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Aspects of lipid metabolism in Oleaginous Yeasts

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by

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by

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on

Aspects of lipid metabolism in Oleaginous Yeasts

1. Phosphoketolase Studies

Pentulose 5-phosphate phosphoketolase (Pu5PPPK) was shown to be induced in nine yeasts grown on xylose. The enzyme was active with both xylulose 5-phosphate (Xu5P) and ribulose 5-phosphate (Ru5P) as assay substrate. Partial purification of the enzyme led to a decrease in activity for Ru5P, but did not distinguish between the possibilities that the enzyme may have a dual substrate specificity and that Ru5P 3-epimerase may be closely associated with the phosphoketolase.

Lipid levels and ATP:citrate lyase activities were similar in oleaginous yeasts grown on xylose and glucose. It was concluded that Pu5PPK does not affect the process of lipid accumulation in these yeasts.

2. Lipid turnover studies

Yeasts of the genera *Candida*, *Trichosporon* and *Rhodosporidium* were able to degrade their storage lipid for cell proliferation under carbon starvation conditions. Yeasts of the genus *Lipomyces* were incapable of this.

All yeasts examined accumulated glycogen simultaneously with lipid and degraded it during carbon starvation conditions.

Lipid turnover was rapid and was accompanied by peroxisome formation and repression of the enzyme ATP:citrate lyase. Other key enzymes in lipid metabolism were also affected. Similar changes occurred when the yeasts were grown on an exogenous lipid source.

3. Triacylglycerol biosynthesis

Triacylglycerol biosynthesis activities were demonstrated in whole sphaeroplasts from Candida curvata D and Lipomyces starkeyi CBS 1809. Biosynthesis was shown to proceed via the α-glycerol 3-phosphate pathway.

The sub-cellular location of biosynthetic activity was not identified but electron microscopy studies indicated that the endoplasmic reticulum may be the location.

Fatty acyl-CoA synthetases were shown to be active with linoleic, oleic and palmitic acids but not with stearic acid. Activity of this enzyme was enhanced during carbon starvation conditions.

4. Lipid globule studies

Globules were isolated from three species of oleaginous yeasts and examined by electron microscopy. They had a rough laminar surface and were 1.3-5 μm in diameter. Their major component was triacylglycerol.

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INTRODUCTION

1.1. Background

Recent developments in technology have demonstrated the possibility of cultivating micro-organisms on extremely large scales for the production of single cell protein. This has led to the realization that micro-organisms can compete on equal terms with cheap plant products provided the scale of operation is sufficiently large. Therefore, there seems little reason why a process for the production of microbial oils and fats should not vie with conventional sources in an attempt to produce a better, more cost-effective commodity.

Approximately 80% of the World's oils and fats are derived from plant oil seeds, the remainder coming from animal and marine sources. These have a multitude of uses, principally as human food stuffs in margarine, cooking oil and fats, salad oils, etc. Technological uses include incorporation into detergents, waxes, paints and soaps.

Ratledge (1982) noted that even with current programmes to expand rapeseed cultivation in the UK and elsewhere in Europe, we are still obliged to import vast quantities at high cost, principally from North America and third world countries. Therefore supplies depend on climatic and political factors which could lead to a continuation in the trend of rising prices.

A complete understanding of the metabolic processes leading to microbial lipid accumulation and lipolysis must be gained before any commercial venture can be truly exploited. It is hoped that the work in this thesis will contribute to a better understanding of these processes.

1.2. Historical Note

Lipid accumulation in micro-organisms was first recognised by Nageli and Loew (1878) who observed the occurrence of lipid bodies in yeast cells. Commercial production of microbial lipid was first considered, albeit unsuccessfully, by Lindner (1922) whose work was prompted by the first World War blockade of Germany and consequent fat and oil shortages. This German scenario was later reviewed by Damn (1943) and Schmidt (1947) but no commercial process was established.

Following the Second World War, normal trading resumed and interest in microbially-produced lipid declined, until Woodbine (1959) produced a review outlining the industrial potential of microbial lipids.

Our laboratory has been one of the few to continue interest in the field and after a decade of research the picture is at last becoming clearer.

1.3. Oleaginicity

An oleaginous micro-organism was defined by Ratledge (1982) as one that is capable of producing 25% or more of its biomass as lipid. However now, thanks to the work of Boulton and Ratledge (1981) oleaginicity can be defined in terms of a biochemical test, i.e. the presence of the enzyme ATP:citrate lyase. This enzyme cleaves citrate into acetyl-CoA and oxaloacetate and hence produces the building blocks for fatty acid and lipid biosynthesis. (However it has been reported that a few strains of yeast exist which have ATP:citrate lyase but only low amounts of

lipid, Boulton, 1982.) Oleaginous micro-organisms include algae, fungi, yeasts and some bacteria. The work in this thesis is confined to yeasts and a number of examples are given in Table 1.

The biochemistry of oleaginous yeasts has been largely ignored, although considerable studies have been made of lipid biosynthesis in the non-oleaginous yeast Saccharomyces cerevisiae, and of physiological aspects of lipid metabolism in Candida utilis, (Lynen, 1961; Babij et al, 1919; Brown and Rose, 1969; Christiansen, 1978, 1979).

Lipid in oleaginous yeasts accumulates in the form of globules which have been isolated from Candida 107 (Gill, 1973) and Lipomyces starkeyi (Uzuko, 1975). The latter demonstrated the presence of a phospholipid monolayer around the globule. The major constituent of such globules is triacylglycerol, which accounts for 90% or more of the total cellular lipid. The remainder of the lipid is made up of sterol, sterol ester and phospholipids including phosphatidyl-choline, phosphotidylethanolamine and phosphotidylinositol. The fatty acid composition of the lipid is essentially the same as plant oils, such as palm oil and groundnut oil, however it may vary with growth conditions (Thorpe and Ratledge, 1972). The major fatty acid component is oleic acid (18:1) with palmitic acid (16:0) and linoleic acids (18:2) following in order of abundancy.

1:4. The process of lipid accumulation

The amount of lipid which an oleaginous micro-organism can accumulate is determined by the cultural conditions.

TABLE 1. Oleaginous yeasts (Ratledge, 1982)

Organism	Lipid content (% w/w)	Reference
Candida curvata D*	58	Moon and Hammond, 1978
Candida lipolytica	36	Zvyagintseva and Pitryuk, 1976
Candida sp. 107	42	Thorpe and Ratledge, 1972
Cryptococcus terricolus	55-65	Pederson, 1962a, 1962b)
Endomycopsis vernalis*	65	Wither et al, 1974
Hansenula saturnus	28	Fahmy et al, 1962
Lipomyces lipofer	37	McElroy and Stewart, 1967
Lipomyces starkeyi	37	Roy et al, 1978
Rhodosporidium toruloides	56	Fuji Oil Co, 1977
Rhodotorula gracilis	57	Enebo et al, 1946
Rhodotorula graminis	36	Fuji Oil Co, 1978
Rhodotorula glutinis	59	Pidoplichko and Zalashko, 1977
Trichosporus cutaneum	45	Moon and Hammond, 1978

*Now reclassified as Trichosporon cutaneum

The intrinsic requirement is an excess of carbon over some other limiting nutrient. Therefore, in batch culture there will be a period of cell proliferation followed by an increase in lipid levels initiated by the exhaustion of the limiting nutrient. The most widely used carbon source for growth of oleaginous micro-organisms is glucose with nitrogen as the limiting nutrient. Lipid accumulation will however also occur with other limiting nutrients such as phosphate (Nielson and Nilsson, 1953), sulphate (Mass-Forster, 1955), iron (Nielson and Rojowki, 1950) and magnesium (Gill et al, 1977).

During lipid accumulation the actual rate of lipid synthesis does not increase above rates observed during cell proliferation, rather the lipid accumulates due to the fact that synthesis of other nitrogenous cellular compounds ceases. A notable exception was reported by Pederson (1962a,b) who observed lipid accumulation in Cryptococcus terricolus even with low carbon to nitrogen (C:N) ratios.

Early studies of lipid accumulation were accomplished either in batch culture or in two-stage continuous culture. It was generally believed that lipid concentrations equal to those attained in batch culture could not occur in a single stage chemostat. However, the work of Gill et al (1977), Hall and Ratledge (1977), Ratledge and Hall (1979) demonstrated that providing dilution rates of 0.03 to 0.15 h^{-1} were used and the medium had a high C:N ratio, oleaginous yeasts could attain lipid concentrations equal to those seen in batch culture. Therefore lipid accumulation occurred because cell proliferation was restricted by nitrogen-limitation

and the residence time of any yeast in the chemostat was such that excess carbon in the media could be converted to lipid.

The chemostat offers a highly controlled environment for the study of lipid metabolism and hence is an invaluable tool in the elucidation of biochemical pathways and controls involved in lipogenesis. Figure 1 illustrates the process of lipid accumulation in batch and continuous culture.

2.0. Biochemistry of lipid accumulation

Although lipid accumulation in micro-organisms has been recognised for many years, studies of the biochemical aspects of the process have been largely confined to the non-oleaginous yeasts, S. cerevisiae and C. utilis. These yeasts contain approximately 8% (w/w) lipid when grown on medium with a high C:N ratio.

Fatty acid biosynthesis has been established as a cytosolic process (VanBaalan and Givin, 1953; Popjak and Tietz, 1955) employing acetyl-CoA as the basic building block (Wakil, 1961). Therefore the study of the mechanism of lipid accumulation can be divided into two basic areas:

(a) Intermediate metabolism leading to the production of cytosolic acetyl-CoA;

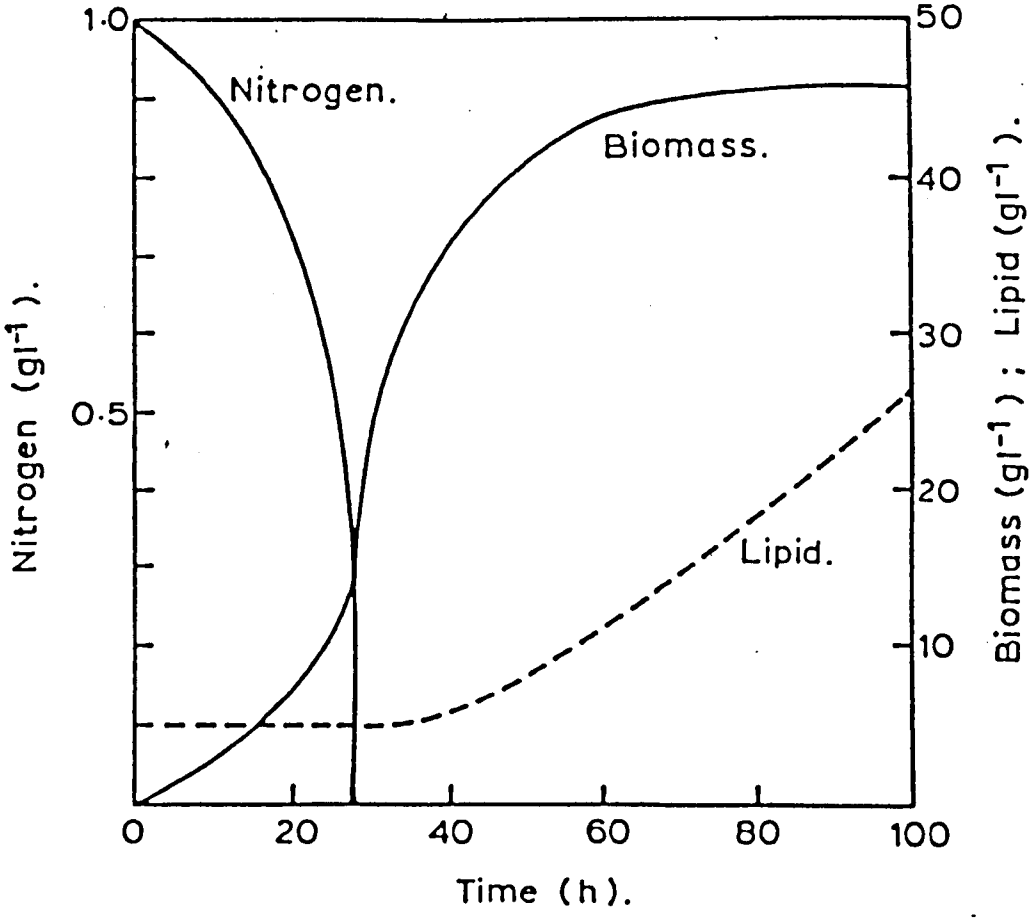
(b) fatty acid biosynthesis and triacylglycerol production.

A working hypothesis of lipid accumulation in oleaginous yeasts is outlined in Scheme 1.

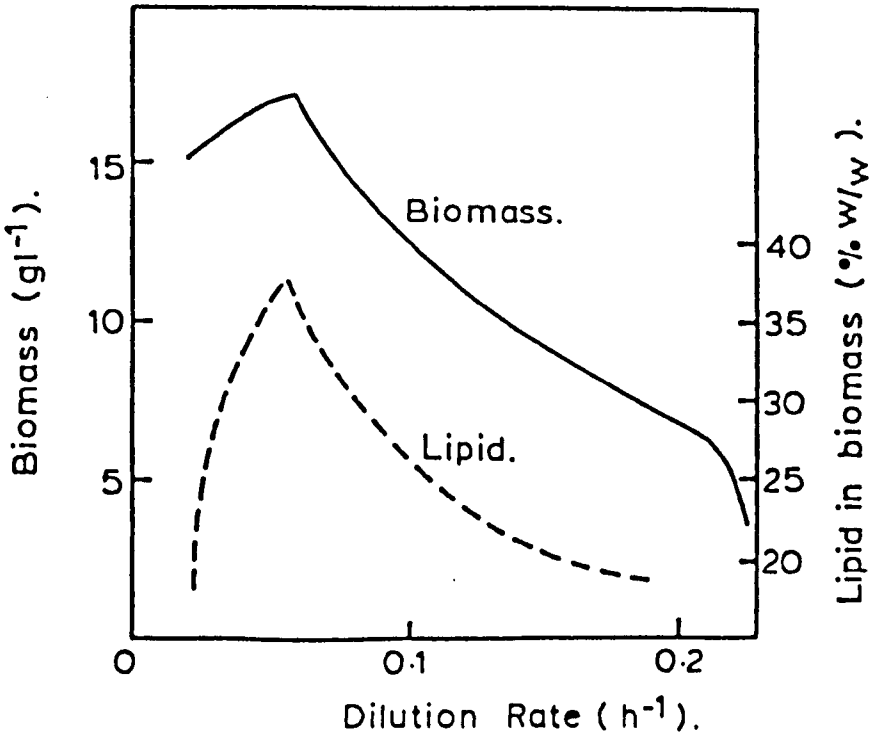
Figure 1. Patterns of lipid accumulation in oleaginous yeasts.

