4	2
0	16	8	0	0	0	2	0	1	0	0
0	4	32	8	2	8	4	4	4	4	4
0	4	4	16	1	0	1	2	1	16	2
8	1	4	2	32	8	8	16	8	2	4
0	1	2	0	4	64	16	128	32	2	0
2	0	2	16	8	8	16	16	16	2	32
0	4	1	4	16	32	8	64	32	8	1
2	2	4	16	8	16	8	16	64	8	4
4	1	0	4	4	8	8	2	4	32	16
0	2	0	8	4	8	4	0	4	8	8

TABLE 14 :

Cross-reactions of Group I antisera in agglutination tests with Group I cell-walls compared with cell-walls of Groups II and III

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Percentage mean value
GROUP I	Number out of 15	8	9	11	11	5	11	8	9	6	0	4	7	12	13	11	6	
	Percentage	53	60	73	73	33	73	53	60	40	0	27	47	80	87	73	40	55
GROUPS II and III	Number out of 10	2	1	5	5	2	0	1	4	1	0	2	1	6	7	4	1	
	Percentage	20	10	50	50	20	0	10	40	10	0	20	10	60	70	40	10	26

values were between 16 and 64 which was significantly higher than any observed with normal rabbit serum where the maximum titre was 2.

Specificity of the yeast anti-cell-wall sera: cross-reactivity

The specificities of the antisera were investigated by testing each of them against the 26 cell-wall suspensions. The extensive array of results obtained by this procedure is set out in Table 12. For simplification, the heterologous titres were subsequently expressed in terms of the corresponding homologous values. Titres which were 25% or more of the homologous value (for a particular antiserum) were recorded as 'R' (reactive); titres of less than 25% were taken as indicating an absence of significant cross-reactivity and were recorded as '.'. The arbitrary 25% threshold was chosen because of the accepted practice of taking a fourfold change of titre as significant in diagnostic microbiology.

The agglutination cross-reactions presented in Table 13 show a pattern of extensive cross-reactivity. This Table shows the number of cross-reactions obtained with each antiserum and with each cell-wall preparation; the maximum number of cross-reactions was 25. The majority of sera cross-reacted with fewer than 50% of the antigens and some sera were extremely specific. For example, antiserum 10 (anti-H. anomala) produced no cross-reactions and antiserum 18 (anti-Sp. roseus) gave cross-reaction only with B. alba cell-walls from the same Basidiomycete group. Antiserum 24 (anti-C. stellatoidea) reacted only with the four Candida species. By contrast, antiserum 14 (anti-Wick. fluorescens) gave cross-reactions with 21 of the 25 cell-walls and antiserum 13 (anti-H'spora valbyensis) cross-reacted with 19 of the 25 cell-walls.

Cross-reactions of Group I antisera with cell-wall preparations

The cross-reactions of the 16 Group I antisera against ascosporogenous yeasts are summarised in Table 14. In order to assess

TABLE 15 :

Cross-reactions of *Saccharomyces* antisera in agglutination tests with *Saccharomyces* cell-walls compared with other Group I and Groups II and III cell-walls

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 2	3	4	5	Percentage mean value
Saccharomyces	Number out of 3	3	2	3	1	
	Percentage	100	67	100	33	75
Rest of GROUP I	Number out of 12	6	9	8	4	
	Percentage	50	75	67	33	56
GROUPS II and III	Number out of 10	1	5	5	2	
	Percentage	10	50	50	20	33

the degree of specificity the proportion of cross-reactions with Group I cell-walls was compared with those obtained using Groups II and III cell-walls. It was found that with each of the 16 Group I antisera there was a higher percentage of cross-reactions with Group I cell-walls (mean value 55%) than with the other yeast cell-walls (mean value 26%). This was most pronounced with antiserum 6 (anti-Schw. occidentalis) which cross-reacted with 73% of Group I antigens and with none of the remaining cell-walls. The Group I antisera showed a definite specificity for Group I cell-walls despite their cross-reactivity with cell-walls of yeasts of other groups.

Cross-reactions observed within a Group I genus

The specificities of the 4 antisera against strains of Saccharomyces in Group I were examined. Table 15 presents the numbers and percentages of cross-reactions of Saccharomyces antisera against :

a) Saccharomyces cell-walls;

b) Cell-walls of other ascosporogenous yeasts (remainder of Group I)

c) Cell-walls of asporogenous yeasts (Groups II and III).

The Table shows that the antisera gave a higher degree of cross-reactivity with Saccharomyces species (mean value 75%) than with either cell-walls of other ascosporogenous yeasts (mean value 56%) or of asporogenous yeasts (mean value 33%). These results showed that Saccharomyces antisera exhibited a higher degree of specificity for cell-walls from within the genus than for antigens derived from other species of yeast.

Cross-reactions of Group II antisera with yeast cell-walls

Antiserum 17 (anti-B. alba) cross-reacted with the other Group II species (Sp. roseus) and with only 2 other yeasts. Antiserum 18 (anti-Sp. roseus) reacted only with B. alba, the other Group II yeast. From the cross-reactions it appeared that these antisera showed a high degree of specificity for Group II antigens.

TABLE 16 :

Cross-reactions of Group III antisera in agglutination tests with Group III cell-walls compared with cell-walls of Groups I and II

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 19	20	21	22	23	24	25	26	Percentage mean value
GROUP III	Number out of 7	5	3	2	6	4	3	4	5	
	Percentage	71	43	29	86	57	43	57	71	57
GROUPS I and II	Number out of 18	4	0	2	2	4	0	1	8	
	Percentage	22	0	11	11	22	0	6	44	15

TABLE 17 :

Cross-reactions of Candida antisera in agglutination tests with Candida cell-walls compared with other Group III and Groups I and II cell-walls

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 21	22	23	24	Percentage mean value
Candida	Number out of 3	2	3	3	3	
	Percentage	67	100	100	100	92
Rest of GROUP III	Number out of 4	0	3	1	0	
	Percentage	0	75	25	0	25
GROUPS I and II	Number out of 18	2	2	4	0	
	Percentage	11	11	22	0	11

Cross-reactions of Group III antisera with yeast cell-walls

The 8 antisera produced against Group III yeast cell-walls were examined in a similar manner. The incidence and percentages of cross-reactions are shown in Table 16. A definite pattern emerged. Each serum showed a higher percentage of cross-reactions with Group III cell-walls than with cell-walls of the other two groups; in some cases the difference was substantial. For example, antiserum 22 (anti-C. guilliermondii) reacted with 86% of Group III cell-walls and only with 11% of the other yeast cell-walls. Antisera 20 (anti-K. apiculata), 24 (anti-C. stellatoidea) and 25 (anti-Tr. cutaneum) reacted with 43 to 57% of Group III yeast cell-walls and only 0 to 6% of the yeast cell-walls in other groups. Thus with Group III, as with the other two groups, there was a greater degree of cross-reactivity of antisera to antigens from within the group (mean value 57%) than with antigens from other groups (mean value 14.5%).

Cross-reactions observed within a Group III genus

As with antisera against Saccharomyces species, the specificities of the 4 antisera against Group III Candida species were examined in relation to :

a) Candida cell-walls;

b) Other Group III cell-walls;

c) Group I and Group II cell-walls.

Table 17 shows that there was a significantly higher percentage of cross-reactions (mean value 92%) with Candida cell-walls than with the remainder of Group III antigens (mean value 25%). In two cases, antisera 21 (anti-C. albicans) and 24 (anti-C. stellatoidea), there were no cross-reactions with Group III cell-walls. The Candida antisera produced a significantly lower incidence of cross-reactions (mean value 11%) against cell-walls of Groups I and II species.

TABLE 18 :

Cross-reactions of Group I cell-walls in agglutination tests with Group I antisera compared with antisera to Groups II and III

ANTISERA	INCIDENCE	Cross-reactivity of cell-wall: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Percentage mean value
GROUP I	Number out of 15	5	10	8	5	11	13	12	6	2	13	8	9	4	6	8	9	
	Percentage	33	67	53	33	73	87	80	40	13	87	53	60	27	40	53	60	54
GROUPS II and III	Number out of 10	0	2	1	2	3	1	1	0	1	1	2	1	1	0	0	2	
	Percentage	0	20	10	20	30	10	10	0	10	10	20	10	10	0	0	20	11

TABLE 19 :

Cross-reactions of Saccharomyces cell-walls in agglutination tests with Saccharomyces antisera compared with other Group I antisera and Groups II and III antisera

ANTISERA	INCIDENCE	Cross-reactivity of cell-walls: 2	3	4	5	Percentage mean value
Saccharomyces	Number out of 3	3	2	1	3	
	Percentage	100	67	33	100	75
Rest of GROUP I	Number out of 12	7	6	4	8	
	Percentage	58	50	33	67	52
GROUPS II and III	Number out of 10	2	1	2	3	
	Percentage	20	10	20	30	20

TABLE 20 :

Cross-reactions of Group III cell-walls in agglutination tests with Group III antisera compared with antisera to Groups I and II

ANTISERA	INCIDENCE	Cross-reactivity of cell-wall: 19	20	21	22	23	24	25	26	Percentage mean value
GROUP III	Number out of 7	2	3	3	5	6	7	3	3	
	Percentage	29	43	43	71	86	100	43	43	57
GROUPS I and II	Number out of 18	4	9	1	8	7	6	7	0	
	Percentage	22	50	6	44	39	33	39	0	29

TABLE 21 :

Cross-reaction of Candida cell-walls in agglutination tests with Candida antisera compared with antisera to the rest of Group III and Groups I and II antisera

ANTISERA	INCIDENCE	Cross-reactivity of cell-wall: 21	22	23	24	Percentage mean value
Candida	Number out of 3	3	2	3	3	
	Percentage	100	67	100	100	92
Rest of GROUP III	Number out of 4	0	3	3	4	
	Percentage	0	75	75	100	63
GROUPS I and II	Number out of 18	1	8	7	6	
	Percentage	6	44	39	33	31

Specificity of the yeast cell-walls: cross-reactions with antiserum

This section deals briefly with the converse comparison of cross-reactivity of individual cell-wall preparations with antisera against species from the three groups. Thus Table 18 shows that cell-walls of Group I gave a higher percentage (mean value 54%) of cross-reactions with Group I antisera than with antisera of Groups II and III (11%).

Similarly, Table 19 shows that the four species of Saccharomyces cell-walls gave a higher percentage of cross-reactions with Saccharomyces antisera (mean value 75%) than with either antisera against the rest of Group I (mean value 52%) or antisera against Groups II and III (mean value 20%).

Table 20 shows that there was a mean value of 57% cross-reactions of Group III cell-walls with Group III antisera and only one of 29% with antisera against species of the other two groups.

Finally, Table 21 shows the much higher cross-reactivity of the four Candida antigens with Candida antisera than with antisera raised against other yeasts.

TABLE 22 :

Homologous Complement Fixation Titres

GROUP	SPECIES	Reciprocal of Homologous Complement Fixation Titres
I	L. starkeyi	320
	S. cerevisiae	160
	S. bayanus	160
	S. rouxii	160
	S. uvarum	640
	Schw. occidentalis	160
	D. hansenii	640
	P. membranaefaciens	320
	W. robertsii	80
	H. anomala	640
	Schiz. pombe	40
	N. fulvescens	160
	H'spora valbyensis	320
	Wick. fluorescens	160
	Nem. coryli	160
	E. capsularis	20
II	B. alba	80
	Sp. roseus	640
III	R. glutinis	160
	K. apiculata	320
	C. albicans	320
	C. guilliermondii	160
	C. krusei	320
	C. stellatoidea	320
	Tr. cutaneum	320
	Trig. variabilis	640

COMPLEMENT FIXING ACTIVITY OF YEAST CELL-WALLS

Yeast cell-wall antisera were examined for the presence of complement-fixing antibodies, since it seemed likely that this technique might yield more definite endpoints than cell-wall agglutination.

Anticomplementary activity of antisera and yeast cell-wall preparations

Each cell-wall preparation was tested for anticomplementary activity by standard procedures. No such activity was detected in any cell-wall preparation at the concentration of 0.1 mg/ml, which was used in the complement fixation tests (CFT). The rabbit antisera, however, were anticomplementary up to a 1/20 dilution; such activity was also present in normal rabbit serum, and was therefore not due to the immunisation.

Complement fixation titres of antisera against homologous yeast cell-walls

Doubling dilutions of antisera from 1/20 to 1/2560 were mixed with 4 HU_{50} of complement and a constant amount of 0.1 mg/ml cell-wall suspension. After holding the mixtures at 4°C for 18 hr, the test was completed by adding sensitised sheep erythrocytes and incubating at 37°C. The endpoint of each titration was taken as the highest dilution of serum showing 50% or less haemolysis. Table 22 presents the reciprocals of the homologous titres of the 26 antisera. The reciprocals of the titres ranged from 20 to 640, with most of the values being 160 or 320.

Specificity of the yeast anti-cell-wall sera: cross-reactivity

Having determined the homologous titres of each serum, the next step was to study all the heterologous reactions. This was done by titrating each of the 26 antisera against constant volumes of 0.1 mg/ml suspensions of cell-walls of each of the 26 yeasts. This gave the

0	20	40	20	20	40	20	80	20	40	20
0	40	40	40	40	80	40	40	40	40	0
0	40	20	20	20	40	40	20	40	20	0
0	20	20	20	80	40	20	40	20	0	0
0	20	40	40	40	80	40	80	20	20	0
0	20	20	20	20	20	20	20	20	40	20
0	80	80	320	320	320	160	40	40	80	20
0	20	20	20	40	40	20	0	20	20	0
0	40	40	40	80	80	40	80	40	80	20
0	20	80	40	40	80	80	0	20	40	0
0	20	40	40	40	80	40	40	40	0	0
0	20	40	40	40	40	40	40	40	20	0
0	40	40	40	80	80	40	40	0	20	0
0	80	80	40	80	80	80	80	40	40	20
0	320	640	160	80	160	160	80	40	80	20
0	40	160	160	40	80	40	20	80	40	20
0	80	40	80	320	160	160	80	40	80	0
0	160	320	40	160	320	160	40	320	320	0
0	80	40	160	160	80	160	80	80	40	0
0	20	40	80	40	80	40	320	80	20	0
0	320	160	80	40	160	80	80	320	80	0
0	80	80	80	160	160	80	20	80	320	0
0	20	20	40	80	40	20	0	0	40	640

.	.	.	R	.	.	.	.	6
.	.	.	.	R	.	.	.	6
R	.	R	R	.	.	.	.	15
.	.	.	R	.	.	.	.	18
.	R	.	.	.	.	.	.	10
R	.	R	R	R	.	.	.	17
.	.	.	.	.	.	.	.	10
R	R	R	R	.	.	R	.	18
.	.	.	.	.	.	.	.	6
R	R	R	R	R	.	R	.	16
R	.	R	R	.	.	.	.	10
E	.	R	R	.	.	.	.	10
R	.	.	R	.	.	.	.	11
R	R	R	R	.	.	.	.	10
R	R	R	R	R	.	.	.	14
R	R	R	R	R	.	R	.	19
R	.	R	R	.	R	.	.	13
R	R	R	R	R	.	R	.	15
R	R	R	R	.	R	R	.	15
R	R	R	R	R	R	.	.	17
R	.	R	R	R	R	.	.	12
R	.	R	R	R	R	R	.	14
R	R	R	R	.	R	R	.	14
R	R	.	.	.	.	.	R	11
17	10	16	19	8	5	6	0	: mean value 12

TABLE 25 :

Cross-reactions of Group I antisera in CF tests with Group I cell-walls compared with cell-walls of Groups II and III

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Percentage mean value
GROUP I	Number out of 15	0	15	13	8	7	5	4	4	4	2	4	14	15	11	5	11	
	Percentage	0	100	87	53	47	33	27	27	27	13	27	93	100	73	33	73	51
GROUPS II and III	Number out of 10	2	10	10	5	1	5	1	2	5	2	3	10	9	9	5	8	
	Percentage	20	100	100	50	10	50	100	20	50	20	30	100	90	90	50	80	54

TABLE 26 :

Cross-reactions of Saccharomyces antisera in CF tests with Saccharomyces cell-walls compared to the rest of Group I cell-walls and Groups II and III cell-walls

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 2	3	4	5	Percentage mean value
Saccharomyces	Number out of 3	3	3	2	2	
	Percentage	100	100	67	67	84
Rest of GROUP I	Number out of 12	12	10	6	5	
	Percentage	100	83	50	42	69
GROUPS II and III	Number out of 10	10	10	5	1	
	Percentage	100	100	50	10	65

extensive array of results set out in Table 23.

As before, the results were simplified by expressing all the CF titres which were 25% or more than the homologous titre of a particular antiserum as reactive 'R', and those less than 25% as non-reactive '.'.

As shown in Table 24, this revealed a pattern of extensive cross-reactions. These cross-reactions were more extensive than those seen in the agglutination tests. For example, 8 of the antisera reacted with 20 or more of the cell-wall preparations in the CF test, whereas only 1 antiserum was so highly cross-reactive in the agglutination test. Furthermore, one antiserum (anti-S. cerevisiae) reacted with all 26 yeast cell-walls in the CF test and 3 other antisera (12, 13 and 17) reacted with all but one of the yeasts. On the other hand, antiserum 26, which had the high homologous CF reciprocal titre of 640, was entirely specific. Antiserum 24 reacted only with Group III cell-walls, while antiserum 7 reacted with none of Group III cell-walls. A majority of the antisera reacted with between 10 and 25 species of cell-walls.

Comparison of the cross-reactivity of antisera

As with the agglutination reactions, comparisons of the specificity of the antisera were made group by group. First, the reactions of the 16 Group I cell-wall preparations were compared with Groups II and III cell-wall preparations (Table 25). The unexpected result which emerged was that Group I antisera gave approximately the same incidence of cross-reactions with antigens of Groups II and III as with antigens of Group I.

Within Group I, the four Saccharomyces antisera (Table 26) showed an average of 84% cross-reactions with Saccharomyces antigens, and 69% and 65% cross-reactions with the rest of Group I and with Groups II and III respectively. The analogous values found in the agglutination cross-reactions were 85%, 56% and 32% respectively.

TABLE 27 :

Cross-reactions of Group III antisera in CF tests with Group III cell-walls compared with Groups I and II cell-walls

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 19	20	21	22	23	24	25	26	Percentage mean value
GROUP III	Number out of 7	7	4	6	6	3	5	3	0	
	Percentage	100	57	86	86	43	71	43	0	61
GROUPS I and II	Number out of 18	10	6	10	13	5	0	3	0	
	Percentage	56	33	56	72	28	0	17	0	33

TABLE 28 :

Cross-reactions of Candida antisera in CF tests with Candida cell-walls compared with the rest of Group III cell-walls and Groups I and II cell-walls

CELL-WALL	INCIDENCE	Cross-reactivity of antiserum: 21	22	23	24	Percentage mean value
Candida	Number out of 3	3	3	2	3	
	Percentage	100	100	67	100	92
Rest of GROUP III	Number out of 4	3	3	1	2	
	Percentage	75	75	25	50	56
GROUPS I and II	Number out of 18	10	13	5	0	
	Percentage	56	72	28	0	39

TABLE 29 :

Cross-reactions of Group I cell-walls in CF tests with Group I antisera compared with Groups II and III antisera

ANTISERA	INCIDENCE	Cross-reactivity of cell-wall: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Percentage mean value
GROUP I	Number out of 15	7	10	4	4	11	6	8	12	9	12	5	9	6	6	8	5	
	Percentage	47	67	27	27	73	40	53	80	60	80	33	60	40	40	53	33	51
GROUPS II and III	Number out of 10	0	3	2	2	4	2	2	5	1	6	1	7	4	4	3	5	
	Percentage	0	30	20	20	40	20	20	50	10	60	10	70	40	40	30	50	32

TABLE 30 :

Cross-reactions of Saccharomyces cell-walls in CF tests with Saccharomyces antisera compared with antisera to the rest of Group I and with Groups II and III antisera

ANTISERA	INCIDENCE	Cross-reactivity of cell-wall: 2	3	4	5	Percentage mean value
Saccharomyces	Number out of 3	3	2	2	3	
	Percentage	100	67	67	100	84
Rest of GROUP I	Number out of 12	7	2	2	8	
	Percentage	58	17	17	67	40
GROUPS II and III	Number out of 10	3	2	2	4	
	Percentage	30	20	20	40	28

TABLE 31 :

Cross-reactions of Group III cell-walls in CF tests with Group III antisera compared with Groups I and II antisera.

ANTISERA	INCIDENCE	Cross-reactivity of cell-wall: 19	20	21	22	23	24	25	26	Percentage mean value
GROUP III	Number out of 7	3	5	5	5	4	5	5	2	
	Percentage	43	71	71	71	57	71	71	29	60
GROUPS I and II	Number out of 18	10	10	10	12	8	9	9	9	
	Percentage	56	56	56	67	44	50	50	50	54

TABLE 32 :

Cross-reactions of Candida cell-walls in CF tests with Candida antisera compared with antisera to the rest of Group III and to Groups I and II.

ANTISERA	INCIDENCE	Cross-reactivity of cell-wall: 21	22	23	24	Percentage mean value
Candida	Number out of 3	2	3	3	3	
	Percentage	67	100	100	100	92
Rest of GROUP III	Number out of 4	3	2	1	2	
	Percentage	75	50	25	50	44
GROUPS I and II	Number out of 18	10	12	8	9	
	Percentage	56	67	44	50	54

The average value for the cross-reactions of Group III antisera with Group III cell-walls was 61% and for other cell-walls was 33% (Table 27).

Within Group III the cross-reactivity of Candida antisera with either Candida cell-walls, or the rest of Group III cell-walls or Groups I and II cell-walls was compared (Table 28). The respective mean percentages of cross-reactivity were 92, 56 and 39%, which may be compared with the values of 92, 25 and 11% obtained in agglutination.

Comparison of the cross-reactivity of cell-walls

The cross-reactivity of the yeast cell-wall preparations with antisera was analysed by the same procedure.

Group I cell-wall preparations gave a mean value of 51% cross-reaction with Group I antisera, and 32% with antisera against other groups (Table 29). Saccharomyces cell-walls gave (Table 30) 84% cross-reactions with Saccharomyces antisera, 40% cross-reactions with other Group I antisera, and 28% cross-reactions with antisera against other yeasts. These figures indicate a more distinct gradation of cross-reactivity than was seen in the corresponding analysis of the antisera (Table 26).

On the other hand, Group III cell-walls showed 60% cross-reaction with Group III antisera and 54% with antisera from the other groups (Table 31). Candida cell-walls gave 92% cross-reaction with Candida antisera 44% with other Group III antisera, and 54% with other antisera (Table 32).

Complement fixation tests using antisera absorbed with heterologous yeast cell-walls

The combined agglutination and complement-fixation test results indicated considerable cross-reactivity. Therefore, it was decided to increase the specificity by absorption. To absorb all the antisera with each combination of heterologous cell-walls was not feasible since this

TABLE 33 :

Reciprocal CF titres against selected cell-walls with absorbed *S. cerevisiae* antiserum

S. cerevisiae antiserum	Reciprocal CF titre with cell-wall: 1	2	3	4	5	7	10	12	13	14	15	18	19	21	25	26
Before absorption	80	160	40	80	160	80	320	160	160	80	80	160	160	160	320	40
Absorbed with *S. bayanus* cell-walls	0	0	0	0	0	0	0	0	0	.	0	0	0	0	0	.
Absorbed with *Wick. fluorescens* cell-walls	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.
Absorbed with *Trig. variabilis* cell-walls	0	40	40	80	0	40	0	0	80	.	80	0	0	0	0	0
Absorbed with *D. hansenii* cell-walls	.	40	0	.	.	0	0	.	.	.	.	0	.	.	.	.

. cell-walls not selected for the absorption study

0 indicates a reciprocal titre less than 20

TABLE 34 :

Reciprocal CF titres against selected cell-walls with *D. hansenii* antiserum absorbed with *H'spora valbyensis* cell-walls

D. hansenii antiserum	Reciprocal titres with cell-wall 1	2	7	8	13	18	22
Before absorption	320	160	640	160	160	160	80
Absorbed with *H'spora valbyensis* cell-walls	0	0	160	0	0	0	0

TABLE 35 :

Reciprocal CF titres against selected cell-walls with absorbed *Sp. roseus* antiserum

Sp. roseus antiserum	Reciprocal CF titres with cell-wall: 2	3	4	5	7	10	13	15	17	18	21	22
Before absorption	40	40	40	40	20	80	80	40	80	640	320	80
Absorbed with *H. anomala* cell-walls	0	0	0	0	0	0	.	0	.	320	0	0

. cell-walls not selected for the absorption study

procedure would yield thousands of absorbed serum samples. Therefore, representative sera were selected for absorption studies on the basis of their apparent antigenic mosaic.

S. cerevisiae antiserum: This reacted with all 26 species of yeast in the CF test. The antiserum was absorbed twice with cell-walls of S. bayanus, Wick. fluorescens, Trig. variabilis or D. hansenii. The antisera were tested before and after absorption with selected yeast cell-wall preparations. Table 33 shows that the antiserum before absorption gave reciprocal titres of 40 to 320 against 16 yeast cell-wall preparations. Absorption with cell-walls of S. bayanus or Wick. fluorescens removed all CF activity, and absorption with D. hansenii cell-walls removed almost all activity to the cell-walls selected. After absorption with Trig. variabilis cell-walls, the activity of S. cerevisiae antiserum against most cell-walls was greatly reduced, with the exception of cell-walls of S. bayanus, S. rouxii and Nem. coryli. The titres against cell-walls of these species were not reduced by absorption of S. cerevisiae antiserum by Trig. variabilis cell-walls.

D. hansenii antiserum: Table 34 shows the result of absorption of D. hansenii antiserum with H'spora valbyensis cell-walls. The absorbed antiserum gave an appreciable homologous titre towards D. hansenii cell-walls but it no longer reacted with any other yeast cell-walls with which it had given reciprocal titres of 80 to 320 before absorption.

Sp. roseus antiserum: A similar increase in specificity was obtained by absorbing Sp. roseus antiserum with H. anomala cell-walls. This antiserum before absorption gave reciprocal titres of 40 or more with the 9 heterologous yeast cell-walls tested; after absorption only homologous activity was retained (Table 35).

TABLE 36 :

Reciprocal CF titres against selected cell-walls with absorbed *C. albicans* antiserum.

C. albicans antiserum	Reciprocal CF titres with cell-wall											
	2	10	12	17	18	19	20	21	22	23	24	25
Before absorption	80	320	80	80	160	80	160	320	160	80	160	160
Absorbed with *K. apiculata* cell-walls	0	0	0	.	0	.	0	80	80	0	80	0
Absorbed with *C. guilliermondii* cell-walls	0	0	0	0	0	0	0	0	0	0	0	0
Absorbed with *C. stellatoidea* cell-walls	0	0	0	.	0	.	0	0	0	0	0	0

. cell-walls not selected for absorption study

TABLE 37 :

Key to chemical characters in Table 39

Character No.	Character	
1	0 - 19%	% Hexose in cell-wall hydrolysates
2	20 - 39%	
3	40 - 59%	
4	> 60%	
5	0 - 24%	% Glucose of Hexose
6	25 - 49%	
7	50 - 74%	
8	> 75%	
9	> 0%	% Galactose of Hexose
10	0 - 24%	% Mannose of Hexose
11	25 - 49%	
12	50 - 74%	
13	> 75%	
14	0 - 0.9%	% Hexosamine in cell-wall hydrolysates
15	> 1.0%	

TABLE 38 :

Key to serological characters in Table 39

Character Aggln.	Character CF	Character ("R" reaction with antiserum to:)
16	42	L. starkeyi
17	43	S. cerevisiae
18	44	S. bayanus
19	45	S. rouxii
20	46	S. uvarum
21	47	Schw. occidentalis
22	48	D. hansenii
23	49	P. membranaefaciens
24	50	W. robertsii
25	51	H. anomala
26	52	Schiz. pombe
27	53	N. fulvescens
28	54	H'spora valbyensis
29	55	Wick. fluorescens
30	56	Nem. coryli
31	57	E. capsularis
32	58	B. alba
33	59	Sp. roseus
34	60	R. glutinis
35	61	K. apiculata
36	62	C. albicans
37	63	C. guilliermondii
38	64	C. krusei
39	65	C. stellatoidea
40	66	Tr. cutaneum
41	67	Trig. variabilis

C. albicans antiserum: The effects of absorbing C. albicans antiserum with other Group III cell-walls is shown in Table 36. Absorption with C. guilliermondii cell-walls removed the CF activity against all the 12 yeast cell-walls tested. Absorption with C. stellatoidea cell-walls produced a similar effect. The specificity of the antiserum for 7 yeast cell-walls was removed by absorption with K. apiculata cell-walls, but some activity against cell-walls of C. albicans, C. guilliermondii and C. stellatoidea remained.

APPLICATION OF NUMERICAL TAXONOMY TO THE CHEMICAL AND SEROLOGICAL RESULTS

Coding of results

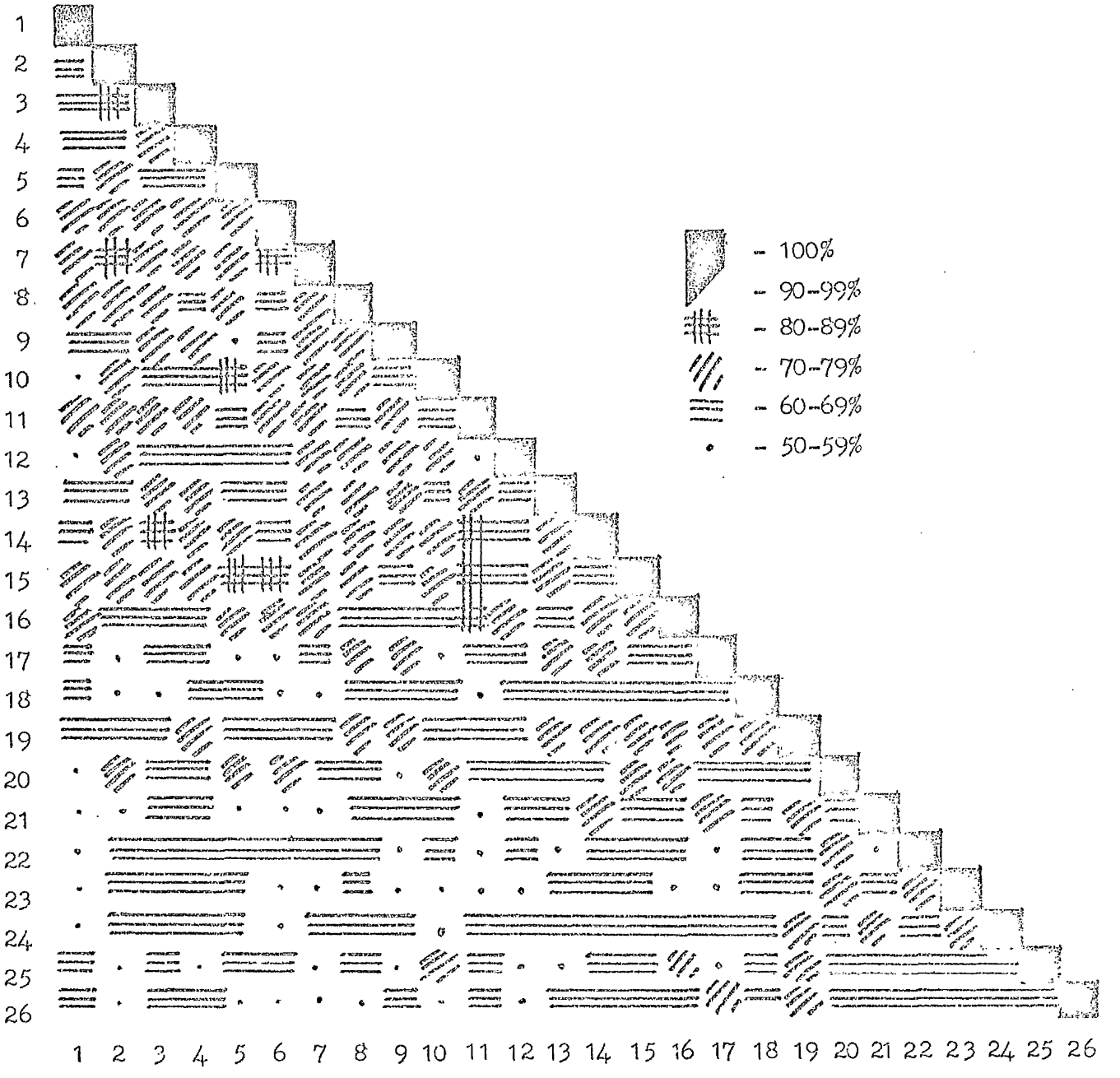
In order to reveal taxonomic relationships in the 26 strains of yeast under study, several of the techniques of numerical taxonomy were applied to the total chemical and serological results. The chemical analyses provided multistate characters which were numbered from 1 to 15 as shown in Table 37. To the 15 chemical characters were added 52 serological characters as shown in Table 38. Characters 16 to 41 represented reactivity with each of the 26 antisera in the agglutination tests, and characters 42 to 67 the reactivity of the same sera in the CF test. The scores of each of the 26 yeasts for each of the 67 characters were rendered in binary form (Table 39). This Table was the basis of the subsequent computer analysis.

Similarity Matrices

The computer printout provided values of the Matching Coefficient and Dissimilarity Coefficient in a matrix. A representation of this matrix

FIGURE 4 :

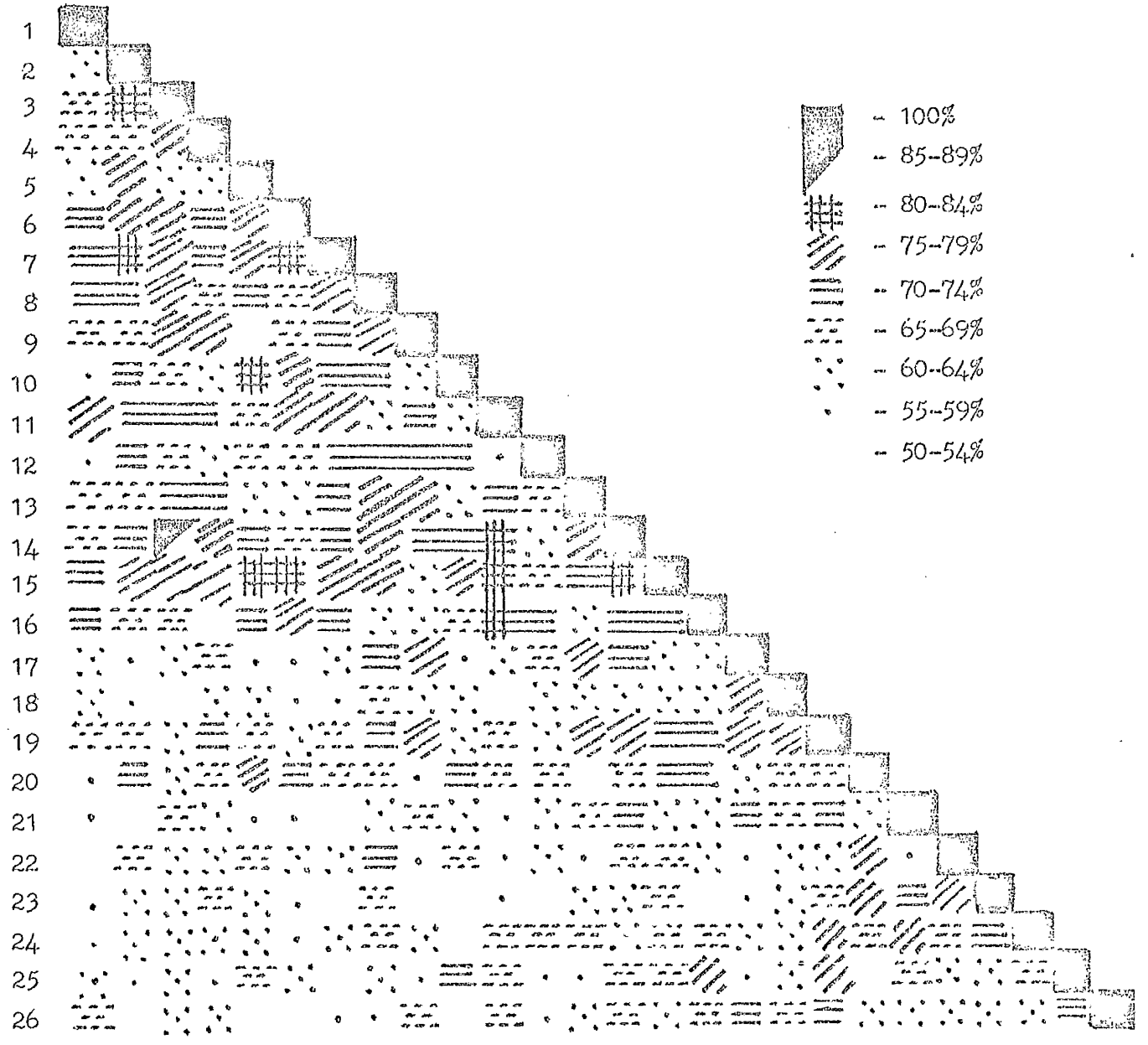
Similarity matrix expressed in 10% intervals based on chemical and serological properties



1 - L. starkeyi
2 - S. cerevisiae
3 - S. bayanus
4 - S. rouxii
5 - S. uvarum
6 - Schw. occidentalis
7 - D. hansenii
8 - P. membranaefaciens
9 - W. robertsii
10 - H. anomala
11 - Schiz. pombe
12 - N. fulvescens
13 - H'spora valbyensis
14 - Wick. fluorescens
15 - Nem. coryli
16 - E. capsularis
17 - B. alba
18 - Sp. roseus
19 - R. glutinis
20 - K. apiculata
21 - C. albicans
22 - C. guilliermondii
23 - C. krusei
24 - C. stellatoidea
25 - Tr. cutaneum
26 - Trig. variabilis

FIGURE 5 :