(from Evans, 1983).

A: Batch culture.



B: Continuous culture.



Key to Scheme 1:

1. Glycolysis
2. Pentose-phosphate cycle.
3. Phosphoketolase.
4. Pyruvate dehydrogenase.
5. Pyruvate carboxylase.
6. Citrate synthase.
7. Aconitase.
8. NAD^+:isocitrate dehydrogenase.
9. Acetyl-CoA hydrolase.
10. Acetyl-carnitine transferase.
11. Acetyl-CoA synthetase.
12. ATP:citrate lyase.
13. Malate dehydrogenase.
14. Malic enzyme.
15. Acetyl-CoA carboxylase.
16. Fatty acid synthetase complex.

G-6-P = Glucose 6-phosphate

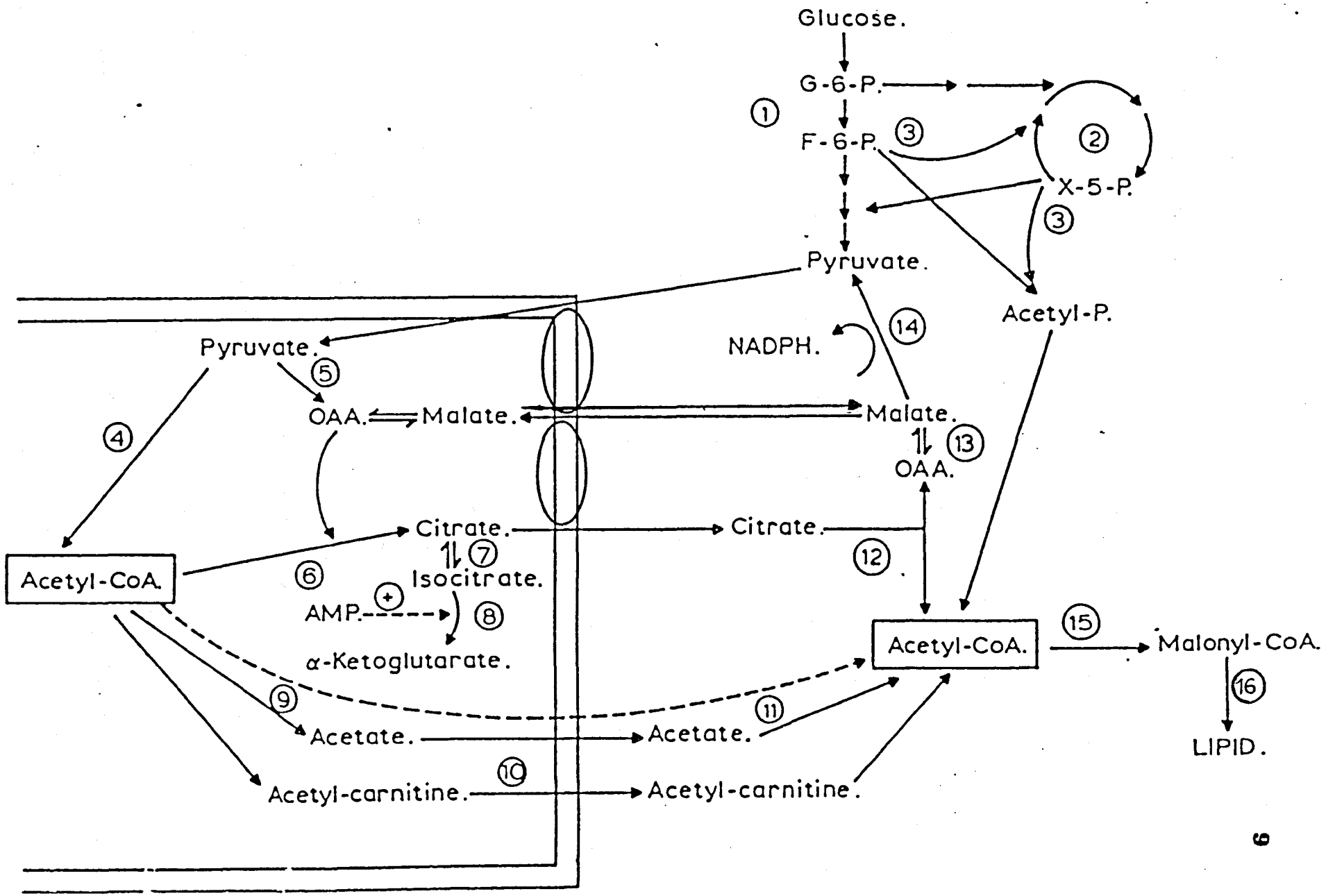
F-6-P = Fructose 6-phosphate

X-5-P = Xylulose 5-phosphate

OAA = Oxaloacetate

N.B. Enzyme 3 only present in xylose-grown cells.

Scheme 1 : Possible mechanisms involved in the supply of cytosolic acetyl-CoA for lipid biosynthesis.



2.1. Intermediate metabolism

Initial investigation into the mechanism of lipid accumulation involved determining the source of cytosolic acetyl-CoA. Primary studies into carbohydrate metabolism failed to detect phosphofructokinase in a number of oleaginous yeasts. Therefore, it was initially assumed that glucose catabolism occurred principally via the pentose phosphate pathway (Brady and Chambers, 1967; Hofer, 1963; Whitworth and Ratledge, 1975a,b). Although this pathway could provide the reducing equivalents for fatty acid biosynthesis, it could not account for the high molar growth and lipid yields in these yeasts (Gill *et al*, 1977) as only 1 mole acetyl-CoA arises from 1 mole glucose. It was suggested, therefore, that this might be supplemented with C_2 units provided by the action of phosphoketolases (Hofer *et al*, 1969, 1971; Hofer, Betz, Becker, 1970), viz:

Xylulose 5-phosphate $\longrightarrow$ glyceraldehyde 3-phosphate + C_2 unit

or fructose 6-phosphate $\longrightarrow$ erythrose 4-phosphate + C_2 unit

These enzymes were detected at low activities in some oleaginous yeasts, by Whitworth and Ratledge (1977). However, in later studies, Boulton (1982), revealed that they had limited distribution and would not provide an explanation for lipid accumulation.

Subsequent studies demonstrated that oleaginous yeasts do possess a labile phosphofructokinase (Mazon *et al*, 1974; Ratledge and Botham, 1977) and hence an operative Embden-Meyerhof pathway.

Uptake of radioactive glucose into Candida 107 was studied by Ratledge and Botham (1977). They concluded that approximately 63% was metabolized via the pentose phosphate cycle, the remainder via glycolysis. This was a surprisingly low flux through glycolysis and hence would still require the action of phosphoketolases to account for the high observed lipid yields; it should therefore be treated with some caution. The pathways of glucose dissimilation in Candida 107 are summarised in Scheme 2.

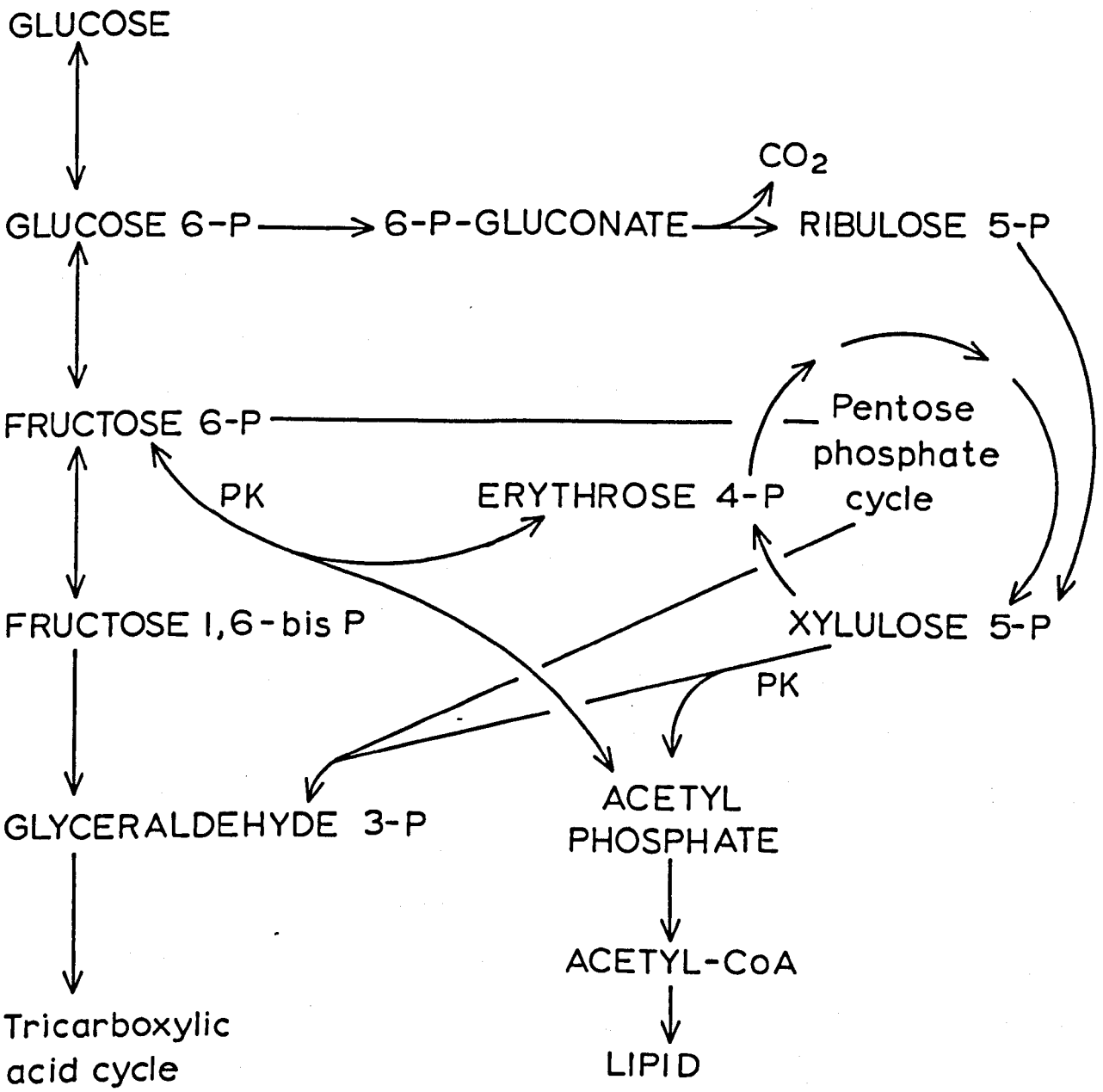
2.2. Origin of cytosolic acetyl-CoA

The net result of the operation of glycolysis and the pentose phosphate pathway is the formation of pyruvate. Metabolism of this intermediate to acetyl-CoA occurs within the mitochondrion by the action of pyruvate dehydrogenase, an enzyme which cannot be detected in the cytosol (Boulton, 1982). The mitochondrial membrane is impermeable to acetyl-CoA, therefore its mode of exit into the cytosol must be discussed. Four mechanisms have been proposed and they are outlined in Scheme 1. These mechanisms are as follows:

(a) Direct transfer of acetyl-CoA. This however has been considered too slow to be a likely mechanism (Purvis and Lowenstein, 1961; Kohlaw and Tan-Wilson, 1977; Spencer and Lowenstein, 1962).

(b) Transfer as acetate. This may be possible as yeasts possess both acetyl-CoA hydrolase and acetyl-CoA synthetase (Klein and Jahnke, 1968; Kohlaw and Tan-Wilson, 1977).

Scheme 2. Pathways of glucose metabolism in Candida 107 (Ratledge and Botham, 1977).



KEY

PK = phosphoketolase

(c) Transfer as acetyl-carnitine. This mechanism has been proposed (Bremer, 1962; Fritz, 1963). However it is now mainly thought that the carnitine mediated system is involved in the transfer of acyl groups into mitochondria, the site of β-oxidation, and not in the genesis of cytosolic acetyl-CoA. Recent work by Ratledge and Gilbert (1985) proposed that this route could be of major significance in some yeasts and observed activities in several yeasts which were accumulating lipid. They also however examined yeasts utilising triacylglycerol as sole carbon source and found considerable induction of the enzyme.

(d) Transfer as carbon atoms 1 and 2 of citrate. This is the most likely method of transport. These carbon atoms are themselves formed from acetyl-CoA and the other four arise from oxaloacetate. Citrate can then be transported across the mitochondrial membrane and acetyl-CoA can be regenerated in the cytosol by the action of ATP:citrate lyase. This mechanism accounts for over 80% of the flux in mammalian systems (Daikuhara et al, 1968; Watson and Lowenstein, 1970). However in non-oleaginous eukaryotic micro-organisms, e.g. Saccharomyces cerevisiae, ATP:citrate lyase has never been detected (Srere, 1972; Botham and Ratledge, 1979; Boulton and Ratledge, 1981). Hence the operation of such a mechanism in these yeasts seems unlikely. In cases where ATP:citrate lyase is detected, there is a greater than usual ability to synthesize lipid (Boulton and Ratledge, 1981), and method (d) is probably the mode in which acetyl-CoA enters the cytosol in these yeasts.

Although the possession of ATP:citrate lyase is essential for the operation of such a mechanism, in oleaginous yeasts its mere presence is insufficient to cause lipid accumulation. There must also be a continuous flux of citrate to the enzyme to enable an adequate supply of acetyl-CoA for fatty acid biosynthesis. Therefore the concerted action of other metabolic activities is necessary to enable citrate to accumulate and prevent its oxidation via the TCA cycle. Reducing equivalents in the form of NADPH must also be supplied for fatty acid biosynthesis.

2.3. Control of Lipid accumulation

The rate of glucose uptake into yeast cells does not limit their growth rate, and negligible lipid turnover has been reported in C. utilis and Candida 107 (Botham and Ratledge, 1979).

Therefore it was concluded that the key to oleaginicity lay mainly in the depletion of AMP in nitrogen limited cells. Botham and Ratledge (1979) demonstrated that in Candida 107 and Rhodotorula glutinis the mitochondrial NAD^+:isocitrate dehydrogenase has a specific requirement for AMP. Therefore as the AMP concentration falls, the enzyme is inactivated leading to build up of isocitrate which will equilibrate with citrate via aconitase and enable the citrate to accumulate.

NAD^+:isocitrate dehydrogenase (ICDH) has long been recognised as a regulatory enzyme in micro-organisms (Hathaway and Atkinson, 1963; Atkinson, 1970; Barnes et al, 1972; Mitsushima et al, 1978). It has been shown to be

sensitive to the prevailing energy charge (Atkinson, 1969) expressed as:

$$\text{Energy charge} = \frac{\text{ATP} + \frac{1}{2}\text{ADP}}{\text{AMP} + \text{ADP} + \text{ATP}}$$

The value of this will vary with prevailing growth conditions.