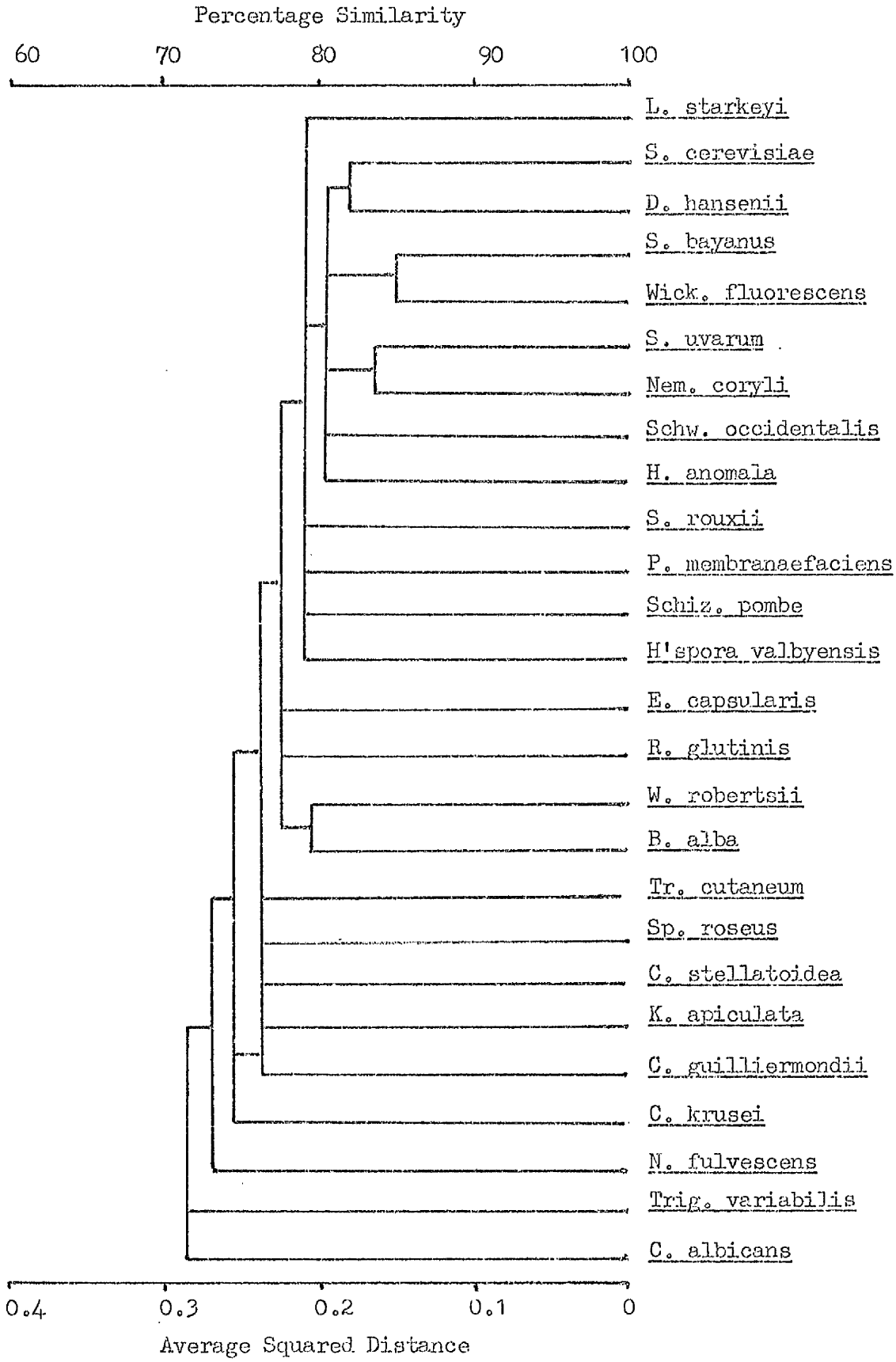
Similarity matrix expressed in 5% intervals based on chemical and serological properties



1 - L. starkeyi
2 - S. cerevisiae
3 - S. bayanus
4 - S. rouxii
5 - S. uvarum
6 - Schw. occidentalis
7 - D. hansenii
8 - P. membranaefaciens
9 - W. robertsii
10 - H. anomala
11 - Schiz. pombe
12 - N. fulvescens
13 - H'spora valbyensis
14 - Wick. fluorescens
15 - Nem. coryli
16 - E. capsularis
17 - B. alba
18 - Sp. roseus
19 - R. glutinis
20 - K. apiculata
21 - C. albicans
22 - C. guilliermondii
23 - C. krusei
24 - C. stellatoidea
25 - Tr. cutaneum
26 - Trig. variabilis

FIGURE 6 :

Single linkage dendrogram based on chemical and serological properties



is given in Figure 4, in which the Matching Coefficients are expressed, according to standard practice, in 10% ranges from 50 to 100%. At this level, within Group I, there was some evidence of clustering and a high proportion of coefficients above the 70% value. The range of similarity values within Group III was more variable than that found in Group I, possibly indicating a more heterogenous population.

As the majority of coefficients were within the range 55-85%, greater differentiation was achieved by expression of the similarity values in 5% intervals as shown in Figure 5. This similarity matrix emphasised the closer relationships within Group I yeasts and the greater contrasts found within Group III yeasts. These groups, especially Group III, were not homogenous and various clustering techniques were used to determine clustering sequences by means of these coefficients.

Alternative Methods of Clustering

The computer program CLUSTAN provides various options of clustering methods for hierarchic fusion based on similarity. These options differ in the criteria which allow an object to join a cluster.

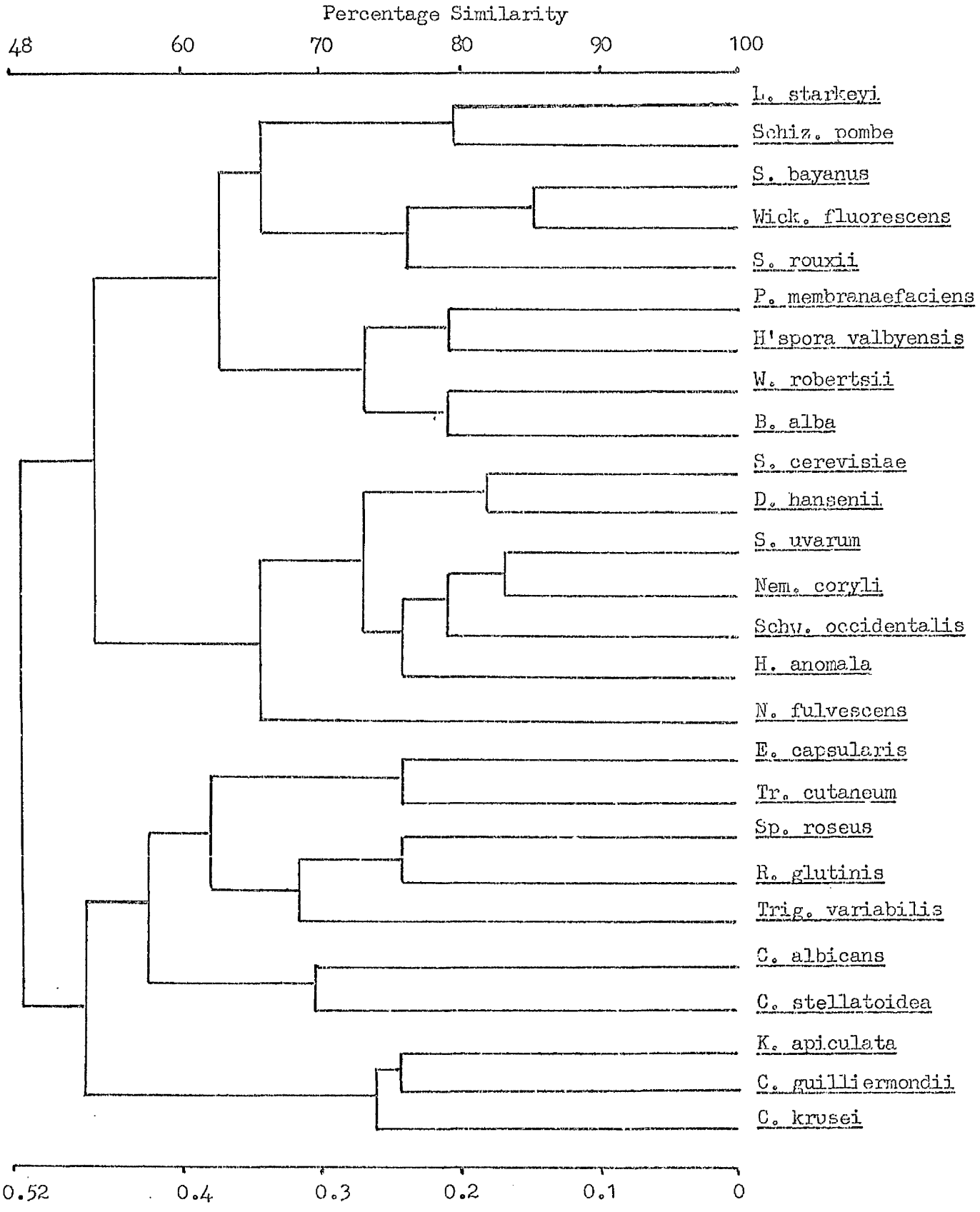
Single linkage

Clustering by single linkage involves the formation of a cluster at the highest similarity level of any two objects. An object can join a cluster if it has the required similarity with only one object within the cluster.

As shown in Figure 6, with the exception of N. fulvescens, Group I strains first clustered together at the 77.6% similarity level; B. alba of Group II and R. glutinis of Group III were included in this cluster. At 71.5% similarity all the yeasts formed one cluster. In general, clustering occurs with the addition of a strain or strains to a cluster in

FIGURE 7 :

Complete linkage dendrogram based on chemical and serological properties



contrast to the fusion of separate clusters. This sequential addition of strains is known as "straggling" or "chaining", and did not facilitate the division of the yeast species into distinct groups.

Complete linkage

Complete linkage is the converse of single linkage. For an object to enter a cluster it must have the same or a higher similarity value with every member of the cluster compared to the similarity level under consideration. For two clusters to join together all the similarities between all the members of the two clusters must be the same or higher than the similarity level being considered.

The dendrogram formed by complete linkage, Figure 7, was composed of distinct clusters which fused at various similarity levels between 49.2% and 85%. The "chaining" effect was not observed as with single linkage and thus groups were easier to define. At the 53.6% similarity level the majority of the Group I and Group III yeasts were separated into two distinct clusters. However, at the 49.2% level these two clusters were fused. Group II was not distinguishable as a single cluster.

Average linkage

Clustering by average linkage is intermediate between single and complete linkage. Average linkage allows an object to join a cluster if the average similarity between it and every member of the cluster is higher than the average similarity between the cluster and any other objects. Two clusters join at the similarity level which is equal to the average similarity values between all members of both clusters. The structure and composition of a cluster are taken into account by calculating the average value, and each member of the group has equal weight in determining if a new member is to be admitted.

FIGURE 8 :

Average linkage dendrogram based on chemical and serological properties

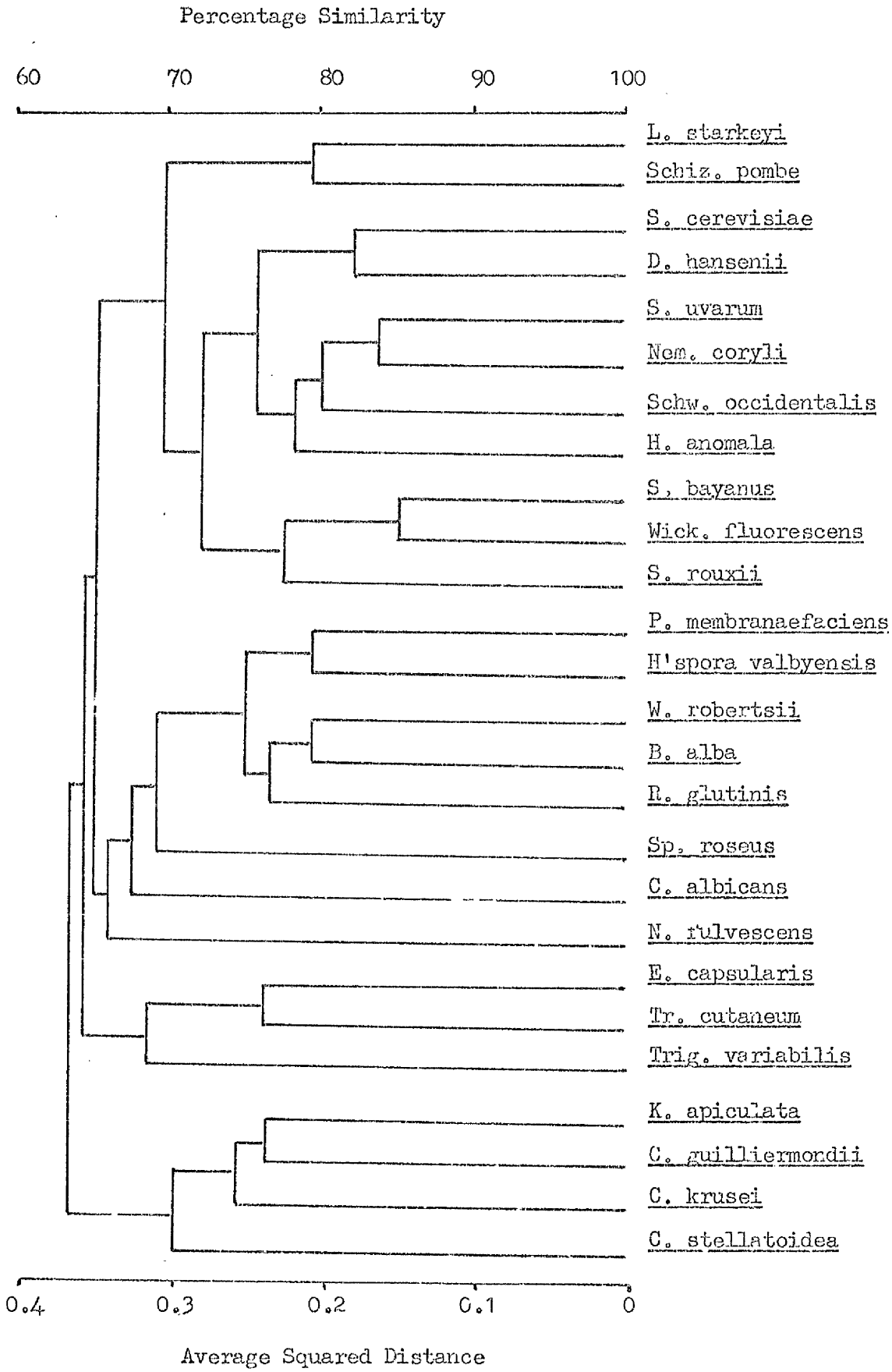


FIGURE 9 :

Dendrogram based on the chemical and serological properties
(Ward's method)

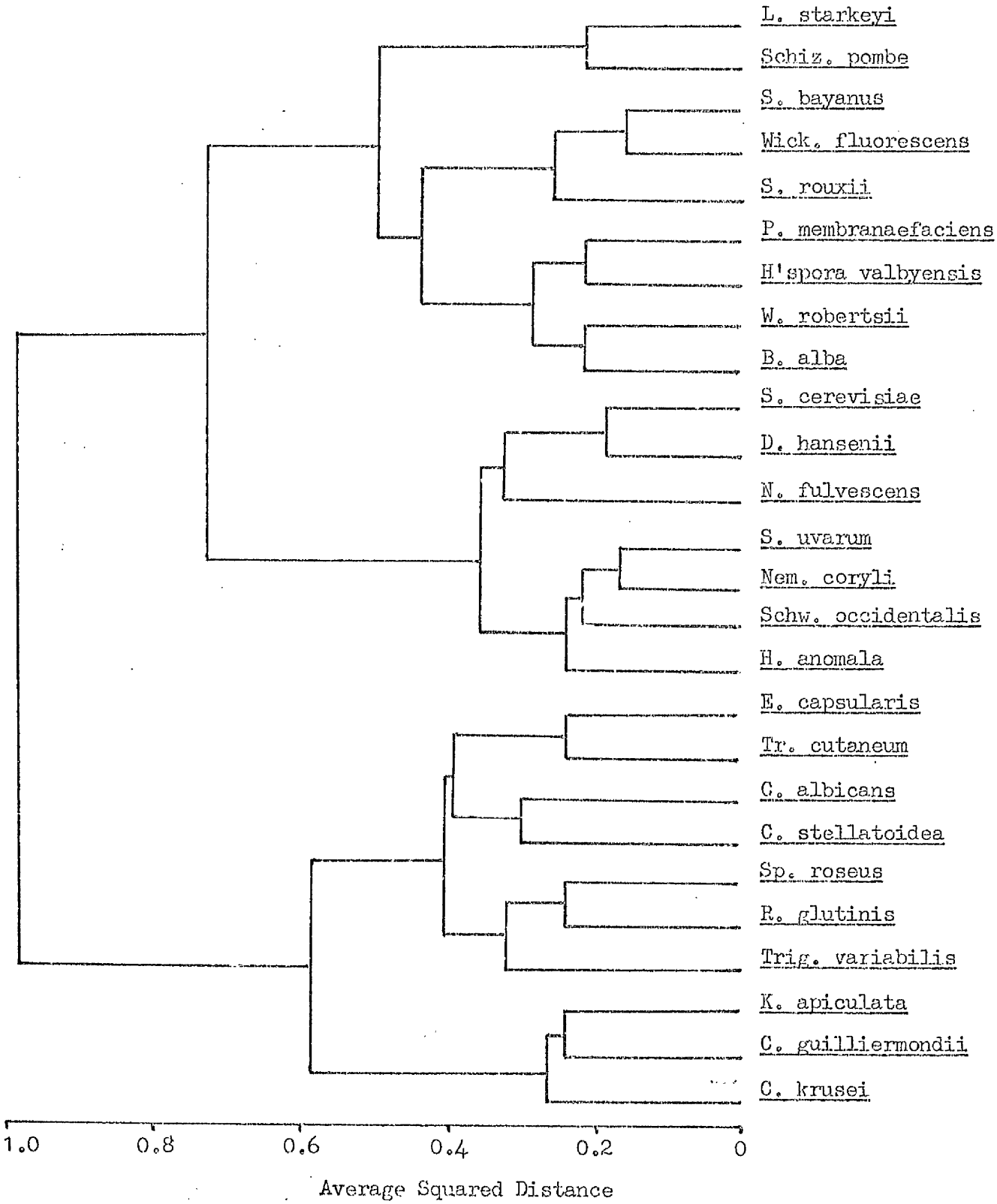


Figure 8 presents the dendrogram formed by average linkage. The "chaining" effect of singly linkage was less evident, and there was less differentiation of cluster levels especially between 60-70%. Indeed, all species were clustered together at the 63% similarity level. The majority of Group I strains occurred in 2 clusters. One cluster at the 69.3% level contained 11 of the 16 Group I strains, and the second cluster at the 66% level contained 4 strains in addition to others. However, a single cluster was not formed with Group III yeasts at any similarity level.

Ward's method of clustering

This is based on the premise that the greatest amount of information is available when a collection of objects is ungrouped. Two of these objects are selected to form a cluster which produces the least impairment of the available information. Admission of objects to clusters and fusion of clusters is also dependent upon this criterion; the procedure continuing until all objects are present in one cluster.

Ward's method of clustering produces the dendrogram in Figure 9, which is similar to that formed by complete linkage clustering, Figure 7. With greater distance, the dendrogram becomes extended at values for average squared distance above 0.4 so that the final cluster is achieved at a distance of 0.98.

Apart from E. capsularis, Group I yeasts were present in the major cluster formed at the 0.73 level. This cluster was divided into 2 smaller clusters, one which occurred at the 0.49 level and the second occurred at the 0.36 level. The Group III yeasts were contained in a single cluster at the 0.59 level and were sub-divided into two clusters at the 0.41 and 0.26 levels.

0	1	1	0	1	1	0	1	1	1	1	0	0	0	1	1	1	1	0	1	0	1	0	1	1	1	51	D-Glucos
0	0	0	0	1	0	0	1	0	1	1	0	0	0	1	0	1	1	0	1	0	0	0	0	0	1	52	
0	1	0	0	1	0	0	1	1	1	1	0	0	1	0	0	1	1	0	1	0	0	0	0	0	1	53	Salicin
0	0	0	0	1	0	0	1	1	1	1	0	0	1	0	0	1	1	0	1	0	0	0	0	0	1	54	
1	1	1	1	1	1	0	1	1	1	0	0	1	0	0	0	1	1	1	1	0	1	0	1	1	0	55	DL-Lactic
1	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	1	0	0	1	0	1	0	1	0	0	56	Acid
1	1	1	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	1	1	0	0	0	0	0	1	57	Succinic
1	0	1	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	0	1	0	0	0	0	0	0	58	
1	1	1	0	1	1	0	1	1	1	1	0	1	0	1	0	1	1	1	1	0	0	0	0	0	1	59	Citric Ac
1	0	0	0	1	0	0	0	1	1	0	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	60	
0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	61	Inositol
0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	62	
0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	63	Glucose
0	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	0	64	
0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	1	0	0	1	1	1	0	0	1	0	65	Galactos
0	0	0	0	1	1	0	0	0	0	0	0	1	0	1	0	1	0	0	0	1	1	0	0	1	0	66	
0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1	1	0	1	1	1	1	1	1	0	67	Sucrose
0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1	1	0	0	1	1	0	1	1	0	68	
0	0	1	0	0	1	0	0	0	0	1	1	0	0	1	1	1	1	0	1	0	1	1	1	1	0	69	Maltose
0	0	1	0	0	1	0	0	0	0	1	1	0	0	1	1	0	1	0	0	1	1	1	1	1	0	70	
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	71	Lactose
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	72	
0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	73	Nitrate
0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	74	Assimila
0	0	0	0	1	0	1	0	0	0	0	0	1	1	0	1	1	1	1	1	0	1	1	1	1	0	75	Osmotic
0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	1	1	0	1	0	0	1	0	0	0	76	Toleranc
1	0	0	1	1	0	1	1	0	1	1	0	0	1	0	1	1	1	1	1	0	0	0	0	0	0	77	Average
0	1	1	0	0	1	0	0	1	0	0	1	1	0	1	0	0	0	0	0	1	1	1	1	1	1	78	Width
1	1	1	0	1	1	1	1	0	1	0	0	0	1	0	0	1	1	0	1	1	0	0	0	0	1	79	Average
0	0	0	1	0	0	0	0	1	0	1	1	1	0	1	1	0	0	1	0	0	1	1	1	1	0	80	Length
1	0	0	1	1	1	1	1	0	1	0	0	1	1	0	1	1	1	0	0	1	1	1	1	1	1	81	Colony
0	1	1	0	0	0	0	1	1	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	0	0	82	Morpholo
1	1	1	1	1	1	0	0	0	0	0	1	0	1	0	1	1	1	1	1	0	1	0	1	1	1	83	Growth
1	0	1	1	1	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	84	at 37°C
0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	85	Pigment
0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	86	Sporulat
0	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0	1	0	1	1	0	87	Pseudomy
0	1	1	1	1	1	0	0	0	0	1	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	88	
0	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	89	Bipolar
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	90	Fission
0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	91	Ballisto
0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	92	True Myc

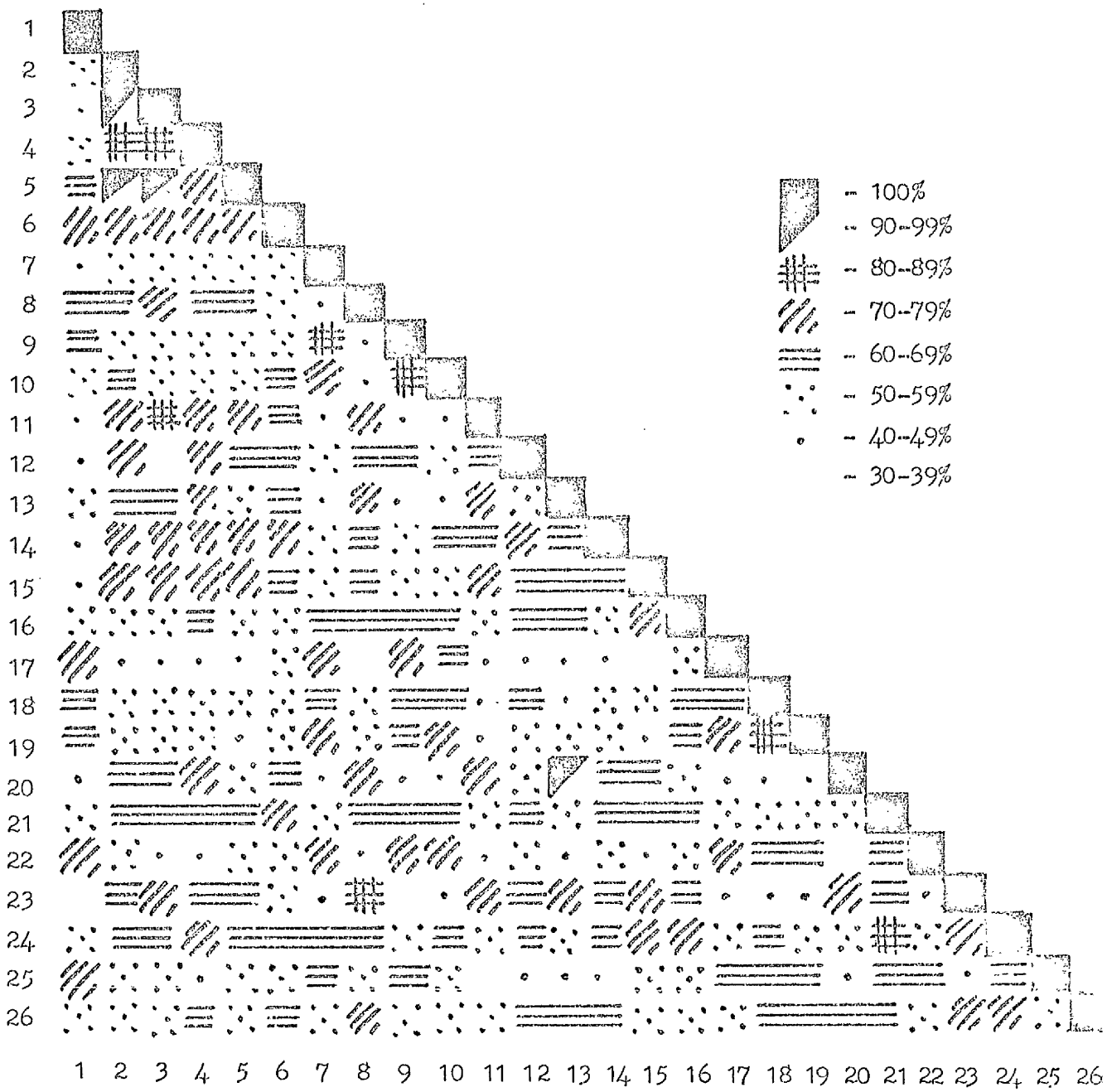
TABLE 41 :

Binary form of yeast characteristics (Lodder, 1970)

Character	Description	Scoring
Assimilation Fermentation Growth at 37°C Tolerance to: 50% glucose or 12% sodium chloride Pseudomycelium	Absent	0 0
	Present	1 1
	Variable	1 0
Average width	$< 4.5\ \mu$	1 0
	$\geq 4.5\ \mu$	0 1
Average length	$< 9.0\ \mu$	1 0
	$\geq 9.0\ \mu$	0 1
Colony morphology	Rough, regular	0 0
	Rough, irregular	0 1
	Smooth, regular	1 0
	Smooth, irregular	1 1
Pigment Ascospores Bipolar budding Fission Mycelium	Absent	0
	Present	1

FIGURE 10 :

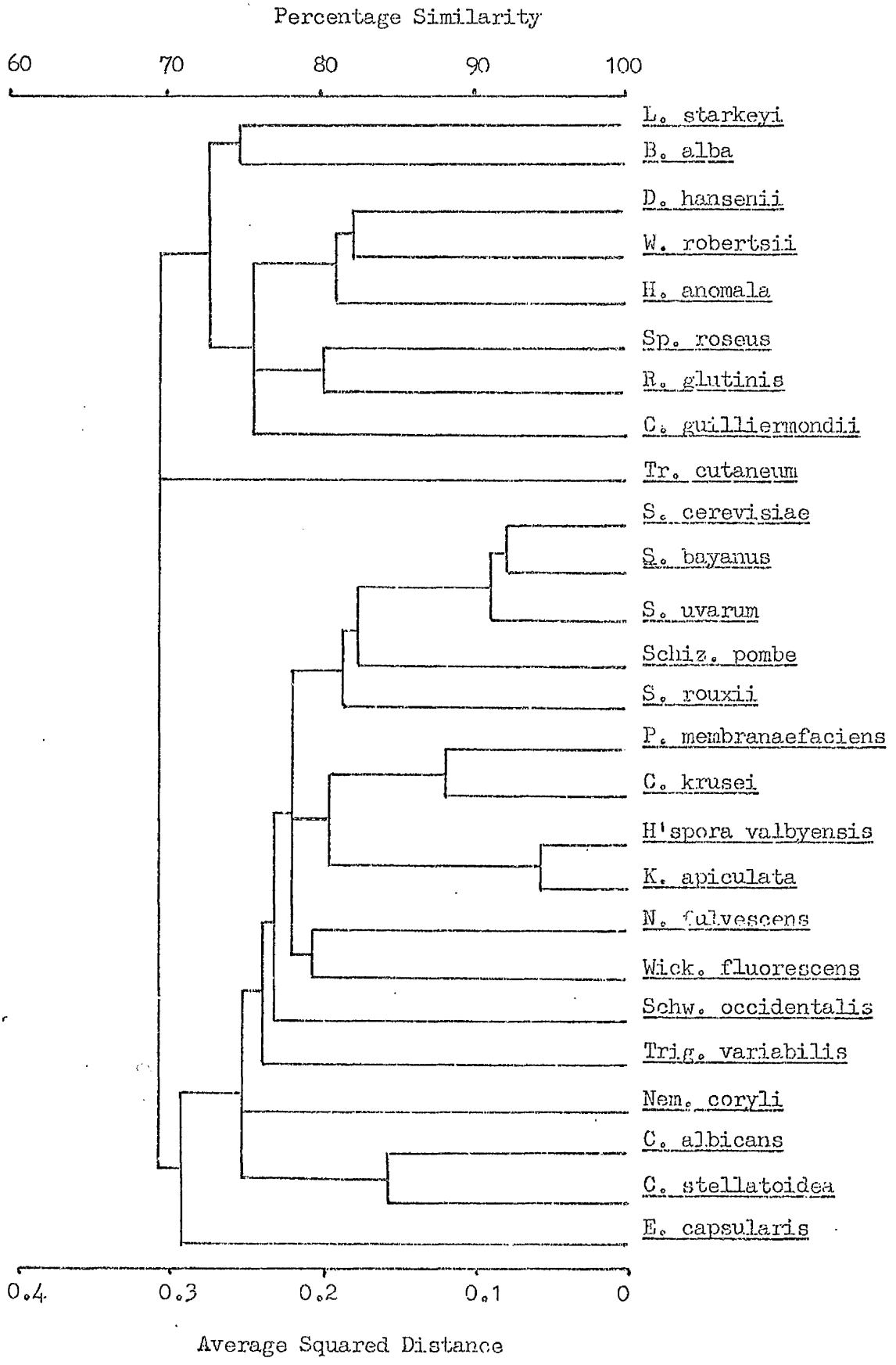
Similarity matrix expressed in 10% intervals based on published properties



1 - L. starkeyi
2 - S. cerevisiae
3 - S. bayanus
4 - S. rouxii
5 - S. uvarum
6 - Schw. occidentalis
7 - D. hansenii
8 - P. membranaefaciens
9 - W. robertsii
10 - H. anomala
11 - Schiz. pombe
12 - N. fulvescens
13 - H'spora valbyensis
14 - Wick. fluorescens
15 - Nem. coryli
16 - E. capsularis
17 - B. alba
18 - Sp. roseus
19 - R. glutinis
20 - K. apiculata
21 - C. albicans
22 - C. guilliermondii
23 - C. krusei
24 - C. stellatoidea
25 - Tr. cutaneum
26 - Trig. variabilis

FIGURE 11 :

Single linkage dendrogram based on published properties



APPLICATION OF NUMERICAL TAXONOMY TO CHARACTERISTICS OF YEASTS USED IN LODDER'S CLASSIFICATION

Coding of Properties

For comparison of the analyses presented above, it was considered of interest to apply the methods of numerical analysis to the yeast characteristics as cited by Lodder (1970). The characters were coded in binary form for each strain (Table 40). The method of scoring is summarised in Table 41. The properties which were described as "variable" were made into 2-state characters so that a "variable" result had 50% similarity with both positive and negative scores. Those properties which are described as "rarely negative" or "occasionally positive" were regarded as being positive and negative respectively.

Similarity Matrix

A similarity matrix was constructed as shown in Figure 10. Group I strains did not form a distinct group, and two areas of relatively higher similarity were evident within the Group. There is also a scatter of relatively high similarities with Group I and Groups II and III resulting in no distinction between the 3 Groups.

To determine the relationships amongst the 26 species it was necessary to produce dendrograms using the various clustering techniques.

Single linkage

The dendrogram formed by single linkage clustering, Figure 11, divides the 26 strains into two major clusters at the 72.8 and 70.6% levels with Tr. cutaneum remaining distinct from each. These clusters fused at the 69.5% similarity level. There was not a high degree of "chaining", although it was still evident. Group I strains were present in both of

FIGURE 12 :

Complete linkage dendrogram based on published properties

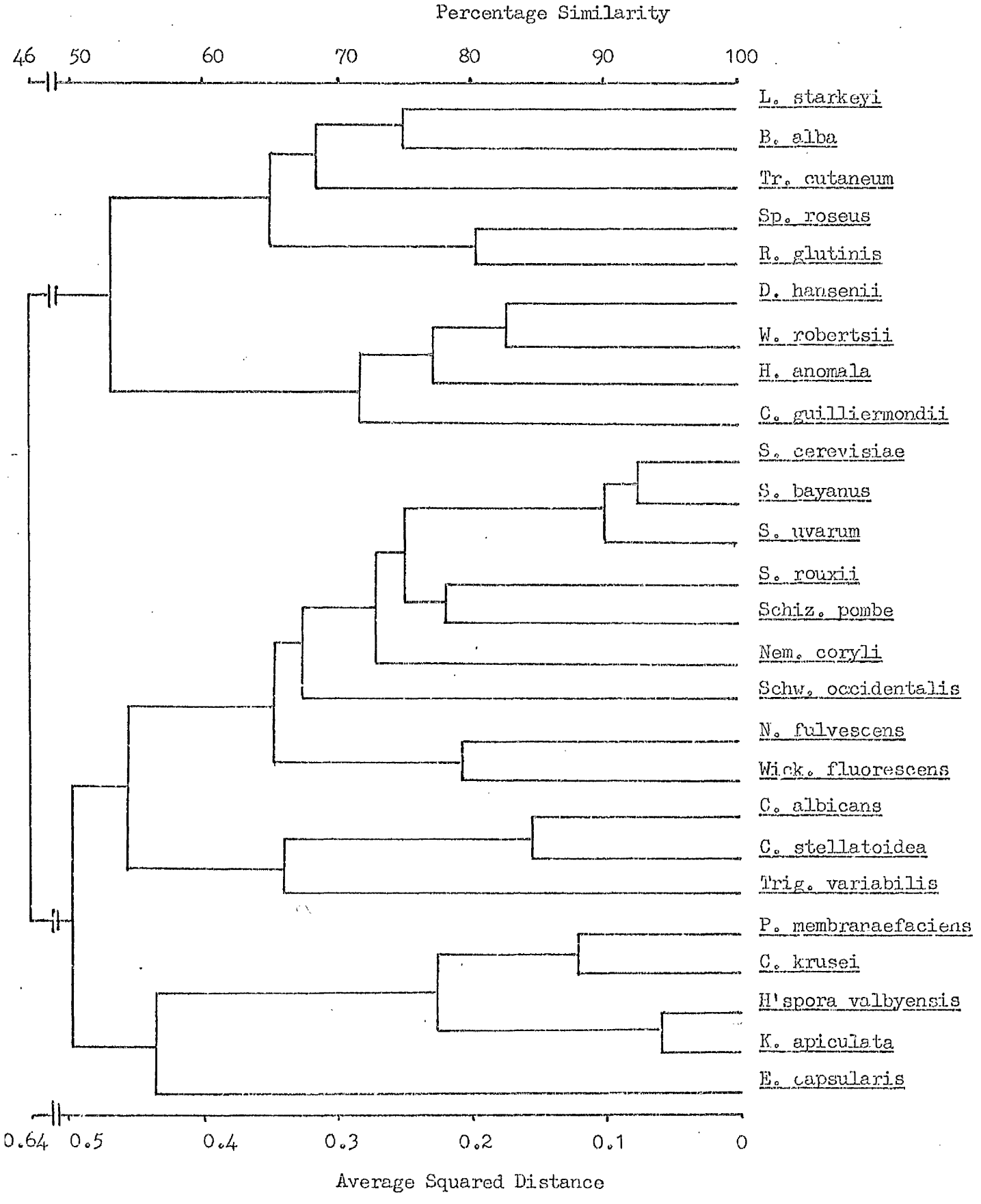


FIGURE 13 :