The enzyme from the non-oleaginous yeast C. utilis has been shown not to be dependent, or activated, by AMP or ADP and so under conditions of nitrogen-limitation, would continue to operate and thus citrate accumulation would not occur.

The findings of Botham and Ratledge (1979) have been reiterated in the work of Boulton (1982). He demonstrated that the activity of NAD^+:ICDH is dependent on the value of the energy charge and the concentration of isocitrate and that increase in lipid levels is preceded by an increase in the energy charge.

Boulton and Ratledge (1980) also demonstrated the improbability of regulation of lipid accumulation at the level of citrate synthase by ATP and ADP, they considered this enzyme to have little regulatory significance in oleaginous yeasts.

2.4. Mitochondrial citrate transport

Transport of citrate out of the mitochondria is thought to be mediated by a specific tricarboxylate carrier in mammalian tissue, this being situated in the inner mitochondrial membrane (Klingenberg, 1970). It catalyses the stoichiometric exchange of tricarboxylate anions and L-malate.

The exchanges are electroneutral, therefore exchange of citrate^{3-} with L-malate^{2-} involves a proton transport in symport with the citrate to maintain electroneutrality (McGiven and Klingenberg, 1971). In mammalian systems, it has been proposed that during lipogenesis citrate effluxes in exchange for malate; the former is then cleaved to acetyl-CoA and oxaloacetate in the cytosol. The oxaloacetate is reduced in the cytosol by malate dehydrogenase, and further exchanges occur (Wit-Peeters et al, 1970).

Until the work of Evans (1983) no studies of citrate transport in oleaginous yeasts has been undertaken. He demonstrated the presence of a carrier system for di- and tricarboxylic acids in oleaginous yeasts which was absent in non-oleaginous yeasts. Citrate efflux was shown to be stimulated by ATP, diminished by AMP and limited by the amount of intra-mitochondrial citrate provided by the action of NAD^+:ICDH.

2.5. ATP:Citrate lyase

This enzyme occupies a key role in lipogenesis in mammalian systems. Originally it was believed that its activity in mammalian systems varied with starving and refeeding (Kornacker and Lowenstein, 1963, 1964, 1965a). Later studies showed that such variations were due to the actual enzyme amounts and not reaction velocity (Yen and Mack, 1980).

Studies in this laboratory over the last six years have greatly advanced the knowledge concerning the microbial enzyme. It has been detected in numerous yeasts (Botham and

Ratledge, 1979; Boulton and Ratledge, 1981a) but its presence gives no indication of prevailing lipid content. However, Boulton and Ratledge (1981) have demonstrated that the ATP: citrate lyase activity does vary with specific rate of lipid synthesis and not with amount of lipid.

Thus, the action of ATP:citrate lyase yields the majority of the cytosolic acetyl-CoA pool. In oleaginous yeasts the metabolism of acetyl-CoA into fatty acids must now compete with other acetyl-CoA requiring reactions e.g. synthesis of sterols, secondary metabolites and pigments.

2.6. Fatty acid synthesis

The priming reaction of this synthesis is catalysed by acetyl-CoA carboxylase; it is biotin dependent and occurs in two stages which can be summarised as follows:

$$\text{Acetyl-CoA} + HCO_3^- + \text{ATP} \longrightarrow \text{Malonyl-CoA} + \text{ADP} + \text{Pi}$$

The enzyme is cytosolic and is thought to be the rate limiting step in fatty acid biosynthesis (Volpe and Vagelos, 1976), this is probably also true for micro-organisms not possessing ATP:citrate lyase. The enzyme is activated by citrate in the oleaginous yeast Candida 107 but not in the non-oleaginous yeast C. utilis (Gill and Ratledge, 1973; Botham, 1978). It is also inhibited by fatty acyl-CoA esters (FACEs) but only at exceptionally high concentrations (up to 200 μM). The latter inhibition may be due to the detergent qualities of FACEs. The importance of this enzyme in control of lipogenesis is still uncertain.

The remainder of fatty acid synthesis involves the action of the fatty acid synthetase multi-enzyme complex, the reactions of which can be summarised as:

$$\text{Acetyl-CoA} + 7\text{Malonyl-CoA} + 14\text{NADPH} + 14\text{H}^+ \longrightarrow \text{Palmitoyl-CoA} + 7\text{CO}_2 + 14\,\text{NADP}^+ + 7\text{CoASH} + 6\text{H}_2\text{O}$$

The terminal acylation reaction of this enzyme in plants and yeasts differs from that of mammalian systems in that there is a terminal acylation to produce FACEs instead of free fatty acids (Bloch and Vance, 1977). This enzyme has not been studied in oleaginous yeasts other than by Gill and Ratledge (1973) who found it to be repressible with no other indication of significant regulatory role.

2.7. Malic Enzyme

This catalyses the oxidative decarboxylation of L-malate to pyruvate and CO_2 and in doing so produces NADPH which is the reducing power for fatty acid biosynthesis. It is a cytosolic enzyme and it has been detected in many oleaginous yeasts (Whitworth and Ratledge, 1975; Boulton, 1982; Evans 1983). It occupies a similar role in mammalian tissues and evidence for this is provided by the fact that dietary and hormonal manipulation of rates of lipogenesis can be positively correlated with the activity of this enzyme (Frenkel, 1975). It is inhibited by α-ketoglutarate in oleaginous yeasts (Evans, 1983).

3. Xylose metabolism in yeasts

There has been much recent interest in the conversion of the pentose sugar, xylose, to ethanol by various yeasts by

virtue of xylose being the principal sugar of hemicellulose. This topic has been reviewed by Gong (1983).

The metabolism of xylose in micro-organisms is believed to involve either the conversion of xylose to xylitol (by an aldoreductase) or to D-xylulose by xylose isomerase (Hofer et al, 1971; Gong et al, 1983). These are both eventually metabolised to xylulose 5-phosphate which is the key intermediate in xylose catabolism in all micro-organisms (Gong et al, 1983). This xylulose 5-phosphate is then channelled into the pentose phosphate pathway, which eventually enters glycolysis to yield pyruvate. Evidence for the metabolism of xylose being solely via the pentose phosphate pathway rests on doubtful assumptions. Evans and Ratledge (1984) have recently shown that such a metabolic route cannot account for the efficiency by which xylose is used by most micro-organisms and have reported the occurrence of an inducible phosphoketolase in various yeasts when grown on xylose.

Pentulose 5-phosphate phosphoketolase (PK) carries out the following reaction:

Xylulose 5-phosphate ⟶ glyceraldehyde 3-phosphate + acetyl-CoA.

There is no loss of CO_2 as would occur if metabolism proceeded via the pentose phosphate pathway.

The presence of PK activity in oleaginous yeasts would result in the production of cytosolic acetyl-CoA directly from xylose. Therefore its presence may cast some doubt on the necessity for the ATP:citrate lyase pathway.

4. Biosynthesis of acylglycerols

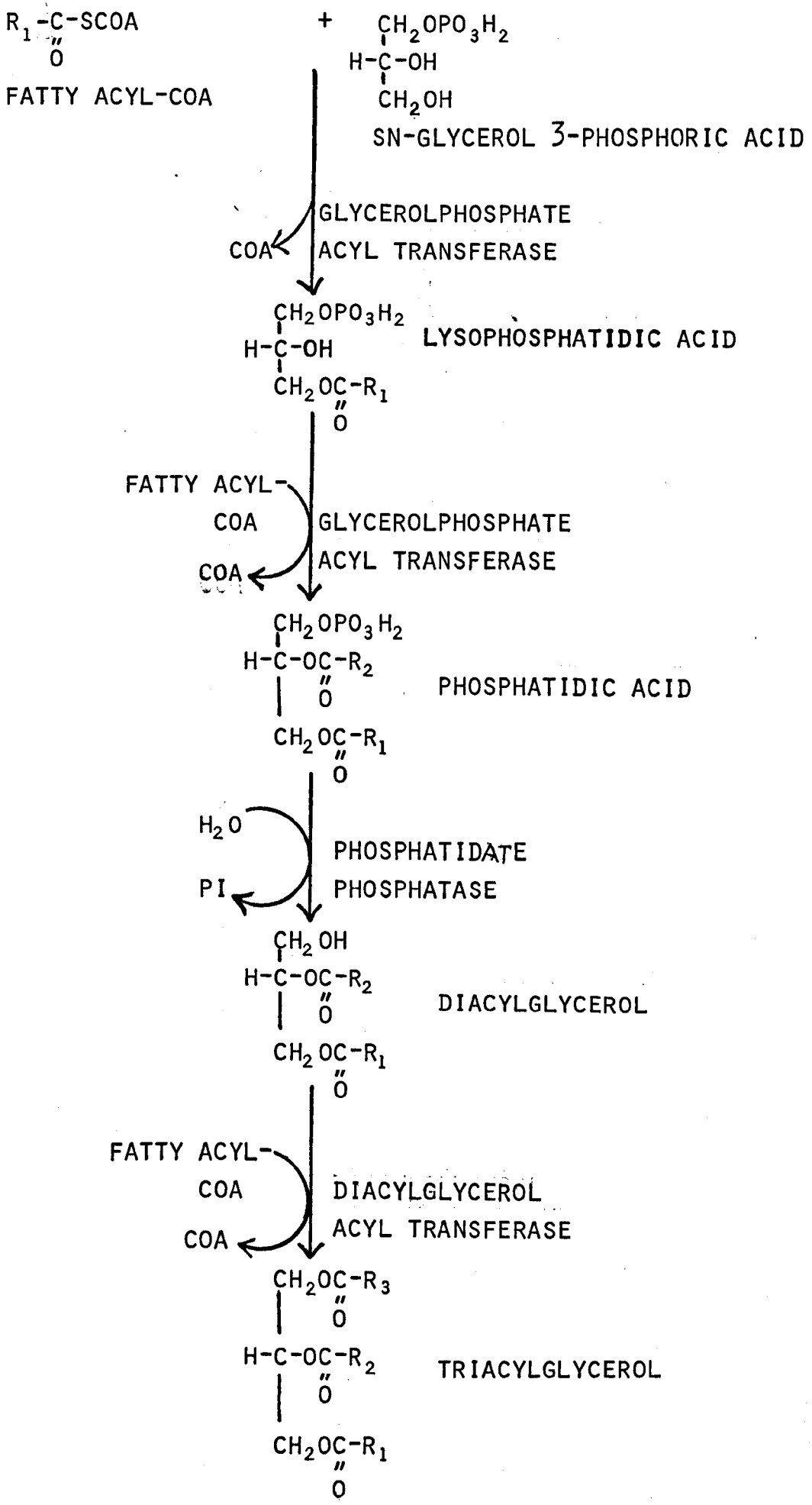
Triacylglycerols are the principal form in which lipid is accumulated in yeasts and so far their biosynthesis has only been studied in the non-oleaginous yeasts S. cerevisiae and Candida tropicalis. The most recent publications on this subject are those of Christiansen (1978) Schlossman and Bell (1978) and Belov and Davidova (1982). The pathways are thought to be similar to those in mammalian systems where the steps involved are fairly well established (Hill and Lands, 1970; Hubscher, 1970; Thompson, 1970; Marinetti, 1970; Van den Bosch, Van Golde and Van Deenen, 1972).

There are two routes for the synthesis of acylglycerols; in one pathway triacylglycerols are synthesized completely from their simplest components and in the other, partial glycerides are re-acylated. These have been termed the α-glycerol phosphate and the mono-acylglycerol pathway, respectively, and are illustrated in Schemes 3 and 4.

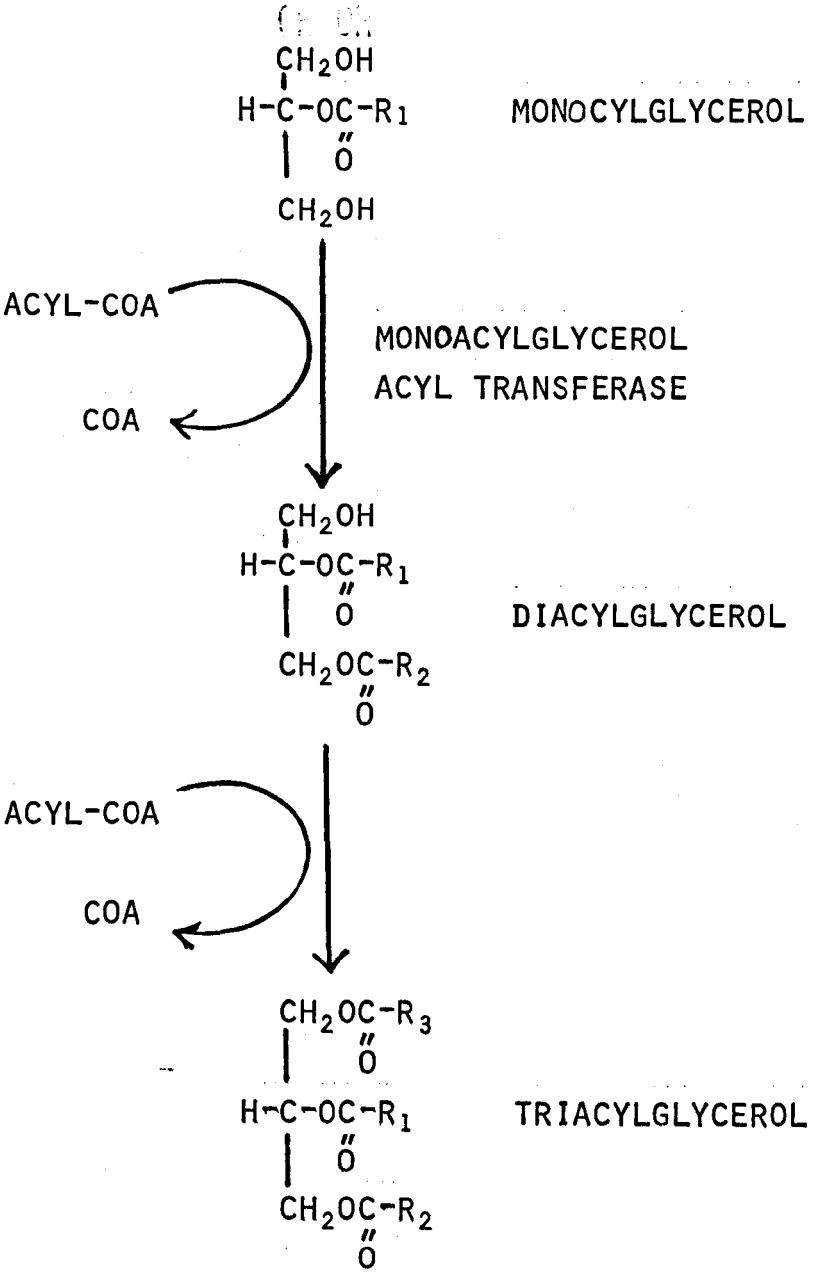
(a) α-Glycerol phosphate pathway

This pathway initially involves the stepwise acylation of α-glycerol phosphate to form L-α-phosphatidic acid. This was first demonstrated in a liver system by Kornberg and Pricer (1953a,b). Studies by Weiss and Kennedy (1956a,b) demonstrated the net synthesis of triacylglycerol in chicken liver preparations by acylation of α,β-diacylglycerol. This suggested a close connection between enzyme synthesis of phospholipids and neutral fats. The enzyme phosphatidic acid phosphatase was later discovered in liver (Smith, Weiss and Kennedy, 1957), and is now known to be the rate limiting step

SCHEME: 3 BIOSYNTHESIS OF LIPIDS VIA THE α-GLYCEROL PHOSPHATE PATHWAY



SCHEME: 4 BIOSYNTHESIS OF LIPIDS VIA THE MONO-ACYLGLYCEROL PATHWAY



in triacylglycerol biosynthesis (Fallon, et al, 1977; Brindley, 1978).

Phosphatidic acid phosphatase activity has been reported to be located in microsomal, mitochondrial, cytosolic and lysosomal subcellular fractions of mammalian systems (Jelsema and Morré, 1978; Wilgram and Kennedy, 1963; Hosaka et al, 1975). Investigations of this enzyme activity have been complicated by this presence in several subcellular fractions, each of which may contain more than one active phosphatase. The specific activity of the soluble and microsomal fractions varies markedly depending on the assay conditions employed (Hosaka et al, 1975). Although magnesium is reported to stimulate (Lamb and Fallon, 1974; Mitchell et al, 1971) or inhibit (Lamb and Fallon, 1974; Sturton and Brindley, 1977) phosphatidic acid phosphatase in various subcellular fractions, van Heusden and Van den Bosch (1978) noted that magnesium dependence is only demonstrated in microsomes prepared in the presence of EDTA. (The reader is referred to a recent review by Bell and Coleman (1980) which discusses the subject more thoroughly.)

Phosphatidic acid phosphatase thus catalyses the conversion of phosphatidic acid into diacylglycerols, which serve as precursors not only for triacylglycerol synthesis, but also for the nitrogenous phospholipids phosphatidylcholine and phosphatidylethanolamine. Rates of synthesis of these compounds have been shown to respond differently to changes in nutritional state in the rat (Park et al, 1972; Groener and Van Golde, 1977).

Phosphatidic acid may also arise from triacylglycerols by the action of lipases to produce diacylglycerols. Thus:

Diacylglycerol + ATP ⟶ phosphatidic acid + ADP

Phosphatidic acid may then be converted into a lipid nucleotide complex by reacting with cytidine triphosphate (CTP) (Kanfer and Kennedy, 1964). This, in turn, may be converted into a variety of phospholipids.

The distribution of fatty acids in phospholipids and triacylglycerols suggests that the stage involving the conversion of α-glycerol phosphate to phosphatidic acid involves two enzymes. The first would have an absolute specificity for unsaturated acids, while the second would be less specific and would esterify acids at the 1-position. These two enzymes have now been identified in both bacterial and animal cells (Cronan and Vagelos, 1972; Yamashita and Numa, 1972). The first enzyme being glycerophosphate acyltransferase, the second monoacylglycerophosphate acyltransferase.

Primary lipid precursors other than glycerol 3-phosphate have been found. Dihydroxyacetone phosphate (DHAP) has been shown to be a precursor of triacylglycerol biosynthesis in mitochondria and microsomes of guinea pig liver (Hajra, 1968). Acyl-DHAP synthesis has also been confirmed in rat kidney, heart, testis, spleen, brain and adipose tissue (La Belle and Hajra, 1972) as well as in Saccharomyces carlsbergensis (Johnston and Paltauf, 1970). The relative contributions of the dihydroxyacetone phosphate pathway and the glycerol

phosphate pathway in glycerolipid biosynthesis remains a controversial topic.

The biosynthesis of yeast lipids has not been investigated to any significant extent, and the majority of study has been in non-oleaginous yeasts.

The enzymes responsible for phosphatidic acid synthesis from sn-glycerol 3-phosphate and acyl-coenzyme A were first isolated from bakers' yeast by Kuhn and Lynen (1965). This study was extended by Steiner and Lester (1972) who demonstrated the biosynthesis of various phospholipids using cell-free extracts of S. cerevisiae. They also demonstrated that CDP-diglyceride is involved in the synthesis of phospholipids by S. cerevisiae.

Early attempts to derive an in vitro synthesis of triacylglycerols employing a system derived from yeasts (Johnston and Paltauf, 1970) were largely unsuccessful. These methods involved attempting to measure incorporation of [^{14}C]labelled fatty acids into triacylglycerols. It was noted that there was always an ATP-dependent formation of [^{14}C]triacylglycerols regardless of the presence of acyl-acceptor.

Later studies by Schlossman and Bell (1978) using membrane protein from S. cerevisiae examined the glycerol-phosphate and DHAP acyl transferase activities. They concluded that these activities represented dual catalytic functions of a single membrane-bound enzyme. They could not detect an acyl-DHAP oxidoreductase activity in yeast membranes and concluded that the DHAP pathway for glycerolipid synthesis may not operate in yeast.

Recent studies by Christiansen (1978) have demonstrated that lipid particles isolated from the non-oleaginous yeast S. cerevisiae can synthesize triacylglycerol de novo, and can introduce fatty acids by acyltransferases into preformed glycerolipid acceptors in the lipid particle. Therefore lipid can be synthesized and its fatty acid composition modified at its site of storage without need for transport. The products of the glycerol 3-phosphate acylation indicate that in S. cerevisiae triacylglycerol biosynthesis proceeds through the phosphatidic acid pathway.

Lipid granules have also been demonstrated to be the site of lipid synthesis in the non-oleaginous yeast C. tropicalis (Belov and Davidova, 1982). These results were in accordance with those of Christiansen (1978).

No previous investigation into triacylglycerol biosynthesis in oleaginous yeasts via the α-glycerol phosphate pathway has been made.

(b) The mono-acylglycerol pathway

The second general route for triacylglycerol biosynthesis was discovered by Hubscher and co-workers (1970). It involves the stepwise acylation of a 2-monoacylglycerol, and occurs mainly in the intestinal mucosa of mammals. The substrate for the pathway arises mainly from the hydrolysis of dietary triacylglycerol by the action of lipases in the intestinal lumen. Monacylglycerols with short chain length saturated acids, or longer chain length unsaturated fatty acids are the best substrates. The pathway is therefore a mechanism by which existing acylglycerols are modified rather than one by which new fat is laid down.

5. Storage and energy reserve compounds

Micro-organisms are subjected to great changes in the nutrient availability in their natural environments. It is vital therefore that they are able to withstand periods of starvation and be able to recover once normal conditions are resumed.

Factors involved in survival of adverse conditions are complicated and involve consideration of many parameters, e.g. the adenylate energy charge, preservation of membrane potential, maintenance energy requirement, possession of storage compounds and the substrates available for endogenous metabolism.

It is not possible to consider all aspects of starvation survival in this introduction, and I shall confine my discussion to a survey of storage and energy reserve compounds. The reader is referred to a recent review (Dawes, 1985) in which other aspects are considered more completely.

5.1. Classes of storage and energy reserve compounds

Three classes of material are classed as potential storage compounds in micro-organisms:

(a) carbohydrates, e.g. polyglucans, glycogen;

(b) lipids, e.g. β-hydroxybutyrate;

(c) polyphosphates.

The quantity of storage material will vary greatly with environmental conditions, however the internal osmotic pressure will not be greatly affected due to the fact that all are of high molecular weight.

Some micro-organisms accumulate more than one storage compound e.g. the bacterium Rhodospirillum rubrum accumulates both glycogen and poly-β-hydroxybutyrate (Stanier et al, 1959) and Mycobacterium smegmatis accumulates both triacylglycerols and glycogen.

5.1.1. Carbohydrates

Accumulation of carbohydrate reserves by micro-organisms in fairly widespread. Most carbohydrate reserves are polysaccharides, and are accumulated when growth is limited by the availability of nitrogen but there is an excess of carbon source. Glycogen is the most common carbohydrate reserve in both eukaryotic and prokaryotic cells. Glycogen synthesis has been well studied in both classes of organism, e.g. in Escherichia coli (Dietzler et al, 1974) and in yeasts (Rothmen and Cabib, 1966). The principal difference between glycogen biosynthesis in yeasts, moulds and fungi and synthesis in bacterial systems is that, in common with mammals, the former utilise UDP-glucose as the glucosyl donor for glycogen synthesis whereas bacteria utilise ADP-glucose.

Other storage polysaccharides include dextrans which are found in both yeasts and bacteria, and mannans which are found in yeasts, moulds, bacteria and higher plants. Yeasts also contain the disaccharide trehalose (Myrback, 1949).

5.1.2. Lipids

Triacylglycerols are ideal storage compounds in many organisms due to the fact that they are hydrophobic and readily catabolised.

Oleaginous plant seeds contain triacylglycerol in oil bodies which are broken down to provide energy via respiration, and carbon skeletons for the growing embryo during germination. Therefore fat can be converted to carbohydrate via the action of the glyoxylate cycle, the enzymes of which are located in the glyoxysome. High activities of lipases can be detected in such germinating seeds, and these break down triacylglycerol along with β-oxidation to provide the energy for germination.

Triacylglycerol is not a storage compound in most yeasts and it is only the oleaginous yeasts which have already been discussed which are capable of accumulating it to an appreciable extent. Moulds are also capable of triacylglycerol accumulation.

Bacteria do not tend to produce high amounts of triacylglycerol and those species that do (Mycobacteria, Corynebacteria, Nocardia) also produce potentially toxic materials. However, many bacteria do accumulate poly-β-hydroxybutyrate (PHB) instead.

Like triacylglycerol, PHB is an ideal storage compound due to its highly reduced state; it is insoluble and exerts negligible osmotic pressure. It is stored in granules 2-8 nm in diameter surrounded by a limiting membrane. It is a helical molecule that is stabilised by interaction of the carbonyl and methyl groups. PHB is synthesized when there is an abundance of carbon and energy source but some other factor, e.g. N_2 or O_2 is limiting.

PHB is thought to have three major roles in survival of adverse conditions:

(a) It is thought to furnish a source of carbon and energy for the sporulation process (Slepecky and Law, 1961);

(b) It can be utilised as a carbon and energy source during encystment (Stevenson and Socolofsky, 1966);

(c) It can be utilised as a carbon and energy source during starvation.

The pathways of synthesis and degradation of PHB in bacteria are well documented and the reader is referred to Dawes and Senior (1973) and Dawes (1985) for a more thorough account of the role of PHB in bacteria.

5.1.3. Polyphosphates

Many bacteria and yeast possess intracellular granules that stain with basic dyes but can also be seen in living cells under the phase-contrast microscope. Such granules are known as "volutin" granules and they were first isolated from a yeast in the late 19th century (Liebermann, 1888). The identity of the volutin granules with polyphosphate (Wiame, 1947) was a crucial advance linking chemical and cytological investigations.

The status of polyphosphates as an energy reserve compound is not clearly established. It is thought that they may simply be involved in metabolism as a reserve of phosphorus, however it is known that in some micro-organisms they can fulfil the role of ATP. Lipmann (1965) advanced the

hypothesis that their physiological function in the microbe is a legacy of their role as the prime energy carrier in the earliest organisms before ATP was evolved.

Conditions which favour polyphosphate accumulation and degradation have been studied. In general, the polyphosphate content is low during rapid growth, but increases markedly when a nutrient imbalance causes the growth rate to decline.

The biosynthesis of polyphosphate appears to follow more than one route of synthesis depending on the organism and many involve either Mg^{2+}-dependent polyphosphate kinase or 1,3-diphosphoglyceratepolyphosphate phosphotransferase. Degradation may be accomplished by a variety of enzymes which have been comprehensively reviewed (Kulaev, 1979), the main one being polyphosphate kinase.

Although polyphosphate may be a storage compound due to the fact that it is accumulated under the described conditions, and due to the fact that it can be utilized when the exogenous energy supply no longer meets demands, the picture is not clear. The polymer may simply have a role as a phosphorus reserve.

6. Aims of investigation

At the outset of this work, triacylglycerol biosynthesis had only been studied in the non-oleaginous yeasts *S. cerevisiae* and *C. tropicalis* (Christiansen, 1978; Belov and Davidova, 1982). It was hoped to extend this previous work by investigating various oleaginous yeasts in an attempt to obtain sub-cellular preparations capable of synthesizing triacylglycerols from sn-glycerol 3-phosphate and fatty acyl-CoA esters (FACEs).

It was also hoped to identify the location of the triacylglycerol synthesizing enzymes. In the non-oleaginous yeast, S. cerevisiae, the location is reported to be the lipid particles (Christiansen, 1978) and it was hoped to isolate these from oleaginous yeasts and perform some electron microscopy studies on them.

It was intended to study the turnover or utilization of the accumulated lipid in a starved yeast in an attempt to ascertain the effects on lipid metabolism. This work would form an important part in understanding if downstream processing in commercial production of microbial oil would lead to unwanted degradation of the lipid.

It was also hoped to study some of the other aspects of lipid accumulation which had not been covered in the theses of Boulton (1982) and Evans (1983), namely the involvement of phosphoketolases and malic enzyme in lipogenesis.

MATERIALS AND METHODS

1. Organisms media and cultural conditions

1.1. Organisms

Lyophilised cultures were obtained from the Central Bureau voor Schimmelcultures (CBS, Baarn, Netherlands) and from the National Collection of Yeast Cultures (NCYC, Food Research Institute, Norwich, UK). Cultures were revived in accordance with the vendors' instructions. Candida curvata D (now reclassified as Trichosporon cutaneum D by Barbara Kirsop NCYC, Norwich, but referred to as C. curvata D throughout this thesis) and Trichosporon cutaneum 40 were the kind gift of Professor E. G. Hammond, Iowa State University, USA.