Average linkage dendrogram based on published properties

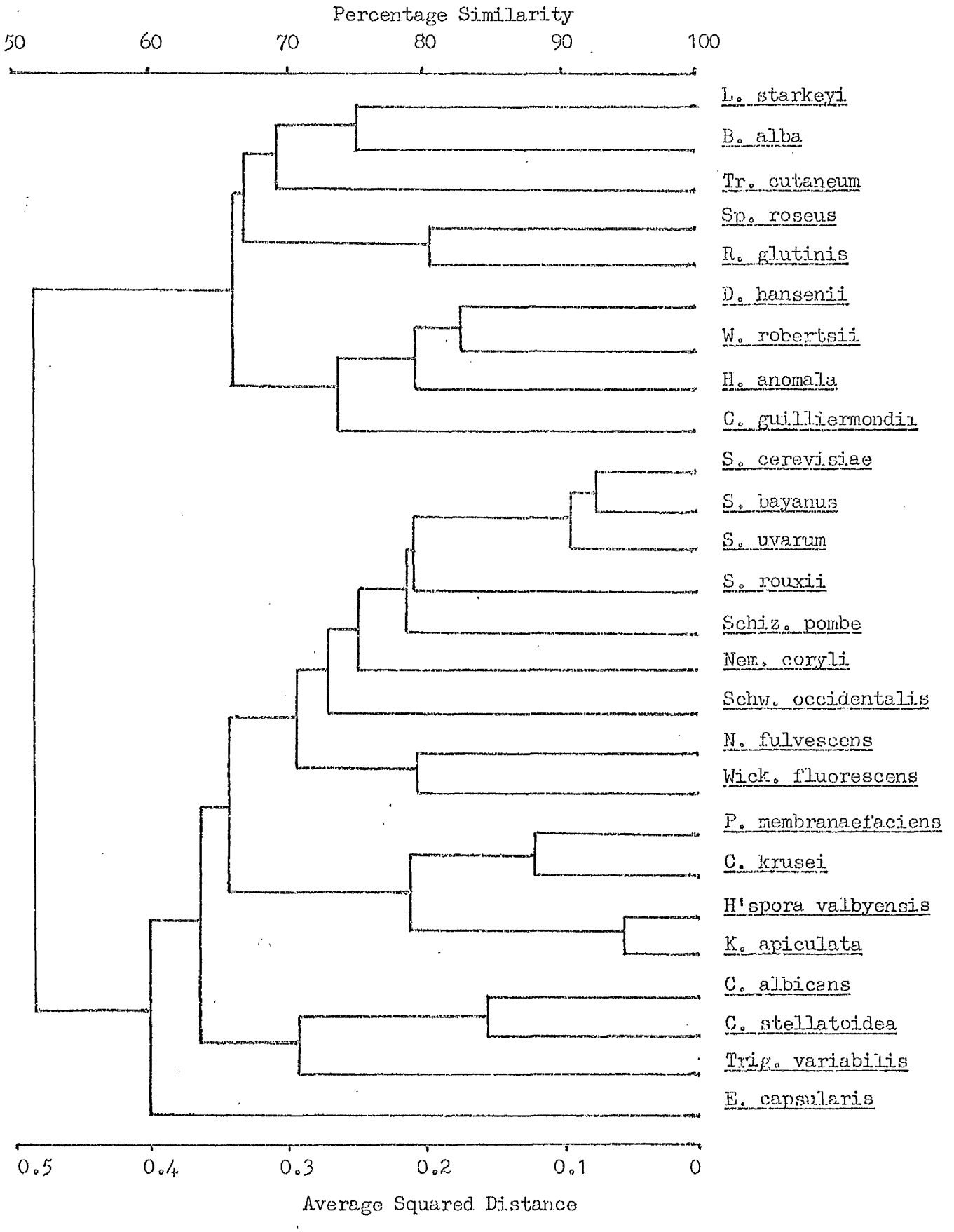
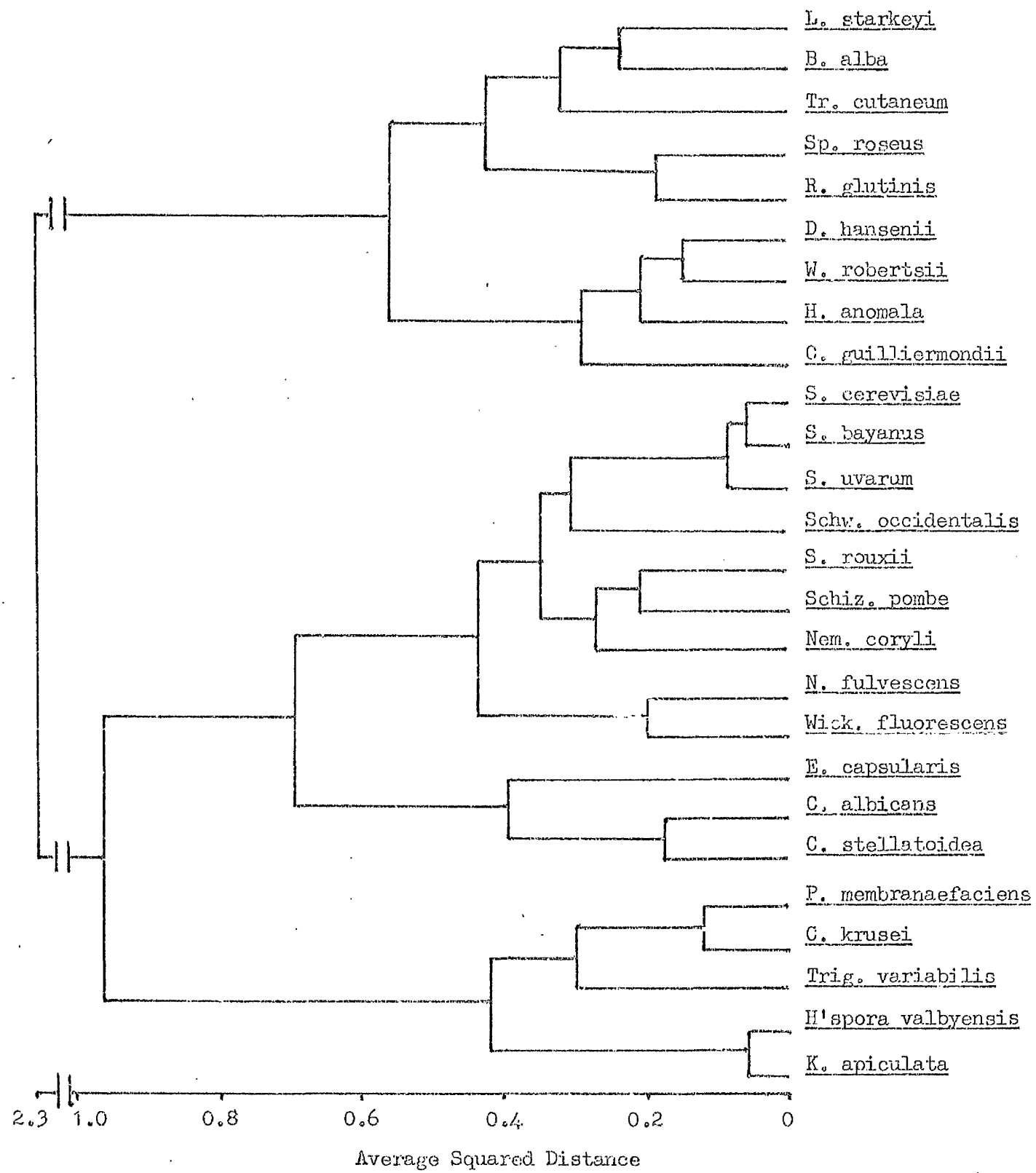


FIGURE 14 :

Dendrogram based on published properties

(Ward's method)



the major clusters, mainly in the larger cluster, which also contained the majority of Group III strains.

Complete linkage

Figure 12 shows the dendrogram formed by complete linkage clustering. Two major clusters were formed at the 53.2 and 50% levels which contained the same yeasts as those in the two single linkage clusters. In addition, Tr. cutaneum was incorporated into one of the clusters. The clusters showed no indication of following the original grouping of the 26 strains, and fused at the 35.8% similarity level.

Average linkage

Clustering by average linkage formed the dendrogram shown in Figure 13, which was similar to the one formed by complete linkage clustering (Figure 12). The 26 yeasts were separated into two major clusters at 66.0 and 60.0% similarity levels; these two clusters fused at the 41.2% level.

Ward's method of clustering

Ward's method of clustering produced the dendrogram in Figure 14. The general appearance was similar to those formed by average linkage (Figure 13) and complete linkage (Figure 12). Because of the increase in "loss of information" as the cluster size increased the dendrogram was extended as fusion of the clusters occurred. The two major clusters contained the same yeasts as the corresponding clusters in both dendrograms formed by average and complete linkage. The two clusters were formed at the average squared distance values of 0.60 and 0.96; these clusters fused at a value of 2.33.

DISCUSSION

At the start of this project it seemed from the literature that the investigation of antigenic relationships of yeasts had been confined to studies of either small groups of related or unrelated species, or a single genus. Where studies had been extended to cover a wider range of yeasts it appeared that severe technical problems cast some doubt on the validity of interpretations of the results.

The aim of the present study was to enlarge the range of yeasts and for this reason it was decided to use the type species of twenty of the genera listed in Lodder and Kreger van-Rij (1952) which was the accepted classification at the start of this study. In addition, because of the economic and medical importance of Saccharomyces and Candida species respectively, several species were examined from these genera. These additional species were also included to test the antigenic homogeneity at the genus level.

The cell-wall composition of yeasts as an aid to taxonomy

During the qualitative examination of the cell-walls of a wide range of Gram positive bacteria Cummins and Harris (1956, 1958) noted that a small number of amino acids were present; not more than 3 or 4 in almost all cases. Each genus was found to possess a characteristic pattern of amino acid components. On the other hand, the monosaccharide components appeared to be characteristic at the species level. Chemical analyses of the cell-walls of the 26 yeast species showed that there was no distinct pattern of amino acid components in any genus. Qualitatively, the major monosaccharides found in the yeast cell-walls were glucose, mannose and galactose. Some quantitative variation was found in the amounts of glucose and mannose, nevertheless these sugars were present in all yeasts examined. Indeed, there was no obvious correlation between the monosaccharide components found in the cell-wall hydrolysates and the major taxonomic

groups. Consequently, yeast cell-wall amino acid and sugar compositions do not in themselves provide taxonomically useful information. However, since many distinct although similar polymers may be constructed from the same few components, the specificity of serological methods was used in an attempt to reveal the differences in the conformation of the polysaccharide polymers.

The serological analysis of yeast cell-walls

It was decided at an early stage to use the cell-walls and not the whole cells of yeasts in the production of antisera and in the serological tests. Cytoplasmic components adhering to the cell-walls were removed by extensive washing. Previously it was shown that the antiserum stimulated by whole yeast cells contained antibodies which were not removed by absorption with the same whole cells. However, if the whole cell disintegrates were used all antibody was absorbed from the antiserum (Hasenclever et. al., 1961). It would seem that the whole cells were partially broken down inside the host, releasing deep-seated antigens which were partially homologous to cell-wall antigens. This effect could cause a greater degree of cross-reactivity and lead to the incorrect formulation of true surface antigens. In this respect the criticism by Richards (1972) of Tsuchiya's work, in which the poor resolution of the finite definition of antigen composition using whole cell slide agglutination tests, is completely relevant. This criticism must not be interpreted as a complete dismissal of the work of Tsuchiya and his colleagues (1957, 1958, 1960, 1961, 1964, 1965). Although more cross-reacting antigens were designated than necessarily present, a number of specific surface antigens were detected by this whole-cell slide agglutination test. The same criticism may be applied to the studies of Campbell (1968, 1970, 1971 a) published during the course of this investigation.

It was noticed that the yeast cell-walls did not stimulate high titre agglutinating antisera even after injection in Freund incomplete adjuvant. The titres of cell-wall agglutinating antibodies were similar to those reputed by other workers (Stewart-Tull et al., 1966). Adjuvant mixtures were not found to stimulate the production of increased antibody levels to pure polysaccharide antigens (Ward, Johnson and Abell, 1959; Stewart-Tull, Wilkinson and White, 1965; Nowotny, 1969). The problem of autoagglutinability of yeast cell-walls in the absence of antibody was overcome by altering the ionic strength of the diluent and by limiting the test incubation period to a maximum of two hours.

Agglutination reactions

The results obtained from cell-wall agglutination tests revealed that antisera against Group I yeasts cross-reacted more extensively with Group I yeast cell-walls than with the cell-walls of Groups II and III yeasts (see Table 14). A significant degree of cross-reactivity was noted between antisera against S. cerevisiae, S. bayanus and S. rouxii and the cell-walls of Saccharomyces species (see Table 15). In addition these antisera cross-reacted more extensively with cell-walls of Group I yeasts than with those of Groups II and III, which indicated a closer serological relationship to Group I species than to Groups II and III. A similar situation was apparent with antisera to Group III species which did not extensively react to Group I and II cell-walls (Table 16). Antisera to Candida species (Table 17) reacted with very few cell-walls of species outwith the genus, suggesting little similarity to other species of Group III as well as to Groups I and II.

The extent of reactivity of cell-walls to all antisera against the species of the three Groups followed a similar pattern to that observed with antisera to all cell-walls (Tables 18, 19, 20 and 21). However, there

is a greater degree of cross-reactivity of cell-walls of Candida species to the rest of Group III antisera than with anti-Candida sera. This may indicate the presence of a common antigen in Group III strains which is of secondary importance in Candida species.

Complement fixation reactions

It was apparent from the literature that the complement fixation test was a more sensitive indicator of serological interactions of yeasts (Negroni, 1969). On average the complement fixing titres were about 10 times higher than those found in the agglutination test. Thus the expectation of higher sensitivity by the CFT was fulfilled. It was evident that the correlation between agglutination and complement fixation was variable. A striking instance was the E. capsularis serum which gave a CF titre of only 20 while its agglutination titre was 32. Based on the 10-fold higher sensitivity of the CF test one might have expected a titre of 320. Conversely, Trig. variabilis antiserum, which gave an agglutination of 8, had a CF titre of 640, which is substantially greater than the value of 80 which might have been expected from the 10-fold sensitivity factor.

Group I antisera reacted to the same extent in complement fixation tests with Groups I, II and III cell-walls (Table 25). It is likely that the presence of a common cell-wall antigen or antigens in all species could account for the degree of cross-reactivity. This is also revealed in the extent of the cross-reactions of Saccharomyces antisera with other Group I cell-walls and with Groups II and III cell-walls (Table 26); the degree of reactivity was the same. Antisera to Group III cell-walls were more extensive in their cross-reactions with Group III cell-walls than with those of Groups I and II (Table 27). Candida antisera exhibited most specificity to Candida cell-walls, but also reacted more extensively with

other Group III cell-walls than with Groups I and II cell-walls (Table 28). In the testing of anti-Candida sera it was noted that the cell-walls of Candida species cross-reacted very strongly. However, the extent of the cross-reaction was less with antiserum against C. krusei than with the other antisera. The specificity of these cross-reactions was not confined solely to the Candida species. Indeed, cell-walls of Groups I, II and III species showed significant levels of cross-reaction. It was interesting that there were only six out of the twenty-two strains which reacted with antiserum against C. krusei and two reacted with antiserum against C. stellatoidea. It would seem that the common antigen(s) suggested by the reaction patterns of Group I antisera is of minor significance in the cell-walls of Group III species. This antigen(s) is of greater immunological importance in species of Group I.

A corresponding situation emerged in the cross-reaction patterns of the cell-walls with all antisera (Table 29, 30, 31 and 32). The most notable feature was the extent of cross-reactivity of Group III cell-walls, which reacted to the same extent with Group III antisera as with antisera to Groups I and II (Table 30). The extent of cross-reactivity of Group III cell-walls with antisera against Groups I and II species adds weight to the proposal that there is a common antigen(s) shared by Groups I, II and III species. This antigen(s) is of greater importance in Group I cell-walls. Thus the major proportion of the antibody response to Group I cell-walls would be directed against this common antigen(s) unlike the response to cell-walls of Group III species which would be directed against other antigenic components.

The distribution of cell-wall antigens

The results which were obtained by agglutination and complement fixation procedures were used to prepare a theoretical allocation of

TABLE 42 :

Theoretical allocation of antigens

	(A	B)	(C	D)	E	F	(G	H)	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
D. hansenii	A	B	C	D			g															
H'spora valbyensis			C	D		F	G															
L. starkeyi			c	d	E																	
P. membranaefaciens		b	C	d		F																
S. cerevisiae		B	C			f	G															
Sp. roseus			c	d			g							N						T		
H. anomala		b	C						I					N								
E. capsularis			c			f	g															
N. fulvescens			C			F	G		I													
Nem. coryli			c			F	g		i	J												
S. bayanus		b	c				g		I													
S. rouxii			c	d			G							n							U	
S. uvarum		b	c			F	G		I									R				
Schiz. pombe			c																			
Schw. occidentalis			c			f	g		I		K											
Wick. fluorescens		B	C	d			G															
W. robertsii			c	d		f						L										
B. alba			c				G		I				M									
C. albicans			C				g	H	I				m	N	O							
C. guilliermondii			C				g	H	I				m	N								V
C. krusei			c			f	g		i							P						
C. stellatoidea			C				g	H	I				m	N								
K. apiculata			C	d									m	N								
R. glutinis			c				g	H	I					n								
Tr. cutaneum			c				g	h	I			l	M	N			Q					
Trig. variabilis			C																S			

TABLE 43 :

Example of cell-wall antigen allocation based on complement fixation and agglutination results

	CELL-WALLS	ANTISERUM	Reciprocal titre CFT	Reciprocal titre Agglutin	Allocation of antigens
I	*D. hansenii*	*D. hansenii*	640	32	
	H'spora valbyensis	*D. hansenii*	160	1	*D. hansenii*: A^+ B^+ C^+ D^+
	D. hansenii	*H'spora valbyensis*	320	16	*H'spora valbyensis*: A^- B^- C^+ D^+
	H'spora valbyensis	*H'spora valbyensis*	320	16	
II	*D. hansenii*	*D. hansenii*	640	32	
	L. starkeyi	*D. hansenii*	320	64	*D. hansenii*: A^+ B^+ C^+ D^+ E^-
	D. hansenii	*L. starkeyi*	40	4	
	L. starkeyi	*L. starkeyi*	320	16	*L. starkeyi*: A^- B^- c^+ d^+ E^+
	H'spora valbyensis	*H'spora valbyensis*	320	16	
	L. starkeyi	*H'spora valbyensis*	160	4	*H'spora valbyensis*: A^- B^- C^+ D^+ E^-
	H'spora valbyensis	*L. starkeyi*	20	2	
	L. starkeyi	*L. starkeyi*	320	16	

cell-wall antigens to the individual species. This formulation was based on the patterns and degree of cross-reactivity between each cell-wall preparation and every antiserum. It was necessary to accept the theory of the specificity of the antigen:antibody reaction as proposed by Landsteiner (1936), from which it was assumed that a structural antigenic component of a yeast cell-wall would stimulate the production of an antibody in an experimental animal which would react with the same antigenic component present on the surface of other yeast cells.

Initially, the results of the CFT for the twenty-six species of yeasts (Table 23) were analysed randomly without division into separate groups. The antigenic scheme thus obtained is shown in Table 42. The procedure used to determine the distribution of cell-wall antigens was based on the sheme shown in Table 43. Initially, the CFT reactions between paired cell-wall preparations and rabbit antisera against these cell-walls were compared. The extent of cross-reactivity was noted in relation to the homologous reaction and antigens were allocated to explain the relationship. For example, D. hansenii cell-walls reacted with antiserum against D. hansenii to a titre of 1/640. The cell-walls of H'spora valbyensis cross-reacted with the same antiserum to a titre of 1/160. Similarly, H'spora valbyensis cell-walls reacted with antiserum against H'spora valbyensis to a titre of 1/320. The cell-walls of D. hansenii cross-reacted with the same antiserum also at a titre of 1/320. These findings were substantiated by the agglutination test results. It was thus apparent that D. hansenii and H'spora valbyensis possessed common antigenic components in their cell-walls. Although D. hansenii possessed the major cell-wall antigens of H'spora valbyensis it was concluded that it also possessed some specific antigens not present in H'spora valbyensis. Therefore, antigens A, B, C, and D were allocated to D. hansenii and antigens C and D were allocated to H'spora valbyensis (see I of Table 43).