1.2. Media

The basal media for batch and continuous culture experiments contained the following constituents (gl^{-1}): KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2 2H_2O$, 0.1; $FeCl_3 6H_2O$, 0.008; $ZnSO_4 7H_2O$, 0.001; $CuSO_4 5H_2O$, 0.0001; $CO(NO_3)_2 6H_2O$, 0.0001; $MnSO_4 5H_2O$, 0.0001, with, for complete media NH_4Cl at 3 gl^{-1} and glucose at 30 gl^{-1}; for nitrogen-limited media NH_4Cl at 0.5 gl^{-1} and carbon source at 30 gl^{-1}; for carbon starvation media NH_4Cl at 3 gl^{-1} and no carbon source; for carbon-limited media NH_4Cl at 3 gl^{-1} and glucose at 10 gl^{-1}; for triolein media NH_4Cl at 3 gl^{-1} and glycerol trioleate at 1.5% (v/v); for oleate media NH_4Cl at 3 gl^{-1} and oleate at 1% (v/v); for ethanol media NH_4Cl at 3 gl^{-1} and ethanol at 1% (v/v). The pH was adjusted to pH 5.5 prior to the addition of carbon source and micronutrients.

1.3. Sterilization of media

Batch culture medium was sterilized in situ by autoclaving at 121°C for 15 minutes.

Medium for continuous culture experiments was sterilized by filtration at 276 kPa through a 142 mm membrane, pore size 0.2 μm (Sartorius-Membrane filter, GmbH, Gottingen, W Germany) fitted with a 127 mm cellulose pre-filter. Medium was filtered into 20 l aspirators. Filters were sterilized in stainless steel holders by autoclaving at 121°C for 15 minutes.

1.4.1. Batch Culture conditions

Batch cultures of yeasts were cultivated in 1 l vortex aerated vessels at 30°C (Marshall et al, 1973).

1.4.2. Continuous Culture conditions

Single stage continuous culture experiments were performed in a 1.5 l chemostat (New Brunswick Scientific Co. Inc., New Jersey, USA), with an operating volume of 1.25 l. Yeasts were grown at 30°C and the culture was maintained at pH 5.5 by the automatic addition of KOH. Foaming was controlled by the timed addition of antifoam (polyglycol P2000, Bevaloid Ltd, Beverley, UK).

1.4.2.1. Sampling procedure

Samples were removed from the chemostat by the standard procedure of allowing the positive pressure inside the vessel to forcibly eject the required volume of culture.

1.5. Monitoring of growth

For routine measurements, growth was monitored by measuring the A_{540} of suitably diluted samples of cell suspensions.

The biomass was then calculated from a calibration curve of A_{540} against cell dry weight. For more accurate measurements, yeast biomass was determined directly. Washed yeast samples were dried in tared vials at 121°C over P_2O_5 *in vacuo* until constant weight.

1.6. Analyses

1.6.1. Determination of glucose

The concentration of glucose in medium samples was determined using a commercially available kit (GOD-Perid, Boehringer-Mannheim GmbH, Mannheim, W Germany).

Suitably diluted medium samples in 0.1 ml were added to 2.5 ml GOD-Perid reagent and, after incubating at room temperature for 25 minutes, the resultant A_{620} nm was read against a reagent blank. The glucose concentration was calculated from the A_{620} of a standard glucose solution (91 μg ml^{-1}) taken through the above procedure. The kit contains glucose oxidase, peroxidase and the chromatogen, 2,2′-azido-di-[3-ethyl-benzythliazoline sulphonate] (ABTS) and the principle is indicated below:

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconate} + H_2O_2$$

$$H_2O_2 + \text{ABTS} \xrightarrow{\text{peroxidase}} \text{coloured complex} + H_2O$$

1.6.2. Determination of NH_4^+

The concentration of NH_4^+ in the medium samples was determined by the method of Chaney and Marbach (1962). A 1 ml sample, containing 5-20 μg NH_4^+, was mixed with 5 ml reagent A (10 g phenol and 50 mg sodium nitroprusside made up to 1 l with distilled H_2O) and 5 ml reagent B (5 g sodium

hydroxide and 0.42 g sodium hypochlorite made up to 1 l with distilled H_2O). After incubating at room temperature for 30 minutes the A_{625} was read against a reagent blank. A calibration curve, constructed using NH_4Cl, was linear at concentrations up to 20 μg ml^{-1} when taken through the same procedure.

1.6.3. Determination of total lipid content of yeast

Total lipid content was determined according to Folch et al (1957). Lipid was extracted from approximately 500 mg of accurately weighed lyophilized yeast by overnight immersion in 150 ml chloroform/methanol (2:1 v/v). Cell debris was removed by filtration through Whatman No. 1 filter paper and the extract washed successively with 30 ml NaCl (1% w/v) and 2 x 30 ml distilled water. The washed extract was dried with anhydrous $MgSO_4$ and after filtration to remove solids, the extract was evaporated to dryness. The lipid residue was dissolved in diethyl ether, transferred to a tared vial, then evaporated to dryness in a stream of nitrogen. Solvent residue was removed by drying in vacuo at 50°C and the lipid weighed.

1.6.4. Determination of protein concentration

The protein concentration of cell-free extracts was determined by the dye-binding method of Bradford (1976). To 0.1 ml sample (containing 10-100 μg protein) was added 5 ml dye reagent (100 mg Brilliant Blue G250 (Sigma London Chemical Co, UK) dissolved in 50 ml 95% v/v ethanol and 100 ml 85% w/v phosphoric acid added and the mixture made up to 1 l with distilled H_2O) and the absorbance of the resultant

colour complex read at 595nm against a reagent blank. A calibration curve was constructed using bovine γ globulin (Sigma London Chemical Co, UK) and this was linear up to 100 μg per 0.1 ml.

1.6.5. Determination of glycogen content of yeast

The glycogen content of cells was determined by the method of Gunja-Smith et al (1977). Yeast cells were weighed (wet weight) in centrifuge tubes (12 ml volume) and suspended in 20% potassium hydroxide (0.05 to 0.4 g ml^{-1}). The tubes were incubated at 100°C for 1 h, and after cooling, the suspensions were adjusted to between pH 6 and 7 with 2.5 M hydrochloric acid. Two volumes of ethanol were added and the resulting precipitates were recovered by centrifugation, washed three times with 67% ethanol, and freed of excess ethanol by inverting the centrifuge tubes over filter paper for approximately 5 minutes. The precipitates were resuspended in water (2 ml) with gentle warming. Portions of the suspensions containing 50 to 100 μg of glycogen were incubated at 37°C for 2 h in digests (1 ml) containing Aspergillus niger glucoamylase (2IU) and salivary α-amylase (1IU), 5 mM calcium chloride and 50 mM sodium acetate buffer, pH 5.0. Glycogen was estimated from the amount of glucose released. The amount of glycogen found in the suspension represented the total glycogen content of the yeast.

Glucose was estimated by the method of Trevelyn and Harrison (1955) using anthrone reagent (2 g of anthrone dissolved in 75 ml water and 950 ml concentrated H_2SO_4). Carbohydrate solution was pipetted into a test tube and water

added to a final volume of 3.8 ml. Anthrone reagent (8.2 ml) was added and the tube shaken and incubated at room temperature for 10 minutes. The tube was then cooled for 5 minutes in running water before reading the resultant A_{620} against a reagent blank. The glucose concentration was calculated from the A_{620} of a glucose standard (0.5 μmole) taken through the above procedure.

2. Methods of yeast disruption used in the preparation of cell-free extracts

2.1. Use of the French pressure cell

Cells were harvested by centrifuging at 5 000xg for 10 minutes at 4°C and washed twice by resuspension in 50 mM KH_2PO_4/Na_2HPO_4 buffer pH 7.5 and centrifuged as before.

Washed yeast was suspended in 30 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5 (0.5 g wet wt per ml) and disrupted by double passage through a pre-cooled French pressure cell at 35 MPa. Whole cells and debris were removed by centrifuging at 48 000xg for 30 minutes at 4°C and the supernatant filtered through Whatman No. 1 filter paper to remove solidified lipid. The filtrate was retained for enzyme assays.

2.2 Use of sphaeroplast disruption

2.2.1. Using Zymolyase 20T

Exponential phase yeast cultures grown on nitrogen limited media were harvested by centrifugation at 5000xg for 10 minutes at 4°C, and washed once with 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5. The cells were then resuspended in Buffer A (50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, containing $MgCl_2$, 1 mM,

EDTA, 1 mM, dithiothreitol, 1 mM) containing 2 M sorbitol. The suspension was then incubated at room temperature for 10 minutes and then centrifuged at 10,000xg for 10 minutes. The pellet thus obtained was resuspended in buffer A containing 2 M sorbitol and 1 mg zymolyase 20T ml^{-1} to a concentration of 20 mg wet weight cell material per ml. The cells were then incubated at 30°C for 1 hour.

Sphaeroplast formation was followed under the phase contrast light microscope, or spectrophotometrically by the decrease in A_{663}.

After complete conversion of whole cells to sphaeroplasts, an equal volume of Buffer A was added. The resultant suspension was allowed to stand on ice for 10 minutes. The zymolyase 20T was then removed by two washes in Buffer A containing 2 M sorbitol.

2.2.2. Using Novozym 234

Exponential phase yeast cultures grown on nitrogen limited media were harvested by centrifugation at 5000xg for 10 minutes at 4°C, and washed twice in citrate-phosphate buffer (0.01 M), pH 5.85, containing KCl, 0.6 M, and dithiothreitol, 5 mM. The cells were then resuspended in MES buffer, 20 mM, pH 6.0 containing sorbitol, 2 M, and 2 mg Novozym 234 per ml, to a concentration of 80 mg wet weight cells per ml. The cells were then incubated at 30°C for 45 minutes. Sphaeroplast formation was followed under the phase contrast light microscope. After complete conversion of whole cells to sphaeroplasts the Novozym was removed by two washes in MES buffer, 20 mM, pH 6.0 containing sorbitol, 2 M.

2.2.3. Lysis of sphaeroplasts

Sphaeroplasts prepared using Zymolyase 20T and Novozym 234 were lysed by diluting the sorbitol concentration of the suspending media from 2 M to 0.25 M. The resultant suspension was centrifuged (2000xg for 5 minutes) to remove whole cells and debris, the supernatant was used in assays.

3. Subcellular fractionation of yeasts cells

3.1. Additional lysis of sphaeroplasts

Sphaeroplasts were lysed as described in Section 2.2.3, additional lysis was brought about by the use of a Potter glass homogeniser. The resultant suspensions were then centrifuged at 2000xg for 5 minutes to remove whole unlysed cells and debris, this fraction being referred to as the pellet.

3.2. Differential centrifugation

The crude supernatant was fractionated further by centrifuging at 20,000xg for 15 minutes to yield a crude mitochondrial pellet and a crude lipid fraction as top layer. The intermediate supernatant was further centrifuged at 48,000xg for 20 minutes to yield an intermediate fraction as pellet and a turbid supernatant which was centrifuged at 85,000xg for 90 minutes to yield a microsomal pellet and a clear supernatant.

3.3. Purification and analysis of lipid globules

3.3.1. Purification of lipid globules

Lipid globules were purified by removing them from the top of the 20,000xg for 15 minutes spin and resuspending them in MES (20 mM) buffer, pH 6.0 containing 1 M sorbitol

and centrifuging at 7000xg for 5 minutes at 4°C. This washing procedure was repeated three or four times. The final top layer consisted of purified oil globules.

3.3.2. Analysis of lipid globules

C. curvata D was grown on nitrogen-limited media in 1 l vortex aerated vessels for 3 days. [1-^{14}C]Sodium acetate (1 ml, 125 μCi; 60 μCi/μmole) was then injected into the growing cultures and after 45 minutes the cells were harvested and lipid globules purified as described in Section 3.3.1.

The oil globule suspension was mixed with an equal volume of chloroform-methanol (2:1 v/v) and lipids were extracted for five hours at room temperature. After two additional extractions, the pooled extracts were washed twice by partition against 0.2 volume of 3 mM $MgCl_2$. The chloroform layer was filtered through Whatman No 1 filter paper to remove debris. The filtrate was evaporated to dryness and taken up in a known quantity of chloroform.

Neutral lipids were analysed by thin layer chromatography (tlc) as described in Section 7.4.3.2. Polar lipids were analysed by a two-dimensional tlc system; the solvents used were firstly chloroform:methanol:water (65:25:4 by vol) and secondly chloroform:methanol:28% ammonia (60:35:5, by vol). Lipid components were located with iodine vapour and their Rf values compared with those of standards. Radioactive samples were counted as detailed in Section 7.4.3.2.

4. Spectrophotometric assays

The Methods in this section were used to assay the various enzymes in cell-free extracts and subcellular fractions.

The cell-free extracts were prepared as described in Section 2.1 unless otherwise stated.

4.1. ATP:citrate lyase

4.1.1. Preparation of extracts

Yeasts were harvested by centrifuging at 2000xg for 10 minutes after 48 hours growth in Stage one on nitrogen-limited media and after 24 hours growth in Stage 2 on carbon starvation media. Yeasts grown on triolein were harvested after 48 hours growth. The yeast was then resuspended in 50 mM Tris/HCl pH 8.0 buffer containing 1 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 20 mM tripotassium citrate and centrifuged as before. French press extracts were then prepared in the above buffer.

4.1.2. Assay for ATP:citrate lyase

(E.C. 4.1.3.8; ATP:citrate oxaloacetate lyase) was assayed at 30°C by the coupled procedure of Srere (1953) in which the oxaloacetate product is reduced to malate by the action of malate dehydrogenase, with the concomitant decrease in A_{340} due to the oxidation of NADH.

The reaction mixture contained in 1 ml: Tris/HCl, pH 8.3, 250 mM; $MgCl_2$, 10 mM; dithiothreitol, 1 mM; ATP, 10 mM; tripotassium citrate, 20 mM; CoA, 0.2 mM; NADH, 0.1 mM; malate dehydrogenase (porcine heart mitochondrial, Sigma London Chemical Co Ltd, UK) 10 units and extract. Reactions were initiated by the addition of CoA.

4.2. Malic Enzyme

4.2.1. Preparation of extracts

Yeasts were harvested as detailed in Section 4.1.1. and French press extracts were prepared in 50 mM Tris/HCl buffer

pH 7.8, containing $MgCl_2$ (5 mM), and dithiothreitol (1 mM).

4.2.2. Assay for Malic Enzyme

[E.C. 1.1.1.40; L-malate: $NADP^+$ oxidoreductase (decarboxylating)] was assayed at 30°C according to Hsu and Lardy (1969) following the reduction of $NADP^+$ at 340 nm.

The reaction mixture contained in 1 ml: triethanolamine buffer, pH 7.4, 67 mM; $NADP^+$, 0.23 mM; $MnCl_2$, 1.6 mM; L-malate (neutralized), 0.5 mM and extract. Reactions were initiated by the addition of L-malate.

4.3. Carnitine acetyltransferase

4.3.1. Preparation of extracts

Yeasts were harvested and washed as detailed in Section 4.1.1. The pellet was resuspended in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5 containing 1.5 mM phenylmethylsuphonyl fluoride and 1.6 M ammonium sulphate, and French pressed. The extract was then stirred at 4°C for 30 minutes prior to centrifuging at 30,000xg for 20 minutes. The extract was then filtered, and dialysed overnight against 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5.

4.3.2. Assay for carnitine acetyltransferase

Carnitine acetyltransferase was assayed at 30°C according to Kohlaw and Wilson (1977) using 5,5'-dithiobis-(2-nitrobenzoate) DTNB as reagent and following the formation of CoA from acetyl-CoA and L-carnitine at 412 nm.

The reaction mixture contained in 1 ml: Tris/HCl buffer pH 7.5, 50 mM; DTNB, 0.4 mM; acetyl-CoA, 0.1 mM; L-carnitine, 1.25 mM; and extract. Reactions were initiated by the addition of L-carnitine.

4.4. $NADP^+$-dependent isocitrate dehydrogenase.

4.4.1. Preparation of extracts

Extracts were prepared as detailed in Section 4.1.1. and dialysed overnight against 50 mM Tris/HCl buffer pH 7.8, containing $MgCl_2$ (5 mM) and dithiothreitol (1 mM) to reduce high endogenous activity.

4.4.2. Assay for $NADP^+$-dependent isocitrate dehydrogenase

[E.C. 1.1.1.42, threo-Ds-isocitrate:$NADP^+$ oxidoreductase] was assayed at 30°C according to the method of Kornberg (1955) by measuring the increase in A_{340} due to the reduction of $NADP^+$.

The reaction mixture contained in 1 ml: KH_2PO_4/Na_2HPO_4 buffer, pH 7.0, 30 mM; $MgCl_2$, 4 mM; $NADP^+$, 0.2 mM; DL-isocitrate, 2 mM; AMP, 0.5 mM; and extract. Reactions were initiated by the addition of DL-isocitrate.

4.5. Isocitrate lyase

4.5.1. Preparation of extracts

Extracts were prepared as described in Section 4.1.1.

4.5.2. Assay for isocitrate lyase

[E.C. 4.1.3.1; threo-DS-isocitrate: glyoxylate-lyase] was assayed at 30°C by the procedure of McFadden (1969) in which the amount of glyoxylate formed from isocitrate was measured using a colorimetric procedure.

The reaction mixture contained in 2 ml: Tris/HCl buffer, pH 7.5, 60 mM; $MgCl_2$, 2 mM; glutathione (reduced, freshly prepared in 50 mM Tris/HCl buffer, pH 7.5), 12.5 mM; DL-isocitrate, 5 mM and extract. Reactions were initiated

by the addition of DL-isocitrate. Mixtures were incubated for 10 minutes at 30°C, then the reaction was stopped by the addition of 1 ml 10% (w/v) trichloroacetic acid.

To 1 ml reaction mixture in a 50 ml flask was added 6 ml of a mixture of five parts oxalic acid (10 mM) and one part phenylhydrazine hydrochloride (1% w/v). The mixture was heated to boiling, then immediately cooled to room temperature. After 5 minutes the mixture was chilled for 2 minutes in an ice bath, then 4 ml concentrated HCl was added, followed by the addition of 1 ml potassium ferricyanide (5%, w/v). The A_{520} of the mixture was read against a distilled H_2O blank after a further 7 minutes.

The yield of glyoxylate (μmoles) per 2 mls of reaction mixture was calculated from the formula:

$$\frac{A_{520} - 0.05}{1.15}$$

4.6. Catalase

4.6.1. Assay for catalase

[E.C. 1.11.1.6, H_2O_2:H_2O oxidoreductase] was assayed at 25°C by measuring the decomposition of peroxide substrate to H_2O and O_2 with the concomitant decrease in A_{240}.

The peroxide substrate solution was prepared by adding 0.1 ml H_2O_2 (30% w/v) to 5 ml KH_2PO_4/Na_2HPO_4, pH 7.0, 50 mM, such that the A_{240} was within the range 0.25-0.52.

The reaction mixture contained in 3 ml: peroxide substrate solution (2.9 ml) and extract. The time taken for A_{240} to decrease by 0.05 of an absorption unit was noted.

This corresponded to the decomposition of 3.45 μmoles of H_2O_2 in a 3 ml solution.

4.7. Citrate synthase

4.7.1. Assay for citrate synthase

[E.C. 4.1.3.7, citrate:oxaloacetate-lyase (CoA acetylating)] was assayed by the procedure of Srere et al (1963). The reaction mixture contained in 1 ml: Tris/HCl buffer, pH 8.0, (100 mM); 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB made up in 20 mM Tris/HCl buffer, pH 8.0), 0.25 mM; oxaloacetate (prepared freshly each day and neutralized prior to use), 200 μM; acetyl-CoA, 100 μM and extract. Assays were performed at 25°C against a blank which contained all components except extract. Reactions were initiated by the addition of oxaloacetate and the appearance of the mercaptide ion formed from DTNB and CoA was followed by measuring the increase in A_{412}.

4.8. NADPH-cytochrome c reductase

[E.C. 1.6.2.3. NADPH-cytochrome c oxidoreductase] was assayed at 30°C according to Sottocasa et al (1967) following the reduction of cytochrome c at 550 nm.

The assay mixture contained in 1 ml: KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, 50 mM; NADPH, 0.1 mM; cytochrome c, 0.1 mM; KCN, 0.3 mM. The reaction was initiated by the addition of extract.

4.9. Pentulose 5-phosphate phosphoketolase

4.9.1. Assay for pentulose 5-phosphate phosphoketolase

[E.C. 4.1.2.9; D-xylulose 5-phosphate D-glyceraldehyde 3-phosphate lyase (phosphate-acetylating)] was assayed by the methods of Goldberg et al (1966).

4.9.1.1. Method A

The formation of glyceraldehyde 3-phosphate was measured by the oxidation of NADH in the presence of glyceraldehyde 3-phosphate isomerase and α-glycero-phosphate dehydrogenase using a two-stage method. In the first stage, aldolase and triose phosphate isomerase were added to convert the C-3 product of the enzyme, glyceraldehyde 3-phosphate, to fructose 1,6-bisphosphate. In the second stage, the fructose 1,6-bisphosphate was determined by adding aldolase, triose-phosphate isomerase and α-glycerophosphate dehydrogenase and measuring the total oxidation of NADH at 340 nm.

The first stage reaction mixture contained in 0.5 ml: KH_2PO_4/Na_2HPO_4 buffer pH 7.0, 30 mM; $MgCl_2$, 2.0 mM; dithiothreitol, 3.0 mM; thiamine pyrophosphate, 0.6 mM; xylulose 5-phosphate, 10 mM; aldolase, 0.5 unit; triose phosphate isomerase, 1.0 unit; α-glycerophosphate dehydrogenase, 0.5 unit and extract. The mixture was incubated at 30°C for 10 minutes and the reaction was stopped by immersion in a boiling water bath for 2 minutes. Precipitated protein was removed by centrifuging at 10,000xg for 3 minutes.

Fructose 1,6-bisphosphate was assayed in the supernatant, in the second stage reaction mixture which contained in 1 ml: triethanolamine buffer, pH 7.5, 50 mM; NADH, 0.1 mM; aldolase, 0.5 unit; triose phosphate isomerase, 0.5 unit; α-glycerophosphate dehydrogenase, 0.5 unit.

4.9.1.2. Method B

The amount of acetyl-phosphate formed from xylulose 5-phosphate was determined by the ferric hydroxamate method

of Goldberg et al (1966). The acetyl-phosphate product was allowed to react with neutral hydroxylamine to give aceto-hydroxamate which in turn reacted with ferric chloride to give a complex with a λ_{max} at 540 nm. The assay was modified according to Botham (1978), firstly, by including hydroxyl-amine in the incubation mixture to prevent further metabolism of acetyl-phosphate to acetate; secondly, by including sodium arsenite in the assay mixture to inhibit pyruvate dehydrogen-ase which could produce interfering acetyl-CoA.

The reaction mixture contained in 1 ml: histidine buffer, pH 7.0, 40 m$\underline{M}$; $MgCl_2$, 0.8 mM; dithiothreitol, 1.4 m$\underline{M}$; xylulose 5-phosphate, 10 m$\underline{M}$; 500 μl 2 $\underline{M}$ hydroxylamine hydro-chloride, pH 5.4 and extract.

Reactions were initiated by the addition of xylulose 5-phosphate and the mixture incubated at 30°C. After 15 minutes, 1.5 ml 20% (w/v) $FeCl_3$ in 0.1 $\underline{M}$ HCl was added and after centrifuging, the A_{540} of the supernatant was compared with a calibration curve produced from acetyl phosphate (0-1 μmol) taken through the above procedure.

4.10. L-Glycerol 3-phosphate dehydrogenase

4.10.1. Assay for L-glycerol 3-phosphate dehydrogenase

[E.C. 1.1.1.8. L-Glycerol 3-phosphate oxidoreductase] was assayed according to Kennedy (1962) at 25°C by measur-ing the increase in A_{340} due to the reduction of NAD^+.

The reaction mixture contained in a final volume of 3 ml: hydrazine-glycine-$MgCl_2$ buffer (prepared by adding 20.8 g hydrazine hydrate to 1.5 g glycine and 0.2 ml of 1 $\underline{M}$ $MgCl_2$, adjusting the pH to 9.8 and making the volume up to 100 ml),

1.8 ml; NAD^+, 0.33 mM; α-glycerophosphate, 1.66 mM. The reaction was initiated by the addition of extract.

4.11. β-Galactosidase

[E.C. 3.2.1.2.3] β-D-Galactoside galactohydrolase was assayed according to the assay procedure described in Biochemica Information (Vol. 2 Boehringer Mannheim GmBH, W. Germany). The assay was carried out at 25°C by measuring the increase in A_{340} due to the reduction of NAD^+.

The reaction mixture contained in a final volume of 1 ml: KH_2PO_4/Na_2HPO_4 buffer, pH 7.0, 81 mM; lactose solution, 32.6 mM; NAD^+, 46 μM; $MgSO_4$, 0.9 mM; β-galactose dehydrogenase, 81 Mu/ml. The reaction was initiated by the addition of extract.

4.12. Urease

[E.C. 3.5.1.5. urea amidohydrolase] was assayed by measuring the amount of ammonia formed by the action of urease on urea. The ammonia was estimated using Nessler reagent according to Umbreit et al (1964) at 490 nm.

Reaction mixtures contained urea solution, 0.5 M in a volume of 2 ml and were initiated by the addition of 50 μl extract. Tubes were incubated at 25°C for 5 minutes, after which time the reaction was stopped by the addition of 1 ml HCl (1 M). A 50 μl aliquot from each tube was mixed with 2 ml Nessler reagent and 3 ml NaOH (2 M). The resultant A_{490} was read against a blank taken through the above procedure using water in place of urea solution. A standard was prepared using 2 ml ammonia solution (1.21 mM) in place of urea solution in the above procedure.

4.13. Phosphorylase a

[E.C. 2.4.1.1. 1,4-α-D Glucan:Orthophosphate α-glucosyltransferase] was assayed according to the assay procedure described in 'Biochemica Information' (Vol. 2. Boehringer Mannheim, GmBH, W. Germany).

Release of glucose-1-P from glycogen by the action of phosphorylase a was determined by a coupled assay procedure:

$$(\text{Glucose})_n + P_i \xrightarrow[\text{Phosphorylase a}]{} (\text{Glucose})_{n-1} + \text{Glucose 1-P}$$

$$\text{Glucose 1-P} \xrightarrow{\text{phosphoglucomutase}} \text{Glucose 6-P}$$

$$\text{Glucose 6-P} + \text{NADP} \xrightarrow{\text{G6P dh}} \text{Gluconate 6-P} + \text{NADPH} + H^+$$

The activity of phosphorylase a was calculated from the increase in A_{340} due to the final reaction step.

The reaction mixture contained in a final volume of 3 ml: KH_2PO_4/Na_2HPO_4 buffer, pH 6.8, 50 mM; EDTA, 0.1 mM; glycogen, 2 mg/ml; NADP, 0.36 mM; glucose-1,6-P_2, 4 μM; $MgCl_2$, 13 mM; phosphoglucomutase, 6.7 U/ml; G6P dehydrogenase, 1 u/ml. The reaction was initiated by the addition of extract.

4.14. Pyruvate kinase

[E.C. 2.7.1.40, ATP:pyruvate phosphotransferase] was assayed according to the coupled assay procedure described in 'Biochemica Information' (Vol. 2 Boehringer Mannheim GmBH, W. Germany). The pyruvate product was reduced to lactate by the action of lactate dehydrogenase with the concomitant decrease in A_{340} due to the oxidation of NADH.

The reaction was carried out at 25°C and the reaction mixture contained the following constituents: triethanolamine buffer, pH 7.6, 87 mM; phosphoenolpyruvate, 0.53 mM; $MgSO_4$, 2.5 mM; KCl, 10 mM; ADP, 4.7 mM; NADH, 0.2 mM; lactate dehydrogenase, 15 units/ml. The reaction was initiated by the addition of extract.

5. Experiments with phosphoketolases

5.1. Growth of yeasts

Yeasts were cultivated on nitrogen-limited medium (1.2) with xylose as sole carbon source (30 gl^{-1}) under batch conditions in 1 l vortex-aerated vessels, at 30°C as described in Section 1.4.1. Yeasts were harvested after 46 hours growth by centrifuging at 5000 xg for 10 min at 4°C, and washed twice by resuspension in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.0.

5.2. Preparation of extracts

Extracts were prepared using the French pressure cell as described in Section 2.1.

5.3. Attempts to devise a continuous assay for xylulose 5-phosphate phosphoketolase

5.3.1. A continuous assay based on the method of Cori et al (1948), which assays glyceraldehyde 3-phosphate dehydrogenase by the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglyceric acid accompanied by the formation of NADH and an increase in A_{340} was attempted.

Two modifications of the original assay were made. Firstly, sodium arsenate was used in place of phosphate as substrate to form NADH and 1-arseno-3-phosphoglyceric acid.

1-Arseno-3-phosphoglyceric acid is unstable at neutral or alkaline pH, and rapidly hydrolyses to 3-phosphoglyceric acid and arsenate. Thus the reaction in the direction of NADH is favoured, since one of the products of the reaction in this direction is removed. Secondly, since one of the products of the reaction is a proton, the assay was performed at alkaline pH to favour NADH formation.

The assay mixture contained in 1 ml: dithiothreitol, 3.0 mM; thiamine pyrophosphate, 0.6 mM; NAD^+, 0.1 mM; sodium arsenate, 8.0 mM; glyceraldehyde 3-phosphate dehydrogenase, 2 units; $MgCl_2$, 2 mM; glycylglycine buffer, pH 8.5, 30 mM; xylulose 5-phosphate, 10 mM, and extract.