When the extent of cross-reactivity between pairs of the species D. hansenii, H'spora valbyensis and L. starkeyi was examined some distinct differences were observed (see II of Table 43). The cells of L. starkeyi cross-reacted with the antiserum against D. hansenii at a titre of 1/320 compared with the homologous titre of 1/640. But, D. hansenii cell-walls cross-reacted with the antiserum against L. starkeyi at a titre of 1/40 compared with the homologous titre of 1/320. It was concluded from these observations that L. starkeyi possessed cell-wall antigens which were present in D. hansenii, but were either very minor in L. starkeyi or weakly immunogenic or haptenic because D. hansenii cell-walls cross-reacted only at a very low titre. The same phenomenon was observed between L. starkeyi cell-walls and the antiserum against H'spora valbyensis which cross-reacted at a titre of 1/160 compared with the homologous reaction of 1/320. The H'spora valbyensis cell-walls cross-reacted with L. starkeyi antiserum at a titre of 1/20 compared with the homologous reaction of 1/320. Thus the same type of relationship exists between D. hansenii and L. starkeyi as exists between H'spora valbyensis and L. starkeyi. For this reason L. starkeyi was allocated haptens c and d. However, since the titre of the homologous reaction between L. starkeyi cell-walls and antiserum against L. starkeyi was 1/320 it was obvious that it possessed a major antigen(s) not present in H'spora valbyensis or D. hansenii, i.e. antigen E.

Throughout this analysis the major antigens are indicated by upper case letters which are underlined. The antigens are indicated by upper case letters and haptens with the corresponding lower case letters. The term hapten is used as defined by Landsteiner (1936) as a specific substance which although reactive in vitro, induces no or only slight antibody responses. As such it is accepted that any designated hapten in this analysis can be agglutinated by antisera directed against the corresponding antigen, i.e. C antibody against c hapten will react positively.

TABLE 44 :

Designation of antigens according to frequency

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
L. starkeyi	a	.	.	.		f	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. cerevisiae	A	B	.	d	E	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. bayanus	a	b	C	.	e	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S. rouxii	a	B	.	.	.	f	.	H	.	.	.	.	.	.	o	.	.	.	.	.	.	.
S. uvarum	a	B	C	D	e	.	.	.	I	.	.	.	.	.	.	.	.	.	.	.	.	.
Schw. occidentalis	a	b	C	d	.	.	.	.	.	J	.	.	.	.	.	.	.	.	.	.	.	.
D. hansenii	A	b	.	.	E	F	.	.	.	.	K	.	.	.	.	.	.	.	.	.	.	.
P. membranaefaciens	A	.	.	D	e	f	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
W. robertsii	a	.	.	d	.	f	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.
H. anomala	A	.	C	.	e	.	.	.	.	.	.	.	.	.	O	.	.	.	.	.	.	.
Schiz. pombe	a	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
N. fulvescens	A	B	C	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H'spora valbyensis	A	B	.	D	.	F	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Wick. flucrescens	A	B	.	.	E	f	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Nem. coryli	a	b	c	D	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	.	.	.
E. capsularis	a	b	.	d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. alba	a	B	C	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.
Sp. roseus	a	b	.	.	.	f	.	.	.	.	.	.	.	.	O	.	.	R	.	.	.	.
R. glutinis	a	b	C	.	.	.	.	.	.	.	.	.	.	N	o	.	.	.	.	.	.	.
K. apiculata	A	.	C	.	.	f	.	.	.	.	.	.	.	.	O	p	.	.	.	.	.	.
C. albicans	A	b	C	.	.	.	.	.	.	.	.	.	.	N	O	p	Q	.	.	.	.	.
C. guilliermondii	A	b	C	.	.	.	.	.	.	.	.	.	.	N	O	p	.	.	S	.	.	.
C. krusei	a	b	c	d	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
C. stellatoidea	A	b	C	.	.	.	.	.	.	.	.	.	.	N	O	p	.	.	.	.	.	.
Tr. cutaneum	a	b	C	.	.	.	.	.	.	.	.	.	.	n	O	P	.	.	.	.	U	.
Trig. variabilis	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	V

However, the antiserum to a cell-wall containing the hapten will not agglutinate any other cell-wall if the sole relationship is by virtue of the corresponding complete antigen, i.e. c antibody against C antigen will not react.

The complement fixing and agglutinating titres of all the twenty-six antisera against yeast cell-walls were analysed by this procedure. It should be stressed that the antigenic and haptenic formula for each cell-wall represents the minimal number of antigens; the formulae do not necessarily represent the complete antigenic mosaics of the yeast cell-walls.

Subsequently, the yeasts were placed in Groups I, II and III (Table 44). The incidence of each antigen was recorded so that the most frequently occurring antigen was re-designated as antigen A; i.e. antigen C of Table 42 corresponds to antigen A of Table 44. The second most frequently occurring antigen was re-designated as antigen B, i.e. antigen G of Table 42 corresponds to antigen B of Table 44, and so on. It appears that antigens/haptens A, B and C are shared by most species of yeast although they seem to be of little importance in L. starkeyi, W. robertsii, Schiz. pombe, E. capsularis and Sp. roseus.

It is extremely interesting that the ascosporogenous yeasts placed together in Group I were seen to share one or more of the antigens/haptens of D, E and F, with the exception of Schiz. pombe which lacks any of these three. The asporogenous yeasts placed together in Group III also lack D, E and F except for C. krusei which possesses hapten d, and K. apiculata which possesses hapten f. It may be significant that H'spora valbyensis has been shown to be the perfect form of K. apiculata and Pichia kudriazevii the perfect form of C. krusei. Therefore, one might expect some sharing of surface antigens between the perfect and imperfect forms. In this respect it is very interesting that H'spora valbyensis possesses the major antigen F, whereas K. apiculata possesses the hapten f. Similarly,

Pichia spp. possess the major antigen D, whereas C. krusei possesses the hapten d. The basidiomycete Sp. roseus, although it possesses hapten f, appears to have more in common with Group III yeasts.

It is also apparent that the asporogenous yeasts of Group III share one or more of N, O and P, with the exception of C. krusei and Trig. variabilis. Table 44 also shows that the Group II basidiomycetes B. alba and Sp. roseus possess antigen P and antigen O respectively, which indicates a close relationship to Group III yeasts. Antigens G - M are considered to be mono-specific antigens of Group I species and Q - V of Group III species.

The detection of additional antigens might be possible using an antiserum against each species extensively absorbed with a variety of different yeast cell-walls. This type of procedure would create an extremely large survey in which the results might be difficult to interpret. Indeed, Hasenclever and Mitchell (1964 b) found that a logical explanation of some of their results obtained with agglutination inhibition presented a challenge of some magnitude; the results were quite paradoxical.

Antiserum to S. cerevisiae after absorption with D. hansenii cell-walls to remove antibody to A, B and E did not react with D. hansenii, H. anomala, S. bayanus and Sp. roseus cell-walls which confirmed the presence of one or more of the antigens in these species. Absorption of S. cerevisiae antiserum with Wick. fluorescens cell-walls also removed the antibody to A, B and E so that no reactivity was found with the above species. It was observed that absorption of S. cerevisiae antiserum with S. bayanus cell-walls which possessed haptens for a, b and e also removed the activity against antigens A, B and E.

The absorption of S. cerevisiae antiserum with Trig. variabilis cell-walls effectively removed the activity for antigen A, leaving antibody to B and E. The absorbed serum did not react with the yeasts Sp. roseus, R. glutinis and Tr. cutaneum which do possess hapten b in an antigenic

mosaic of five or six antigens. The absorbed serum cross-reacted with S. cerevisiae, S. bayanus, S. rouxii, S. uvarum, D. hansenii, H. anomala, H'spora valbyensis and Nem. coryli which was expected due to the presence of B or E. In some instances the cross-reacting titre was lower than anticipated and it was expected that N. fulvescens would react as it possessed antigen B.

The absorption of Sp. roseus antiserum with H. anomala was done to test the presence of antigen O in the latter species. The absorbed serum failed to react with cell-walls of H. anomala, S. rouxii, C. albicans and C. guilliermondii.

Antiserum to D. hansenii absorbed with H'spora valbyensis cell-walls to remove antibody to A, b and F did not cross-react with H'spora valbyensis, L. starkeyi, S. cerevisiae, Sp. roseus and C. guilliermondii; some reaction was expected with S. cerevisiae due to its possession of antigen E.

After absorption of C. albicans antiserum with cell-walls of C. stellatoidea no reaction was observed with C. albicans nor with the other cell-walls tested. This was unexpected since antibody to Q should have remained in the serum after absorption, and therefore the presence of Q (as a major antigen) is questionable in C. albicans. Similarly, absorption of C. albicans antiserum with C. guilliermondii cell-walls again removed all complement fixing activity in spite of the presence of Q in C. albicans. Conversely, it could be argued that antigen Q is present in C. guilliermondii and C. stellatoidea, but the paired cross-reactions of the unabsorbed sera do not indicate this.

C. albicans antiserum absorbed with K. apiculata cell-walls retains antibody to N and Q. The absorbed antiserum did not react with cell-walls of C. krusei, H. anomala, N. fulvescens or S. cerevisiae confirming the absence of these antigens. The presence of hapten n on Tr. cutaneum cell-walls was not sufficient for a reaction with the absorbed serum. The

slight reaction observed with Sp. roseus cell-walls was not considered to be of great significance, although it possibly indicated a close relationship of this species to C. albicans. C. albicans, C. guilliermondii and C. stellatoidea cell-walls reacted to the same extent with the absorbed antiserum as expected since N is present in all three.

Computer analysis of the chemical and serological results obtained with the twenty-six species

Numerical taxonomy has been successfully applied in the classification of various groups of micro-organisms, including yeasts, (Kocková-Kratochvílová, Šandula, Sedlárová, Vojtková-Lepšíková, and Kasmanova, 1969; Kocková-Kratochvílová, Sedlárová, Vojtková-Lepšíková and Šandula, 1970; Campbell, 1971 b, 1972). Consequently, it was decided to analyse the data obtained during this investigation to determine the relationships based on cell-wall composition and serological properties of yeast cell-walls. Different analytical methods were used in order to discover whether one clustering method was capable of differentiating species.

Single linkage

In the single linkage dendrogram the Saccharomyces species, excluding S. rouxii, form a cluster with five other species at the 80.5% level, see Figure 6. However, the Saccharomyces species do not show a close relationship with each other. It seems that the yeasts are grouped in pairs - S. cerevisiae with D. hansenii, S. bayanus with Wick. fluorescens, S. uvarum with Nem. coryli. Schw. occidentalis and H. anomala are added at the 80.5% level after which five species enlarge the cluster at the 79.1% level, namely, L. starkeyi, S. rouxii, P. membranaefaciens, Schiz. pombe and H'spora valbyensis. The rest of the Group I species, E. capsularis and W. robertsii, join at the 77.6% level

in addition to R. glutinis and B. alba.

Of Group II species, B. alba is most closely related to W. robertsii, whereas Sp. roseus joins the Group I cluster at the 76.1% level together with Tr. cutaneum and C. stellatoidea. The remaining species of Group III (in addition to N. fulvescens of Group I) are added sequentially to this cluster. K. apiculata and C. guilliermondii cluster with each other prior to joining the main cluster and the last two species, Trig. variabilis and C. albicans, join at the 71.6% level.

The majority of Group I species are clustered together in the 85 to 77.6% similarity range and the remaining Group II and III species join this cluster in the 77.6 to 71.6% level. Thus although the species represent a wide range of different yeasts they are clustered within a relatively narrow similarity range. The clustering of the Group I species at the higher similarity values and the chaining effect of the addition of Group III species at the lower values provides an indication of the greater heterogeneity of the latter species compared to the former.

Complete linkage

Group I species, apart from E. capsularis, are contained in one of the two major clusters formed at the 53.6% similarity level, Figure 7. The Group I cluster (which also contains D. alba of Group II) is in turn divided into two discrete clusters at the 62.6% and 66.1% levels. S. bayanus and S. rouxii occur in the former cluster and S. cerevisiae and S. uvarum occur in the latter. As with single linkage, each Saccharomyces species is more closely related to species outside the genus. Further subdivision of these clusters occurs until, in the majority of cases, clusters containing only two species are formed. The most closely related species are S. bayanus and Wick. fluorescens whose similarity level is 85%. By contrast, the strain least related to Group I species is N. fulvescens, which does not join a cluster until the 66% level. The most noticeable

feature of the Group I cluster is the division into two subgroups which join at the relatively low level of 53.6%.

Of Group II strains, B. alba is most closely related to W. robertsii (79% similarity) and is situated in the Group I cluster in contrast to Sp. roseus which is most closely related to R. glutinis (76% similarity) and is situated in the Group III cluster which also contains E. capsularis. Within this major cluster there are two smaller clusters. One at the 74.6% level contains K. apiculata, C. guilliermondii and C. krusei. The other at the 59.7% level contains the rest of Group III species in addition to Sp. roseus and E. capsularis. The latter cluster can be sub-divided. The first of these sub-divisions contains E. capsularis and Tr. cutaneum (76% similarity), the second contains Sp. roseus, R. glutinis and Trig. variabilis (68.6% similarity), and the third contains C. albicans and C. stellatoidea (70% similarity).

In addition, and unlike single linkage, the dichotomous branching of each major cluster reveals the relationships and grouping of the twenty-six yeast species at all similarity levels.

Average linkage

At the lower similarity levels a chaining effect is seen in the dendrogram for average linkage clustering, Figure 8. There is also little evidence of dichotomy. However, at the 69% level the majority of Group I species are contained in a single cluster. Outwith this cluster, but at the same level, P. membranaefaciens, H'spora valbyensis and W. robertsii are clustered with the two Group II species (B. alba and Sp. roseus) together with R. glutinis. The remaining Group I species, E. capsularis, is most closely related to Tr. cutaneum (76.1% similarity) and occurs in a cluster containing Group III strains. The Saccharomyces species are, as before, more closely related to species outside the genus. However, all the Saccharomyces species do occur in the same cluster. At

higher similarity levels, the relationships which exist are similar to those found by complete linkage. At the 79.1% level L. starkeyi and Schiz. pombe cluster as do Schw. occidentalis and H. anomala together with S. uvarum and Nem. coryli. N. fulvescens remains distinct from other strains until it joins a cluster at the 66% level.

Both Group II strains, B. alba and Sp. roseus, are members of the same cluster at the 69% level, B. alba first clustering with W. robertsii. In addition to R. glutinis, C. albicans is the other Group III strain in the cluster and joins after Sp. roseus at the 67.6% level. The rest of Group III yeasts are placed in two smaller clusters. One contains E. capsularis (Group I) and Tr. cutaneum clustered at the 76.1% level and to which Trig. variabilis joins at 68.6% similarity level. The second cluster comprises K. apiculata and C. guilliermondii linked at the 76.1% level below which C. krusei enters the cluster (74.6% similarity), followed by C. stellatoidea at the 70.1% level.

Thus average linkage clustering forms four clusters; the first containing eleven Group I species; the second containing four Group I species, both Group II species and two Group III species; the third containing two Group III species and one Group I species; and the fourth containing four Group III species. The first two clusters combine at the 65.1% similarity level, closely followed by the third cluster at the 64.3% level and lastly the fourth cluster joins at the 63% level.

The dendrogram formed by average linkage does not divide the two major Groups I and III. There is an overlap consisting of species of both these Groups as well as Group II. Average linkage clustering occurs between 63 and 85% similarity levels. By comparison, complete linkage clustering occurs between 49.1 and 85% similarity levels. Thus there is less distinction of clusters by the average linkage method. However, as average linkage clustering is based on all the members of a cluster when a species is considered for entry, it should produce a more "natural" sequence

of clusters than those formed by complete and single linkage which are based on the similarity value of one species within the cluster and the proposed member.

Ward's linkage method

Two major clusters were formed at the 0.72 and 0.59 values of the average squared distance, Figure 9. The former cluster can be divided into two smaller clusters. The first contains L. starkeyi and Schiz. pombe which form a distinctly separate cluster on their own. The remainder of the species in this cluster includes the Group II strain B. alba and two of the Saccharomyces species, S. bayanus and S. rouxii which are most closely related to Wick. fluorescens at the 0.3 value. P. membranaefaciens, H'spora valbyensis, W. robertsii and B. alba form the same relationships observed by complete linkage analysis (Figure 7). The second cluster containing Group I strains, S. uvarum, Nem. coryli, Schw. occidentalis and H. anomala shows the same relationship as found by complete linkage analysis (Figure 7). In this cluster N. fulvescens clusters with S. cerevisiae and D. hansenii at the 0.32 value which is the most specific relationship for N. fulvescens found by any of the clustering methods.

B. alba of Group II also occurs in the major cluster containing Group I species, whereas, Sp. roseus occurs in the cluster consisting of mainly Group III species. In addition, the latter is most closely related to R. glutinis at the 0.24 value. A major cluster consisting of mainly Group III species is formed at the 0.59 value, in which K. apiculata, C. guilliermondii and C. krusei form a smaller cluster at the 0.26 level. The second subdivision of this major cluster contains three smaller clusters - (i) E. capsularis and Tr. cutaneum (0.24 value), (ii) C. albicans and C. stellatoidea (0.3 value) and (iii) Sp. roseus, R. glutinis and Trig. variabilis (0.32 value).

Ward's method of linkage is recommended by the CLUSTAN manual as being possibly one of the best options, although it has not been used by numerical taxonomists in the field of microbiology. It has, however, the disadvantage that, as the clusters increase in size, the "loss" of information becomes increasingly greater as two clusters combine, resulting in an extended dendrogram.

Assessment of linkage methods

Single linkage, because of the "chaining" effect, makes it difficult to interpret relationships amongst the twenty-six yeasts. Complete linkage provides distinct clusters, but has the same disadvantage as single lirkage by not taking into account the structure of a cluster, when the addition of another species is considered. The average linkage method takes the structure of the cluster into account and produces distinct clusters which are not as well differentiated as those formed by complete linkage. Ward's linkage method is based on the data shared by all the members of a cluster and in this respect is better than the average linked method. The clusters formed are distinct although fusion of larger clusters results in extension of the dendrogram. The dendrogram formed by Ward's method is preferred as it has the advantages of both average linkage and complete linkage with regard to cluster structure and differentiation.

A comparison of the analysis by Ward's method (Figure 9) with the antigenic analysis (Table 44) revealed some interesting relationships. At the 0.73 and 0.59 values for the average squared distance, it is seen that the twenty-six species are divided into two main clusters, in which B. alba and E. capsularis are placed in different clusters than expected. It is seen from the antigenic analysis (Table 44) that E. capsularis is related to Group I yeasts by possession of hapten d. Similarly, B. alba

is related to Group III yeasts by possession of antigen P. After consideration of computer analyses it would seem feasible to propose that there are two distinct antigenic groups. It is difficult to place E. capsularis, H. anomala, B. alba and C. krusei into one of these groups without some reservations. However, since the formation of ascospores is a natural characteristic and since the majority of the ascoporogenous yeasts are in Antigenic Group I, E. capsularis and H. anomala are also included. For the same reasons, C. krusei was placed in Antigenic Group II, although possessing hapten d. Although Sp. roseus, a basidiomycete, was found to be antigenically related to the asporogenous yeasts, B. alba was included in the Antigenic Group I on the weight of the computer analysis.

The two type species Schiz. pombe and Trig. variabilis did not appear to fit into either antigenic group and even after consideration of the computer analysis it was considered that they are sufficiently distinct from either Antigenic Group to be excluded from both. However, it is not suggested that there is a positive relationship between these two species.

Computer analysis of the published properties (Lodder, 1970) of the twenty-six species

The properties of the species published by Lodder (1970) were analysed in the computer. A comparison with the relationships revealed by antigenic analysis and numerical analysis of the chemical and serological results was made to determine whether the use of a wide range of characteristics would yield better differentiation. The dendrograms formed by single, complete and average linkage clustering, Figures 11, 12, 13 and 14, revealed the same characteristics of clustering as found in the corresponding dendrograms based on the chemical and serological results. Ward's method of clustering was therefore chosen for purposes of comparison.

Ward's linkage method

Two distinct major clusters, originating at average squared distance values of 0.59 and 0.97, are apparent in the dendrogram formed by Ward's method of clustering, Figure 14. Fusion of these clusters occurs at a value of 2.33 indicating considerable difference of the properties of the members. The first cluster is divided into two parts, one of which contains L. starkeyi, B. alba and Tr. cutaneum clustering at 0.25 and 0.35 successively, and is joined at the 0.43 level by Sp. roseus and R. glutinis which previously clustered at a value of 0.19. The second part of the cluster contains D. hansenii, W. robertsii, H. anomala and C. guilliermondii which are sequentially clustered at successive values of 0.18, 0.22 and 0.3.

The second major cluster can be divided into three smaller clusters. In the first of these S. cerevisiae, S. bayanus and S. uvarum are clustered at the 0.1 value and are joined by Schw. occidentalis at the 0.32 value. S. rouxii forms a cluster with Schiz. pombe at the 0.22 level which is enlarged by the addition of Nem. coryli at the 0.27 level and fuses with the cluster containing the Saccharomyces species at the 0.35 level. The first of the three small clusters is completed when N. fulvescens and Wick. fluorescens join the above species at the 0.45 value, after first clustering together at a value of 0.21.

The second of the smaller clusters contains C. albicans and C. stellatoidea clustering at the 0.17 value and E. capsularis which joins these at the 0.4 value. This cluster joins the one containing the Saccharomyces species at the 0.72 level.

The third cluster, which completes the major cluster, contains five species. P. membranaefaciens and C. krusei which cluster at the 0.12 level and are joined at the 0.3 level by Trig. variabilis. H'spora valbyensis and K. apiculata, the most closely related species,

form a cluster at the 0.06 value and enter the cluster containing the three species (P. membranaefaciens, C. krusei and Trig. variabilis) at a value of 0.42. These are joined to the remainder of the species in this major cluster at the 0.97 level.

There is little ordering of the species with regard to the original Groups I, II and III. The smaller of the two main clusters contains four Group I strains, both Group II strains and three Group III species. The division of the larger cluster into three parts reveals that the first of these contains ten Group I species, the second one Group I and two Group III, and the third two Group I and three Group III species.

Of the Saccharomyces species, S. cerevisiae, S. bayanus and S. uvarum are closely related, whereas S. rouxii remains distinct. C. albicans and C. stellatoidea are most closely related to each other and are only distantly related to C. guilliermondii and C. krusei which are in turn widely separated from each other.

The differentiation of genera in accepted classification is restricted in most cases to one or two properties which are frequently morphological. The inclusion of physiological properties and the reduction of the emphasis on these one or two properties, results in the relationship shown in Figure 14. It is difficult to see how the present system of classification (Lodder, 1970) relates to this distribution.

CONCLUSIONS

This analysis of yeast cell-walls has shown that it is possible to detect three types of antigenic component. The first of these is widely shared among all the species and is of little taxonomic value. The second broadly divides the type-species into two groups, one of which contains the ascosporogenous yeasts and the other which contains the asporogenous yeasts. The third type of antigenic component appears to be specific at the species level.

Examination of several Saccharomyces and Candida species indicated that antigenic differentiation cannot be achieved at the genus level. This point was confirmed by the computer analyses, since in no instance were the Saccharomyces species and Candida species grouped at the genus level. In this respect the results of this investigation have been confirmed by the reports of other workers (Tsuchiya et al., 1965; Campbell, 1968) who in general used small groups of yeasts.

It is interesting that a compilation of the published results of Tsuchiya et al., 1965; Murray and Buckley, 1966; Sweet and Kaufman, 1970; and Richards, 1972 (Table 45), using slide or tube agglutination also shows the presence of shared and specific antigens. These authors also agree that there is considerable variation within the genera Saccharomyces and Candida. C. krusei was placed in the same antigenic group as P. membranaefaciens by Tsuchiya et al., (1965). As mentioned previously, the latter species has the major antigen D and the former has the haptenic equivalent which may reflect this relationship. It is interesting that Schiz. pombe was also shown to be antigenically indistinct by Tsuchiya et al., (1965). Further evidence for the relationship between Sp. roseus and R. glutinis was provided by the antigenic analysis, although Tsuchiya et al., (1957, 1969) did not find that they cross-reacted with

TABLE 45 :

Antigenic formulae proposed by different authors

	Tsuchiya et al., (1965)	*Richards (1972), Sweet and Kaufman (1970)
L. starkeyi		
S. cerevisiae	1 2 3 10 (14) 18 31	*1 2 3 5 6 7 11 12
S. bayanus		
S. rouxii	1 (8) (10) (28) 32	
S. uvarum	1 2 3 10 (14) 18 31	*1 2 3 4 5 6 7 8 9 10 11 12 13 14
Schw. occidentalis	1 2 3 4 (14) 9	
D. hansenii	1 2 3 4 9 (14)	
W. robertsii		
P. membranaefaciens	1 2 5 11 12	
H. anomala	1 2 14 15 16 (17) 20	
Schiz. pombe	i iii	
N. fulvescens		
H'spora valbyensis	1 8 10 28	
Wick. fluorescens		
Nem. coryli		
E. capsularis		
B. alba		
Sp. roseus	I VIII IX	
R. glutinis	I II V VIII	
K. apiculata	1 8 10 28	
C. albicans	1 2 3 4 5 6 7	1 2 3 4 5 10 11
C. guilliermondii	1 2 3 4 9	1 2 3 5 9 10 11
C. krusei	1 2 5 (11)	1 6 11
C. stellatoidea	1 2 3 4 5 (10)	1 2 5 10 11 12
Tr. cutaneum		
Trig. variabilis		

C. albicans	1 2 3 4 6 7 10 13 14 15	Murray and Buckley (1966)
C. guilliermondii	1 2 3 4 9 10 13 15	
C. krusei	1 2 5 11	
C. stellatoidea	1 2 3 5 16	

any other species.

The criticisms by Richards (1972) of the slide agglutination test cannot be totally accepted since useful information on the antigenic behaviour of yeast cells has been obtained. The detailed antigenic formulae involve both cell-wall and cytoplasmic components which may give rise to a number of overlapping antigens. In addition, the initial experiments done with absorbed sera indicated that a further study involving many yeasts would be a vast undertaking. To conclude, in my opinion it would seem logical to develop procedures for the enzymatic digestion of yeast cell-walls and subsequent fractionation of specific antigenic components. A range of antisera produced against these structural components would be extremely useful for the antigenic identification of the yeasts.

These investigations have shown that until such information becomes available the antigenic analysis of yeast cell-walls provides useful additional taxonomic data, but in itself does not provide a complete yeast classification.

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SUMMARY

Chemical and serological properties of the cell-walls of twenty-six species of yeast, representing twenty genera, were examined to determine whether such properties might prove to be of value in taxonomy.

Procedures for the large-scale production of yeast cell-walls and preparation of rabbit anti-yeast cell-wall sera were developed.

Chemical analyses showed that, unlike Gram positive bacterial cell-walls which possessed a limited range of amino acids, yeast cell-walls contained a complete range of amino acids. The monosaccharides in yeast cell-walls, whether analysed qualitatively or quantitatively, failed to yield information of value in taxonomy.

Agglutination and complement fixation tests with yeast cell-walls and rabbit antisera revealed an extensive and complex pattern of cross-reactions between the species. From this it was possible to construct a scheme of antigen distribution in the different species. This scheme broadly separated the ascosporogenous yeasts from the asporogenous yeasts. Examination of several Saccharomyces and Candida species indicated that antigenic differentiation cannot be achieved at the genus level.

A common antigen(s) was found in all the yeast cell-walls. The ascosporogenous yeasts possessed antigens which were not found in the asporogenous yeasts and vice versa. A major antigen in cell-walls of Candida species was also found in cell-walls of Trichosporon cutaneum, Kloeckera apiculata, Rhodotorula glutinis and Sporobolomyces roseus, but not in Candida krusei which had greater antigenic similarity to the ascosporogenous yeasts.

Trigonopsis variabilis and Schizosaccharomyces pombe were antigenically distinct from the remainder of the yeasts and from each other.

Numerical analyses of the serological and monosaccharide properties of the cell-walls confirmed the distinction between ascosporogenous and asporogenous yeasts. It also showed that little differentiation could be achieved at the genus level, since in no instance were the Saccharomyces and Candida species grouped in their respective genera.

In contrast, a similar computer analysis of the standard taxonomic characteristics failed to reveal a division of the yeasts into an ascosporogenous and an asporogenous group. Instead the yeasts emerged as a heterogenous population of species, indicating that physiological properties are unsuitable for classification above the species level.

It was concluded that fractionation of antigenic determinants of the cell-wall and subsequent serological investigation of these components would be necessary for the construction of a comprehensive classification scheme for yeasts in which these properties would form an integral part together with physiological and morphological characteristics.

APPENDIX

Anthrone Reagent

Anthrone	2 g
95% H_2SO_4	1 l

Ferric alum reagent

Ferric ammonium sulphate	0.99 g
conc. HCl	1 l

Orcinol reagent

Orcinol	1 g
95% ethanol	10 ml

Acetylacetone reagent

Sodium carbonate	2.302 g
Sodium bicarbonate	0.276 g
Sodium chloride	0.584 g
Distilled water	100 ml
Redistilled acetylacetone	1 ml

Aldehyde reagent

p-dimethylaminobenzaldehyde	0.8 g
Ethanol	96.5 ml
conc. HCl	3.5 ml

Glucose oxidase enzyme reagent

"Glucostat" enzyme kit (Worthington Biochemical Corp., New Jersey, U.S.A.) containing:

(i) Glucose oxidase

(ii) Peroxidase

(iii) O-dianisidine

0.1 M phosphate buffer pH 7.0 50 ml

0.1 M Phosphate buffer pH 7.0

Monopotassium phosphate	5.621 g
Disodium phosphate	10.452 g
Distilled water	1 l

Galactose oxidase enzyme reagent

"Galactostat" enzyme kit (Worthington Biochemical Corp., New Jersey, U.S.A.) containing:

(i) Galactose oxidase

(ii) Peroxidase

(iii) O-tolidine

0.1 M phosphate buffer pH 7.0 50 ml

0.25 M glycine buffer pH 9.7

Glycine	13.175 g
Sodium chloride	10.256 g
Sodium hydroxide	2.98 g
Distilled water	1 l

FIGURE 6 :

Single linkage dendrogram based on chemical and serological properties

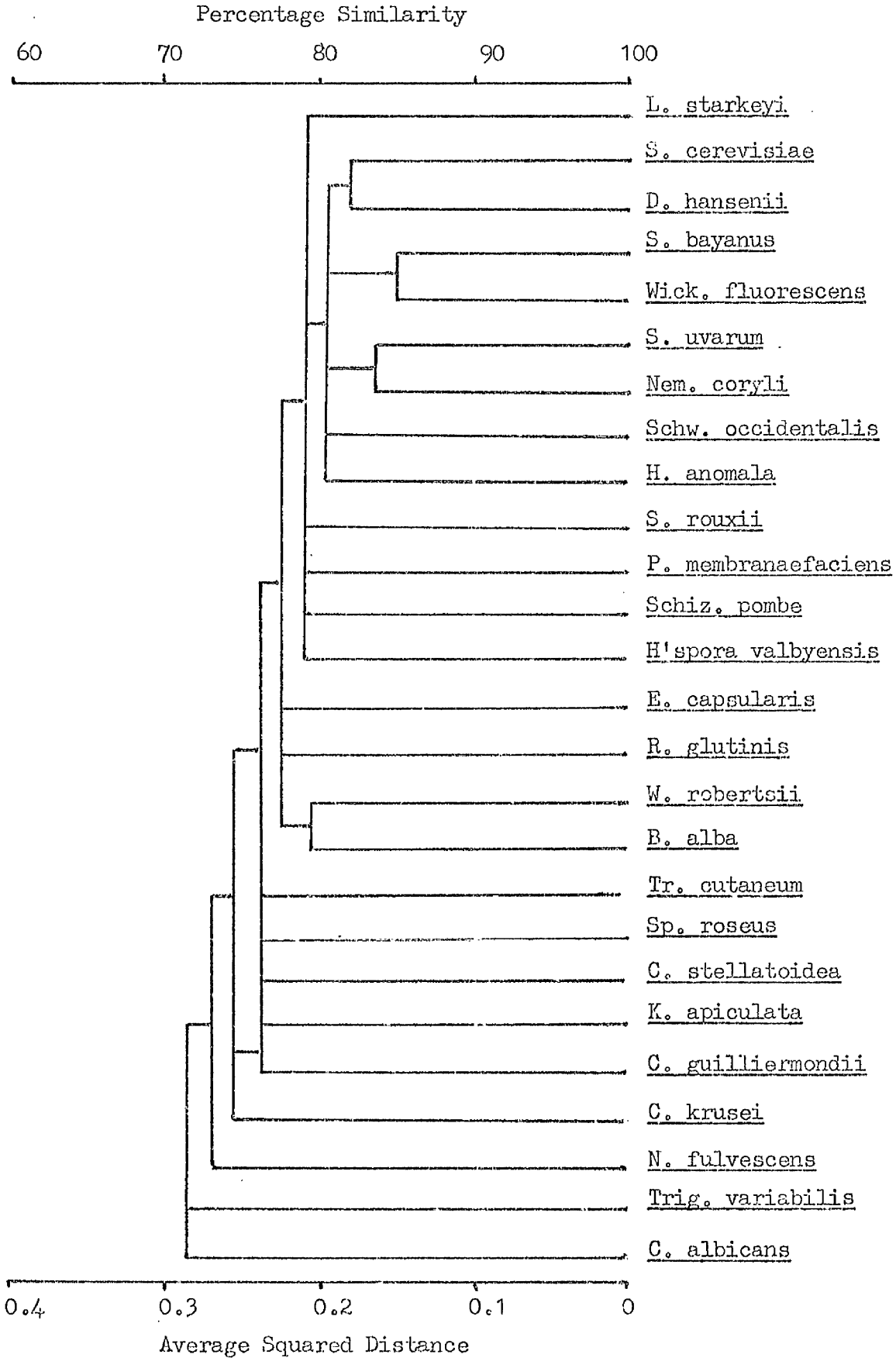

FIGURE 7 :

Complete linkage dendrogram based on chemical and serological properties

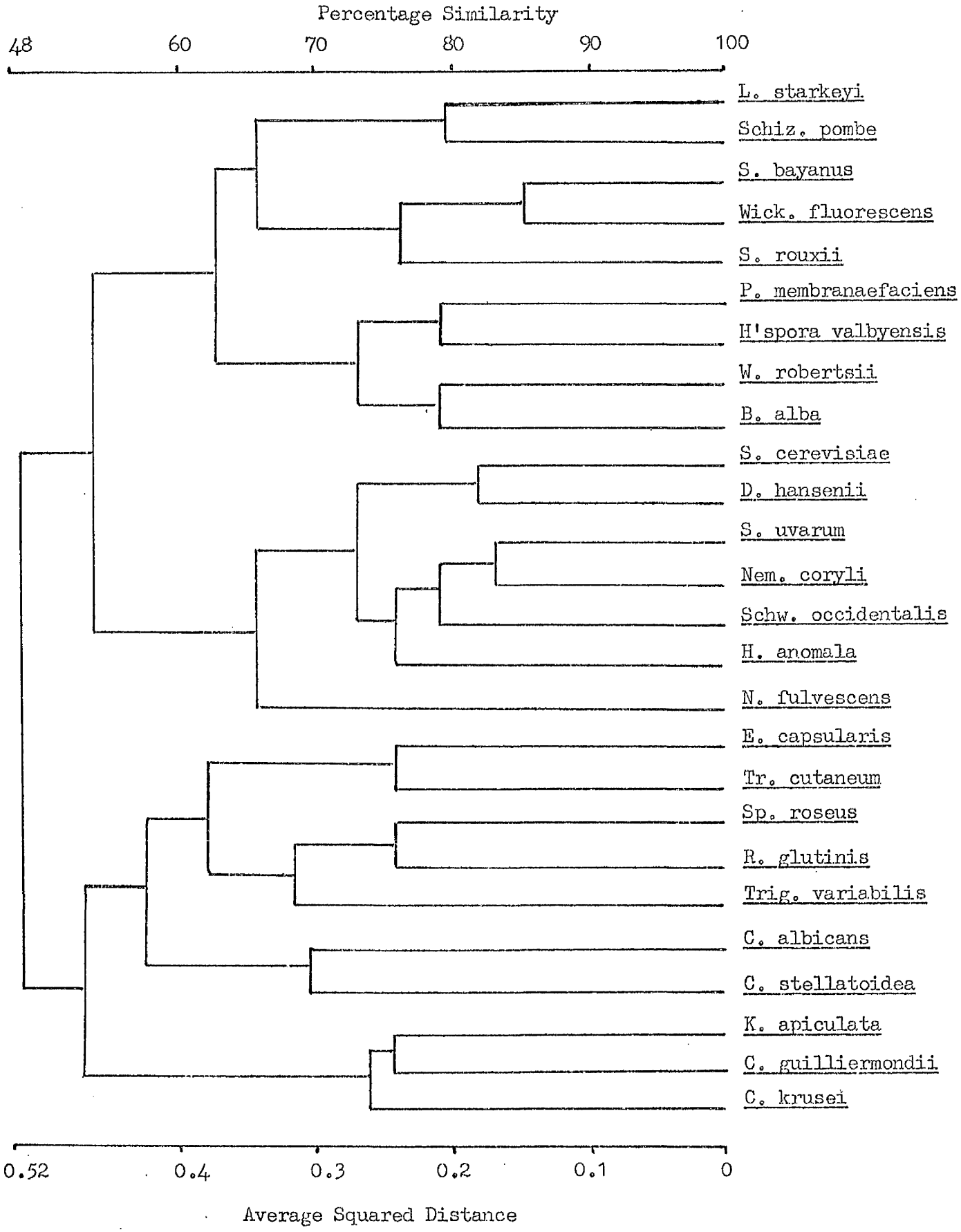

FIGURE 8 :