5.3.2. Another assay which involves the conversion of glyceraldehyde 3-phosphate, formed from xylulose 5-phosphate by phosphoketolase, to dihydroxyacetone phosphate by triose phosphate isomerase, and then to glycerol 3-phosphate by α-glycerophosphate with the concomitant oxidation of NADH was also attempted.

The reaction mixture contained in 1 ml: KH_2PO_4/Na_2HPO_4 buffer, pH 7.0, 30 mM; $MgCl_2$, 2 mM; dithiothreitol, 3.0 mM; thiamine pyrophosphate, 0.6 mM; NADH, 0.1 mM; α-glycerophosphate dehydrogenase, 0.5 unit; triose phosphate dehydrogenase 0.5 unit; xylulose 5-phosphate, 10 mM and extract.

5.4. Enzyme purification

Phosphoketolase was partially purified for Candida famata grown in batch culture at 30°C on nitrogen limited media as previously described. A crude cell-free extract was prepared as described (2.1). All procedures were carried out at 4°C.

Solid $(NH_4)_2SO_4$ was added to the stirred extract over 15 minutes to give 50% saturation. After a further 15 minutes equilibrium period the mixture was centrifuged at 30,000 xg for 10 min and the precipitate was discarded. The supernatant was taken to 75% saturation in a similar manner and the resulting precipitate dissolved in a minimum volume of 10 m$\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.0.

The $(NH_4)_2SO_4$ fraction was applied to a Sepharose-6B column (2.5 x 42 cm) equilibrated in 10 m$\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.0. After elution with similar buffer the active fractions were combined and heated at 50°C for 15 min, then centrifuged at 5000 xg for 10 min and applied to a DEAE cellulose column (2.5 x 40 cm) equilibrated in 10 m$\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.0. The enzyme was eluted with a linear gradient (total volume 500 ml) of KCl in 10 m$\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.0, from 0-0.75 $\underline{\underline{M}}$. Active fractions were combined.

The $(NH_4)_2SO_4$ fractionation step was repeated, and the 70-100% fraction was retained, it was then dialysed overnight against 10 m$\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.0, and stored at -20°C until required.

5.5. Kinetic analyses

Kinetic analyses and inhibitor studies were performed as detailed in the relevant results section using a recording spectrophotometer (Model SP 8-100 Pye-Unicam Co. Ltd., Cambridge, U.K.).

5.6. Calibration of sepharose-6B column for the determination of the molecular weight of phosphoketolase from Candida famata

For the purpose of calibration the column (2.5 x 42 cm) was equilibrated in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.0. The void volume of the column was determined by loading 2 ml 0.2% (w/v) blue dextran (Pharmacia Uppsala, Sweden) and eluting in the above buffer, collecting 3 ml fractions. The peak of the eluted dye was determined spectrophotometrically at 630 nm.

The following proteins, in the quantities indicated were loaded on to the column in a volume of 2.5 ml and eluted in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.0: β-galactosidase (Sigma, grade VI), 3 mg, (mwt = 520 K, Zipser, 1963); urease (Sigma, type III), 30 mg, (mwt = 490 K, Creeth and Nichol, 1960); phosphorylase a, (Sigma) 5 mg, (mwt = 370 K, Madson and Cori, 1956); pyruvate kinase (Sigma, type II), 5 mg, (mwt = 237 K, Morawiecki, 1960). Peak activities were detected using the assay procedures described in Section 4.

On a separate occasion thyoglobulin (Sigma, type I), 30 mg (mwt = 695 K, Steiner and Edelhoch) in 5 ml KH_2PO_4/Na_2HPO_4, pH 7.0, 50 mM was loaded on to the column and eluted in a similar buffer. Thyoglobulin was detected spectrophotometrically at 280 nm.

6. Lipid turnover studies

6.1. Growth of yeasts

6.1.1. Two-stage batch experiments

Oleaginous yeasts were grown in 1 l vortex-aerated vessels containing 700 ml of medium for 5 days, the biomass

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and lipid content of the yeasts were monitored. An amount of the culture was then aseptically transferred to a similar vessel containing 400 ml of carbon starvation medium, a biomass sample was taken immediately after transfer in order to calculate the amount of culture transferred, and hence its lipid content, initial glucose and NH_4^+ concentrations were also determined by analysis of the supernatant. Yeasts were grown in the second vessel for a further 36 h prior to determination of biomass, lipid content, residual glucose and NH_4^+ concentration.

6.1.2. Two-stage continuous culture experiments

For growth in two-stage continuous culture, the first vessel was a 1.5 l Chemostat (New Brunswick Scientific Co. Inc., New Jersey, U.S.A.), with an operating volume of 1.25 l. Yeasts were grown at 30°C and the culture was maintained at pH 5.5 by the automatic addition of KOH. Foaming was controlled by the timed addition of antifoam (polyglycol P2000, Bevaloid Ltd., Beverley, U.K.). The second vessel was a similar unit with a working volume of 3.75 l. The units were connected such that the entire output of vessel 1 was pumped directly into vessel 2. The hold-up time of yeast in the connecting tubing was less than 1 minute. Fresh carbon-free medium was also pumped into vessel 2.

6.1.3. Single-stage chemostat transition studies

6.1.3.1. Non-radioactive transition study

C. curvata D was grown at 30°C on the nitrogen limited media described in 1.2. in a chemostat at a dilution rate of 0.05 h^{-1}. The chemostat had an operating volume of 1.25 l

and the yeast was maintained at pH 5.5 by the automatic addition of KOH. Foaming was controlled by the timed addition of anti-foam (Polyglycol P2000, Bevaloid Ltd., Beverley, Yorkshire).

The incoming media was switched from nitrogen limited to carbon starvation (Methods 1.2) at the transition point, this being recorded as zero time. The transition from nitrogen limitation to carbon starvation media was studied and lipid concentration, biomass, glucose concentration and NH_4^+ concentration were monitored in the chemostat.

6.1.3.2. Radioactive transition study

C. curvata was grown in a chemostat under the same conditions as for the non-radioactive transition study.

[1-^{14}C] Sodium acetate (1 ml, 125 μCi; 60 μCi/μmole) was injected into the growing culture via a syringe and needle inserted into the chemostat. After 45 min, the incoming media was switched from nitrogen limited to carbon starvation, this being recorded as zero time. Cell samples were taken at regular time intervals after the transition point.

The expelled air from the chemostat was passed through two Dreschel bottles containing 20%-KOH in order to absorb any labelled CO_2 before being led to the atmosphere.

6.2. Determination of radioactivity in lipid, whole cells and culture medium from the radioactive transition experiment

6.2.1. Radioactivity in lipid

Lipid was extracted by the method of Folch et al (1957) as described in Section 1.6.3. The extracted lipid was then

taken up in 1 ml of toluene. A sample (100 µl) was transferred to a toluene-based scintillation fluid (10 ml), containing 4 g 2,5-diphenyloxazole in 1 l toluene, and counted.

6.2.2. Radioactivity in whole cells

A known volume of culture from the chemostat was centrifuged at 10,000 xg for 5 min, washed once and resuspended in a minimum volume of water. The pellets were dried at 121°C over P_2O_5 in a vacuum oven until most of the water had evaporated. One ml of Soluene-100 (Packard Instrument Co. Inc.) tissue solubiliser was added, and the vial was incubated at room temperature for 2 h. Scintillation fluid (10 ml) containing: 4 g, 2,5-diphenyloxazole; 60 g naphthalene; 20 ml ethanediol; 100 ml methanol and 880 ml dioxan, was added. The vials were stored in a dark cupboard overnight to reduce chemiluminescence and then counted.

6.2.3. Radioactivity in aqueous samples

Aqueous radioactive samples were counted by adding 1 ml to 10 ml of dioxan-based scintillation fluid (4 g, 2,5-diphenyloxazole; 60 g naphthalene; 20 ml ethanediol; 100 ml methanol; 880 ml dioxan) held in a plastic vial. A standard to give counting efficiency and a blank to give background information were also counted for all determinations.

7. Triacylglycerol biosynthesis studies

7.1. Growth of yeasts

Yeasts were grown in 1 l vortex-aerated vessels on nitrogen-limited or carbon-limited media with glucose as sole carbon source as detailed in Sections 1.2 and 1.4.1.

7.2. Preparation of cell-free extracts

Extracts were prepared by sphaeroplast disruption using Novozym 234 as detailed in Section 2.2.2. In some cases whole sphaeroplasts were used as protein source in assays, in others sphaeroplasts were lysed prior to use. Assays were also performed using sub-cellular fractions as detailed in Section 3.

7.3. Preparation of sn-[2-^{3}H]glycerol 3-phosphate from sn-[2-^{3}H]glycerol for use in triacylglycerol biosynthesis assays

sn-[2-^{3}H]Glycerol 3-phosphate was prepared enzymically from sn-[2-^{3}H]glycerol and purified as described by Chang and Kennedy (1967).

The incubation mixture contained in a total volume of 5 ml: Tris/HCl buffer pH 8.0, 50 m$\underline{\underline{M}}$; [2-^{3}H]glycerol, 4 m$\underline{\underline{M}}$; specific activity approximately 200 mCi/mmole; ATP, 20 m$\underline{\underline{M}}$; $MgCl_2$, 20 m$\underline{\underline{M}}$; mercaptoethanol, 20 m$\underline{\underline{M}}$; serum albumin, 1 mg/ml; and glycerol kinase, 0.01 mg/ml.

After incubation for 3 h at 37°C the mixture was heated in a boiling water bath for 5 min and centrifuged briefly.

The supernatant was passed over a Dowex AG1-X2 (formate) column (1.3 x 16 cm); the glycerol 3-phosphate was eluted with a linear gradient (total volume 500 ml) of formic acid from 0-4 $\underline{\underline{M}}$. Fractions (10 ml) were collected; and each was assayed for radioactivity. A single radioactive peak was eluted from the column in fractions 23-30.

The peak fractions were pooled and taken to dryness under vacuum in a rotary evaporator at a 10°C bath temperature.

The dried material was dissolved in 3 ml distilled H_2O. Aliquots were counted, and the content of glycerol 3-phosphate was determined by assay with L-glycerol 3-phosphate dehydrogenase as detailed in Section 4.10.

The radiochemical purity of the L-glycerol 3-phosphate was checked by paper chromatography in the following solvent system: ammonium acetate, pH 7.5, 1 M, absolute ethanol; 35:65 (v/v). This showed a single radioactive spot with an Rf of 0.27, identical to that of authentic DL-glycerol 3-phosphate.

7.4. Radiochemical assay procedure for enzymes involved in triacylglycerol biosynthesis

Assay systems were as described by Christiansen (1978).

7.4.1. Basic incubation mixture

The basic incubation mixture for all assays contained the following constituents: Tris/HCl, pH 7.4, 100 mM; ATP, 15 mM; $MgCl_2$, 15 mM; CoA, 1 mM; β-mercaptoethanol, 8 mM; oleic acid, 0.4 mM, bound to 0.1 mM defatted bovine serum albumin (fraction V, Sigma). When added, the concentration of glycerol 3-phosphate was 5 mM. The reactions were carried out in a shaking water bath at 27°C and initiated by the addition of extract.

7.4.2. Acyl-CoA synthetase

7.4.2.1. Assay

The conversion of 0.4 μmole [1-^{14}C]oleate (specific activity 0.64 Ci/mole) to [1-^{14}C]oleoyl-CoA was measured in a total reaction volume of 0.25 ml basic incubation mixture. The reaction was started by the addition of protein (10-40 μg),

carried out for 4 minutes and stopped by the addition of 1 ml dole reagent (isopropanol:heptane:1 M H_2SO_4, 40:10:1), 0.35 ml H_2O and 0.6 ml n-heptane. The specific activity was determined with two different concentrations of protein.

7.4.2.2. Extraction and scintillation counting of the product

The extraction and scintillation counting of the product, acyl-CoA was carried out as described by Lloyd-Davies and Brindley (1975).

The tube contents were mixed and separated and the heptane layer removed. The lower phase was washed 3 times with 0.6 ml portions of n-heptane.

The radioactivity in a 0.5 ml sample of the lower phase was measured by scintillation counting after the addition of 0.1 ml H_2O and 5 ml Triton X100-xylene (1:2 v/v) containing 5.5 g/l 2,5-diphenyloxazole, and 0.1 g/l 1,4,-bis(5-phenyloxazol-2-yl benzene).

7.4.3. sn-Glycerol 3-phosphate acylation

7.4.3.1. Assay

The biosynthesis of triacylglycerol was determined by measuring the rate of incorporation of sn-[2-^{3}H]glycerol 3-phosphate (spec. act. 0.1 Ci/mol) or [U-^{14}C]glycerol 3-phosphate (spec. act. 0.2 Ci/mol) into lipids in a total reaction volume of 1 ml. The reaction was started by the addition of 0.1-0.3 mg protein and carried out for 60 min. The reaction was terminated by the addition of 1.5 ml butanol followed by 2 ml H_2O. After shaking, the mixture was centrifuged and most of the butanol phase was pipetted

off, washed once with 3 ml butanol-saturated H_2O to remove unreacted glycerophosphate. The butanol was removed under reduced pressure and the lipid dissolved in a known amount of chloroform. Aliquots were taken for counting total radioactivity and for chromatography.

7.4.3.2. Analysis of product

Neutral lipids were analysed by thin-layer chromatography on silica gel plates (Silica Gel G, 250 microns, manufactured by Analtech.) in petroleum ether (b.p. 60-80°C)/diethyl ether/acetic acid 60:40:1 by vol). In this system triacylglycerol, free fatty acids, diacylglycerol and monoacylglycerols are separated from phospholipid and acyl-CoA which remain at the start. Phospholipids were separated from the neutral acyl-CoA (which remains at the start) in the solvent system chloroform:methanol:acetic acid:water, 80:15:2:2 (by vol).

Lipid classes were detected by exposure to iodine vapour and their Rf values compared with those of standards. After the iodine had evaporated the spots were cut or scraped from the plates and the radioactivity determined.

7.4.3.3. Scintillation counting of product

Samples were counted in 7.5 ml of a scintillation mixture containing 0.3 g 1,4-bis(5-phenyl oxazol-2-yl benzene), 7.0 g 2,5 diphenyl-oxazole, 100 g naphthalene, 200 ml of water and 1 l dioxan.