Average linkage dendrogram based on chemical and serological properties

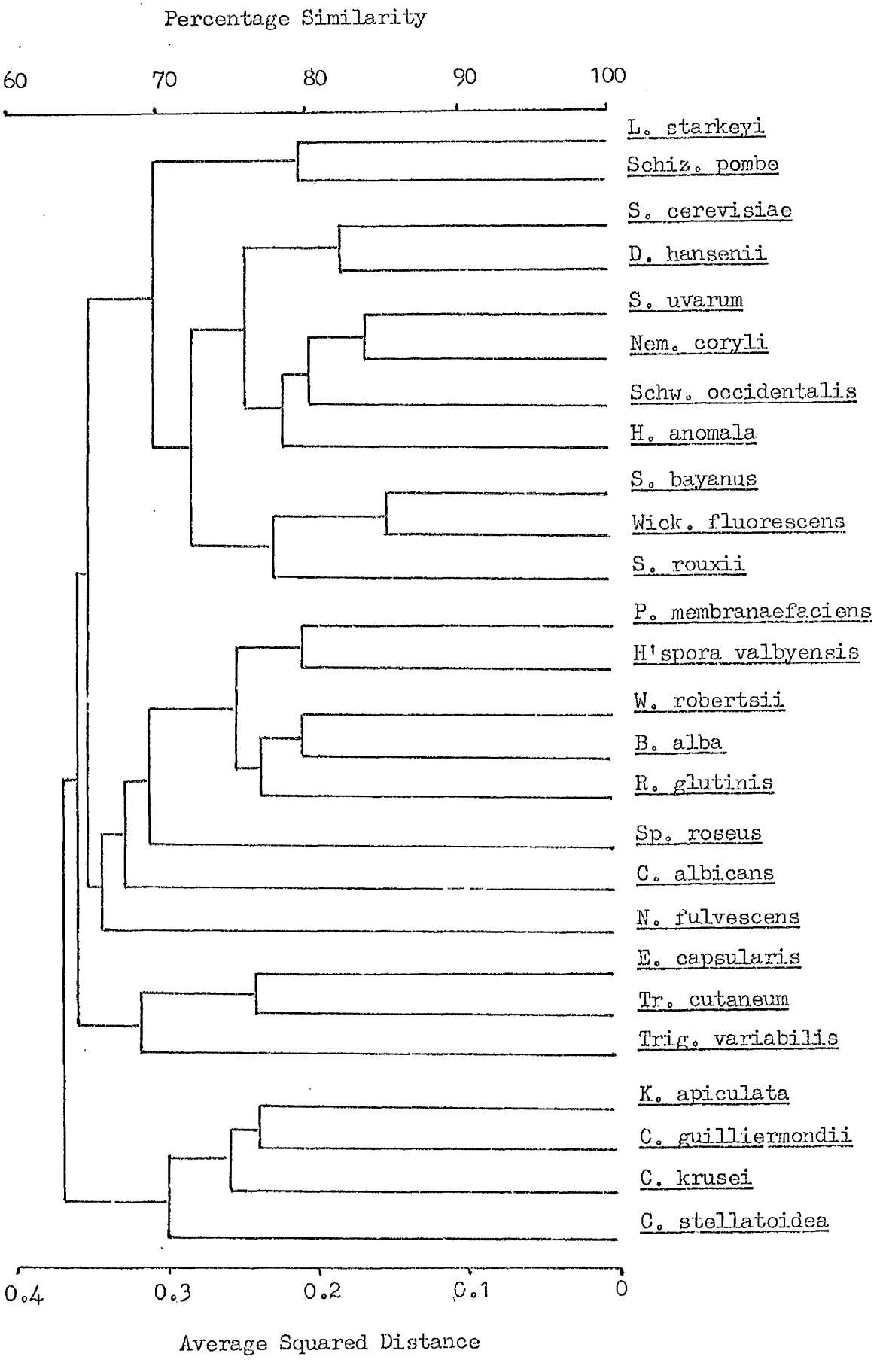

FIGURE 9 :

Dendrogram based on the chemical and serological properties
(Ward's method)

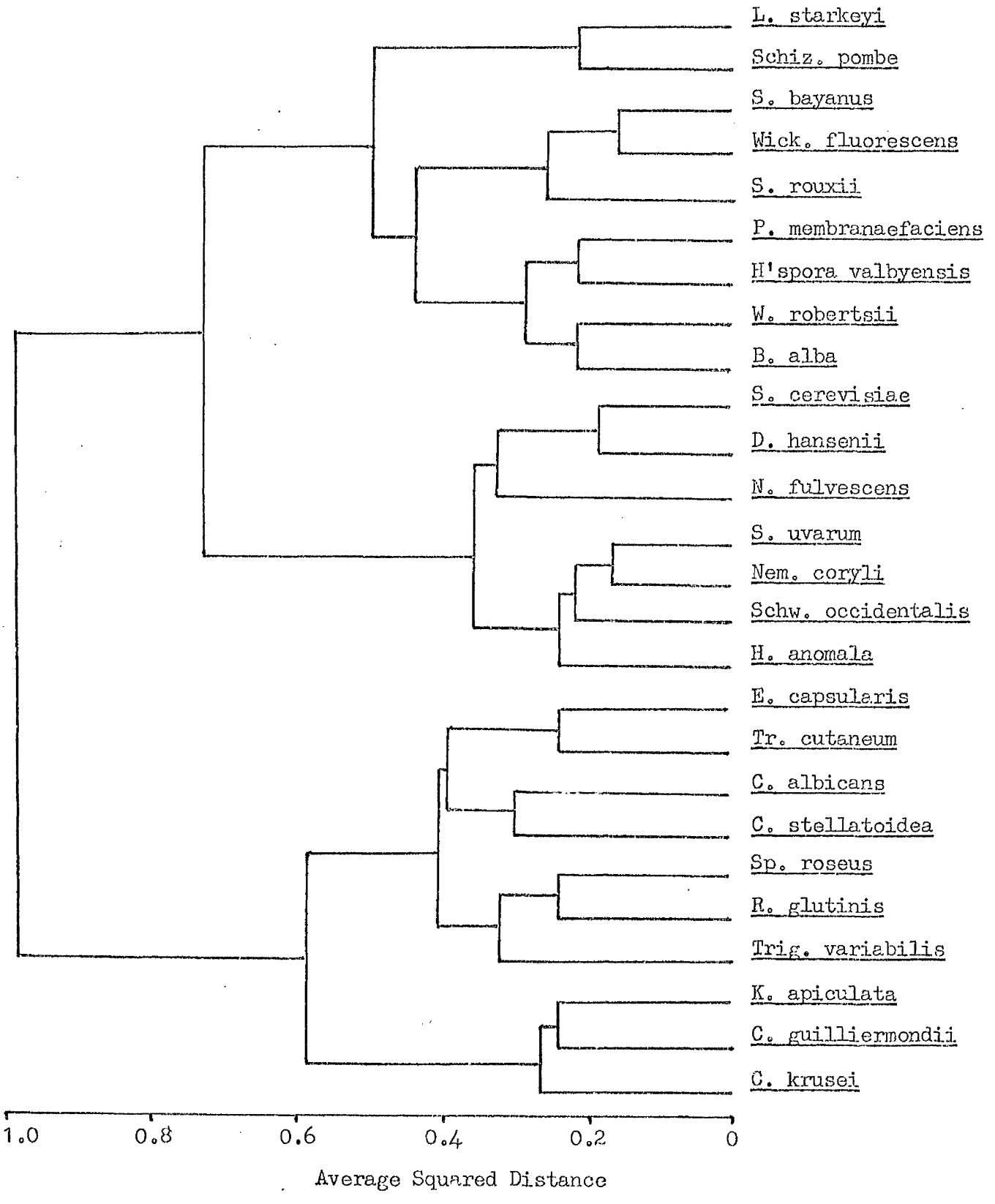

FIGURE 11 :

Single linkage dendrogram based on published properties

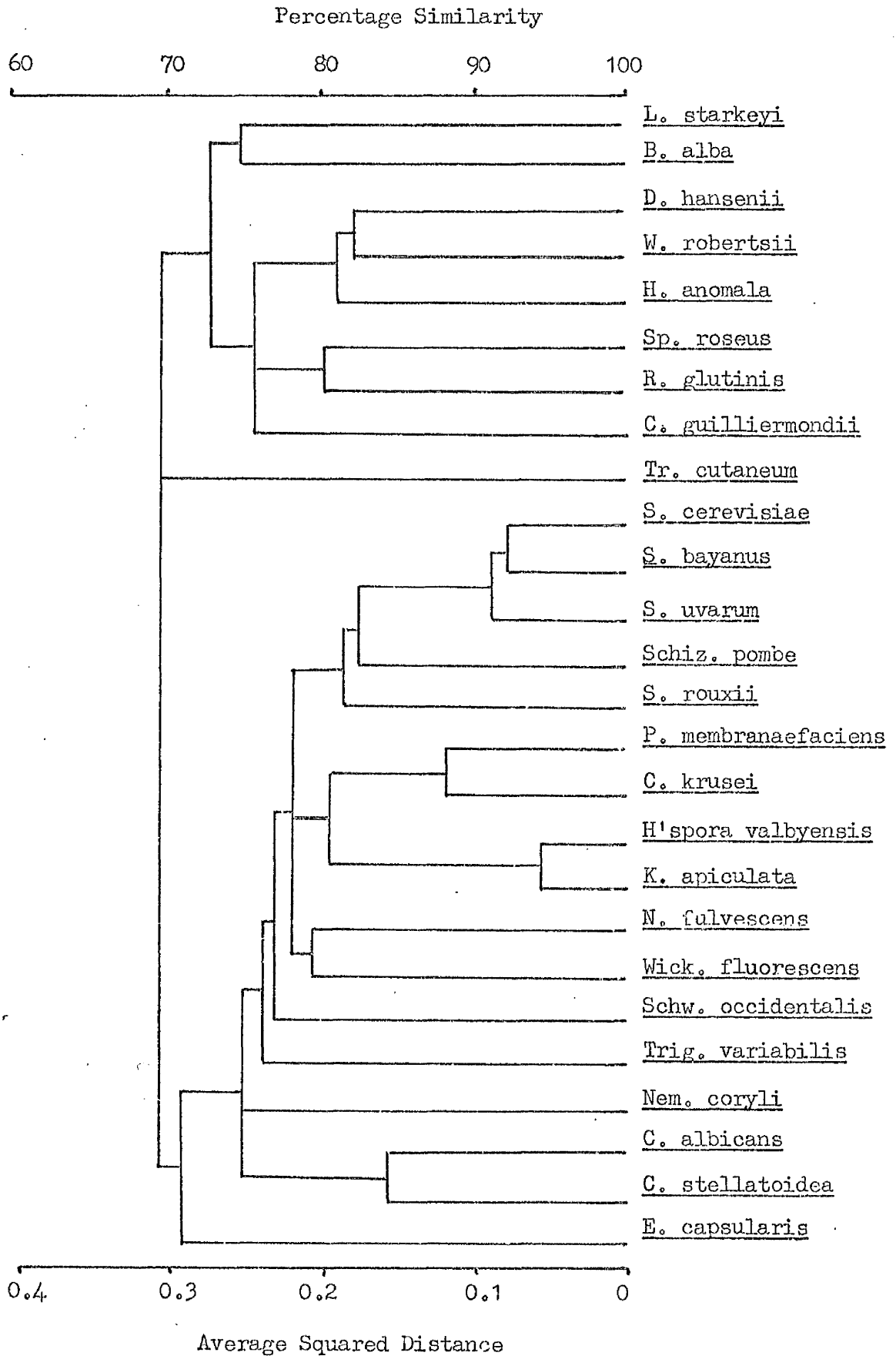

FIGURE 12 :

Complete linkage dendrogram based on published properties

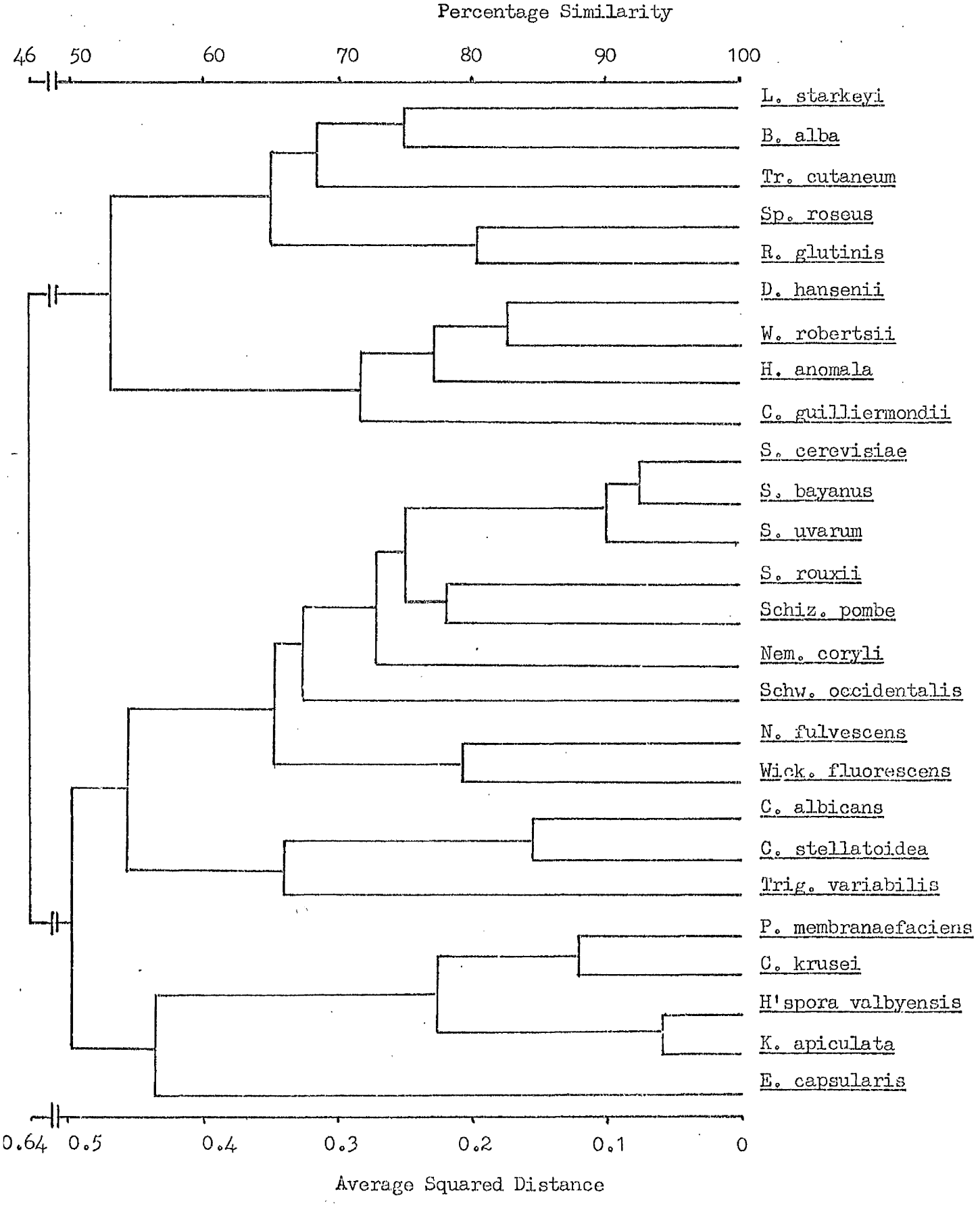

FIGURE 13 :

Average linkage dendrogram based on published properties

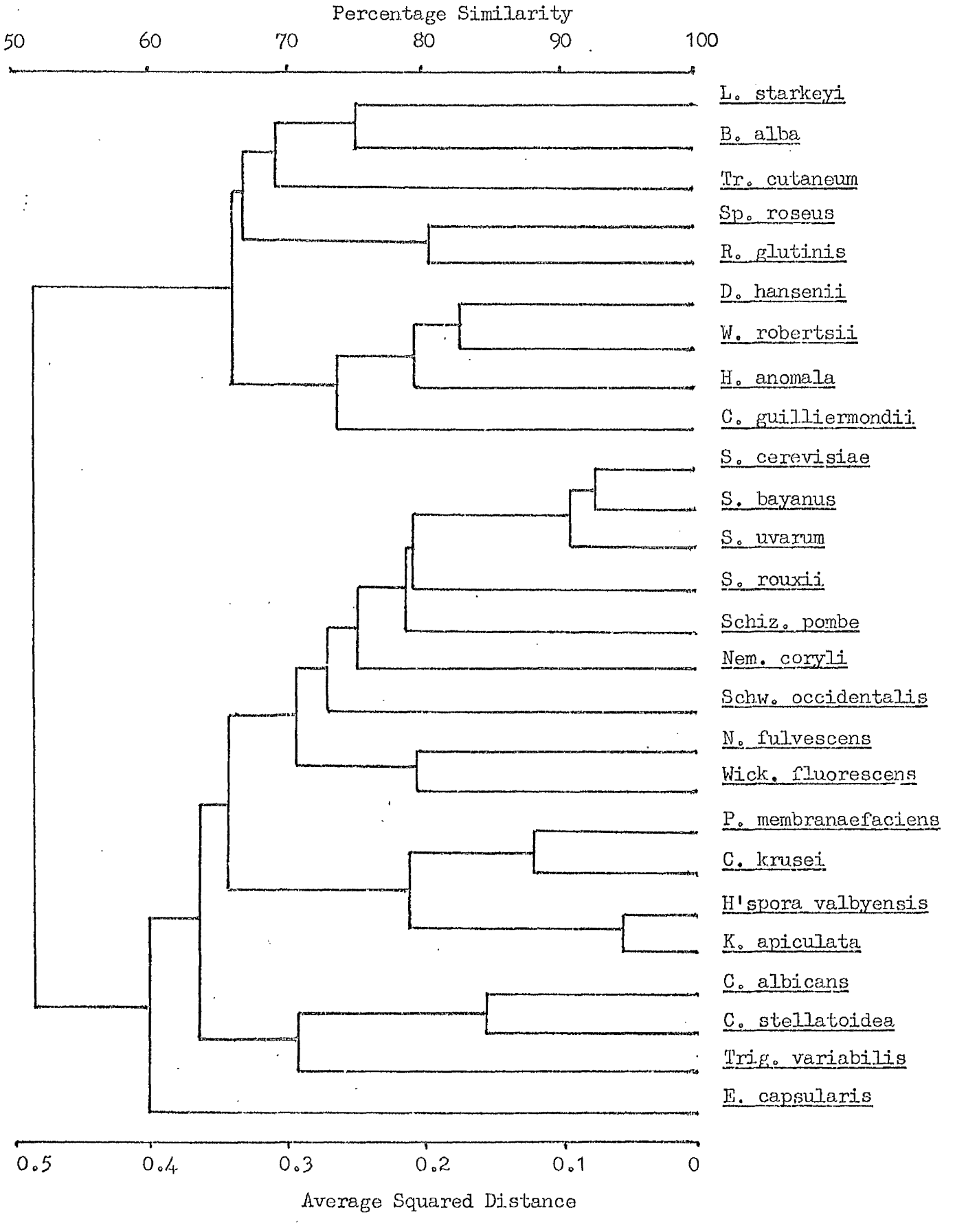

FIGURE 14 :

Dendrogram based on published properties
(Ward's method)