7.4.4. Oleate esterification studies

(i) With added glycerol 3-phosphate as acyl acceptor

Oleate esterification was measured by incubating 0.4 μmol [1-^{14}C]oleate (spec. act. 0.64 Ci/mol) with 5 μmol glycerol

3-phosphate in a total reaction volume of 1 ml of basic incubation mixture. The reaction was started by the addition of 0.1-0.3 mg protein, and carried out for 60 min. The reaction was stopped by the addition of 20 ml chloroform: methanol (2:1, v/v). After standing for 15 min, precipitated protein was removed by filtering the extract through Whatman No. 1 filter paper. The test tube and the filter were rinsed with 15 ml chloroform:methanol (2:1 v/v), followed by 15 ml chloroform:methanol (1:2, v/v). The combined extracts (50 ml) were evaporated to complete dryness in a rotary evaporator. The dried residue was extracted with small amounts of chloroform. The chloroform extracts were pooled and made up to a total volume of 0.5 ml. This extract will, in the following, be termed the chloroform extract. The more polar residue in the evaporation flask was dissolved in 1 ml chloroform: methanol:water, 4:10:2 (by vol), and is referred to as the methanol extract. If the two extracts were combined, phase separation and/or precipitation would occur. Therefore, aliquots representing equal-volume percentages of the two extracts were used for determination of total radioactivity and for chromatography. For determination of total radioactivity, equal-volume percentages were placed in the same vial, and the solvent was blown off in a stream of nitrogen. Samples were counted as for glycerol 3-phosphate studies.

Lipids were separated by thin-layer chromatography, and aliquots representing equal-volume percentages were applied in one spot to the chromatogram which was developed and counted as for glycerol 3-phosphate studies.

(ii) With endogenous acyl acceptor

[1-^{14}C]Oleate was incubated under exactly the same conditions as stated above, except that no glycerol 3-phosphate was added. The extraction of the incubation mixture and the radioactive determination was also as above.

8. Triacylglycerol lipase assay system

The amount of fatty acid released by the enzymic hydrolysis of glyceryl tri([1-^{14}C]oleate) was measured radiochemically as [1-^{14}C]oleic acid released. The substrate suspension was prepared immediately prior to use: 100 µl of tri [1-^{14}C]oleoyl-glycerol (specific activity 6 µCi/mmol, 0.885 g/ml) and 3 ml 7% defatted bovine serum albumin (Sigma, fraction V) were sonicated on ice for 4 x 1 minute periods at 2 minute intervals. The assay mixture contained in a final volume of 500 µl: 150 µl of substrate suspension; 200 µl 0.3 $\underline{\underline{M}}$ Tris/HCl, pH 7.5 and protein. The reaction was initiated by the addition of yeast protein and incubated at 30°C for 30-120 minutes. The reaction was stopped by the addition of 100 µl 1 $\underline{\underline{M}}$ H_3PO_4 with rapid cooling. The lipid was extracted analysed and counted as outlined for sn-glycerol 3-phosphate acylation studies (Section 7.4.3.).

9. Electron microscopy studies

9.1. Transmission electron microscopy protocols

Method A. Yeast cells were fixed according to the method of Kopp (1975). One part glutaraldehyde (2.5% w/v) in KH_2PO_4/Na_2HPO_4 buffer (50 m$\underline{\underline{M}}$), pH 7.0, was added to 9 parts of growth medium containing cells. After 15 min on ice, the samples were mixed with an equal volume of the fixative and

stored overnight at 4°C. The cells were washed twice with KH_2PO_4/Na_2HPO_4 buffer (50 mM), pH 7.0, followed by secondary fixation with osmium tetroxide (1% w/v) in 0.1 M phosphate buffer, pH 7.0 for 2 h at 25°C.

After fixation, cells and protoplasts were harvested by centrifugation (2000 xg for 5 min) and the pellets were then mixed with molten agar (45°C, 2% w/v, Oxoid No. 1). After setting, the solid agar containing yeast cells was cut into cubes (1 mm) which were dehydrated in a graded ethanol series followed by 100% propylene oxide (2 x 10 min).

The agar cubes were infiltrated with 50:50 propylene oxide: Araldite C7212 resin mixture for 4 h at 25°C. This was followed by further infiltration by Araldite C7212 mixture (100%) for 24 h (25°C). Finally the agar cubes were placed in BEEM embedding capsules (size 00) and covered with fresh Araldite mixture (100%) and were allowed to stand for a further 24 h (25°C) before polymerisation at 60°C for 72 h.

Thin sections were cut from the blocks on a Reichart OMu2 ultramicrotome using a diamond knife. The sections were floated on to copper specimen grids (400 mesh), and stained with aqueous uranyl acetate (2% w/v) for 30 min and Reynolds lead citrate for 10 min (Reynolds, 1963). The sections were then stabilized by carbon coating in an Edwards 12t6 vacuum coating unit prior to examination under the electron microscope (Philips EM 400T).

Method B. Yeast cells were harvested and washed once with water. The cells were fixed with $KMnO_4$ (1.5%) for 20 min at room temperature and washed twice in distilled H_2O.

The cells were then dehydrated in a graded ethanol series, and stained with uranyl acetate (1% w/v in water). They were infiltrated with 3:1 propylene oxide:Epon 812 resin mixture for 4 hours at room temperature, followed by 1:1 propylene oxide:Epon 812. The yeasts were finally embedded in Epon 812 and allowed to stand for 12 hours prior to polymerisation at 100°C for 24 hours. The material was cut on an LKB ultramicrotome with a diamond knife. Electron micrographs were taken with a Philips EM300. Freeze etch replicas were made in a Balzer's unit as described by Moor (1964).

9.2. Scanning electron microscopy protocol

Lipid globules were isolated as described in Section 3.3. The purified oil globules were fixed with osmium tetroxide (2.5 % w/v) in 0.1 $\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.4 for 30 min at room temperature. After fixation the oil globules were washed twice with distilled water by centrifugation (5 min at 5000 xg).

The globules were filtered on to polycarbonate nuclepore filters and mounted on stubs. Each stub was coated with gold and carbon on a rotary turntable to obtain a uniform coating approximately 20 nm thick. Observations were made with a Jeol JFM 35 CF scanning electron microscope.

RESULTS

Chapter 1: Phosphoketolase studies

The enzyme pentulose 5-phosphate phosphoketolase catalyses the reaction:

Xylulose 5-phosphate ⟶ glyceraldehyde 3-phosphate + acetyl-CoA.

Although xylulose 5-phosphate (Xu5P) has always been used as substrate in the assay for phosphoketolase in yeasts (Whitworth and Ratledge, 1977; Boulton, 1982; Evans and Ratledge, 1984) there is no reason why ribulose 5-phosphate (Ru5P) should not also be considered as substrate. The difference between Ru5P and Xu5P lies in the stereospecific orientation at $C\text{-}_3$ which is lost when this carbon atom becomes the $C\text{-}_1$ of glyceraldehyde 3-phosphate (G3P). Ru5P would readily arise from Xu5P via ribulose 5-phosphate epimerase but, as cell extracts will inevitably contain this enzyme no matter which substrate was used in the assay of phosphoketolase, both would engender activity.

An attempt to resolve this difficulty was made by surveying a number of yeasts for the presence of phosphoketolase when grown on glucose or xylose using both xylulose 5-phosphate (Xu5P) and ribulose 5-phosphate (Ru5P) as assay substrate.

1.1. Survey for the presence of Xu5P phosphoketolase and Ru5P phosphoketolase in yeasts grown on xylose and glucose as sole carbon source

Ten yeasts, both oleaginous and non-oleaginous, were cultivated in 1 l vortex aerated batch cultures (Methods,

Section 5.1) for 46 h on nitrogen limited media with glucose or xylose as sole carbon source. Assays for the presence of Ru5P and Xu5P phosphoketolases were then carried out (Table 2).

The yeast Candida famata NCYC 33 used in this study was originally classified as Saccharomyces cerevisiae NCYC 33. However, due to its ability to utilise xylose as sole carbon source it was reclassified as Candida famata NCYC 33 (Barbara Kirsop, NCYC, Norwich, personal communication).

No significant phosphoketolase activity with Xu5P as substrate was detected in any yeast grown on glucose as sole carbon source. The activity of phosphoketolase with Ru5P as substrate was not examined in any yeast grown on glucose except C. famata NCYC 33 due to the extremely high cost of the substrate, which necessitated that the number of assays be kept to a minimum wherever possible. However, results using Xu5P as assay substrate were in accordance with those of Evans and Ratledge (1984), and they showed conclusively that the enzyme was not significantly active in yeasts grown on glucose as sole carbon source.

Significant phosphoketolase activity was detected in all oleaginous yeasts grown on xylose. The non-oleaginous yeast S. cerevisiae NCYC 74 did not grow on xylose and showed no phosphoketolase activity when grown on glucose. The other non-oleaginous yeast examined, namely C. famata NCYC 33, showed significant activity of phosphoketolase when cultivated on xylose.

Table 2. A survey of Xu5P and Ru5P phosphoketolases in yeasts grown on glucose and xylose as sole carbon sources

Enzyme assays were performed according to Golberg et al. (1966) using Method A (Methods Section 4.9).

Yeast	XYLOSE GROWN		GLUCOSE GROWN	
	Specific activity of phosphoketolase (nmol.mg protein^{-1}min^{-1}) with:			
	Substrate 1 Xu5P	Substrate 2 Ru5P	Substrate 1 Xu5P	Substrate 2 Ru5P
Candida boidinii CBS 5777	13	46	1	ND
Candida curvata D	37	41	1	ND
Candida famata* NCYC 33	39	38	0	1
Lipomyces starkeyi CBS 1809	72	64	2	ND
Pachysolen tannophilus NCYC 614	25	17	0	ND
Pichia media CBS 5521	29	41	1	ND
Rhodotorula glutinis NCYC 59	25	24	1	ND
Rhodosporidium toruloides CBS 14	55	27	2	ND
Saccharomyces cerevisiae NCYC 74	DNG	DNG	0	ND
Yarrowia lipolytica CBS 2074	2	23	1	ND

ND = not determined DNG = did not grow

*Originally classified as Saccharomyces cerevisiae NCYC 33

The apparent dual substrate specificity of phosphoketolase was demonstrated with nine yeasts cultivated on xylose. In five cases (Candida curvata D, C. famata, Lipomyces starkeyi, Rhodotorula glutinis and Pachysolen tannophilus) the enzyme was approximately equally active with either substrate. With C. boidinii, Pichia media and Yarrowia lipolytica, there was a decided preference for Ru5P as substrate. Only with Rhodosporidium toruloides did there appear to be a decided substrate preference for Xu5P over Ru5P.

This apparent dual substrate specificity of the pentulose 5-phosphate phosphoketolase (Pu5PPK) in the various yeasts could be either genuine or due to the presence of Ru5P-3-epimerase interconverting the two substrates. Therefore it was decided to attempt to purify the Pu5PPK in order to resolve the question and ascertain some of the properties of the enzyme.

Current assay methods for phosphoketolase are as described in Methods 4.9. These comprise a time-consuming two stage method which involves an incubation for 30 min or a colorimetric method which takes 15 min. Therefore prior to attempting purification of the enzyme it was decided to attempt to devise a continuous assay method.

1.2. Attempts to assay phosphoketolase from Candida famata by a continuous method

Two attempts to devise a continuous assay method were made. These are detailed in Methods 5.3.

The first involved the conversion of glyceraldehyde 3-phosphate (formed from xylulose 5-phosphate by the action

of phosphoketolase) to 1,3-diphosphoglyceric acid by the action of glyceraldehyde 3-phosphate dehydrogenase. The second involved the conversion of the glyceraldehyde 3-phosphate to dihydroxyacetone phosphate and thence to glycerol 3-phosphate by the action of triose phosphate isomerase and α-glycerophosphate dehydrogenase respectively. This latter procedure being used to assay for aldolase which could be considered an analogous reaction in that it also yields glyceraldehyde 3-phosphate as product. The results are shown in Table 3. Unfortunately in both cases the added commercial dehydrogenases also acted directly on the xylulose 5-phosphate, presumably converting it to xylitol 5-phosphate. Therefore large apparent enzyme activities were also observed in the absence of phosphoketolase and so neither assay gave a valid measurement of phosphoketolase activity.

Therefore partial purification of phosphoketolase was attempted using the established assay methods of Goldberg (1966) (Methods 4.9).

1.3. Partial purification of phosphoketolase from Candida famata

C. famata was selected for purification due to the fact that it was the only non-oleaginous yeast examined either in this study, or in previous work (Whitworth and Ratledge, 1977; Boulton, 1982; Evans and Ratledge, 1984) that demonstrated phosphoketolase activity. Therefore once the enzyme had been purified there was a possibility that its genes could be genetically manipulated into other non-oleaginous yeasts which could not grow on xylose (e.g. Saccharomyces species) enabling them to utilise this cheap and abundant

Table 3. Attempts to devise a continuous procedure for the assay of Xu5P phosphoketolase

Assay procedures were as described in Methods 5.3.1 (Method 1) and 5.3.2. (Method 2).

Method 1:

Assay contents	Specific activity of Xu5P phosphoketolase (nmol.mg protein^{-1}.min^{-1})
Blank (a) (minus substrate)	35
Blank (b) (minus enzyme)	7
Test (containing enzyme and substrate)	36

Method 2:

Assay contents	Specific activity of Xu5P phosphoketolase (nmol.mg protein^{-1}.min^{-1})
Blank (a) (minus substrate)	27
Blank (b) (minus enzyme)	120
Test (containing enzyme and substrate)	160

carbon source.

The yeast was grown and purified according to Methods Section 5.

It was not possible to assess whether or not the extract was free of Ru5P 3-epimerase, as assay, for it depended on having a pure phosphoketolase, which was unfortunately not commercially available. Assay for transketolase was also impossible as this depended on the same principle as Method A for phosphoketolase, i.e. detection of glyceraldehyde 3-phosphate by its conversion to glycerol 3-phosphate with the concomitant oxidation of NADH.

The activity of PK towards Xu5P appeared to be more heat stable than the activity displayed with Ru5P as substrate (Figure 2) and a heat treatment step was therefore introduced into the purification procedure.

As purification continued, the pK activity with Ru5P as substrate gradually decreased to a low value (Table 4). At this point the extract was considered to be sufficiently pure to continue with kinetic analyses. The progress of a typical purification is shown in Table 4. The preparation was used in all subsequent work.

1.4. Variation in the initial velocity of phosphoketolase from Candida famata with substrate concentration

The usual Michaelis-Menten relationship was established when initial velocity was measured as a function of xylulose 5-phosphate concentration (Figure 3). The apparent K_m and V_{max} for xylulose 5-phosphate were calculated to be 2.4 mM and 0.71 μmol min^{-1} (mg protein)$^{-1}$, respectively.

FIGURE 2

Denaturation profile for partially purified phosphoketolase from Candida famata NCYC 33.

Portions of the extract were incubated at each temperature for 15 minutes prior to assay (Goldberg, 1966, Method A).

○ Xylulose 5-phosphate substrate

● Ribulose 5-phosphate substrate

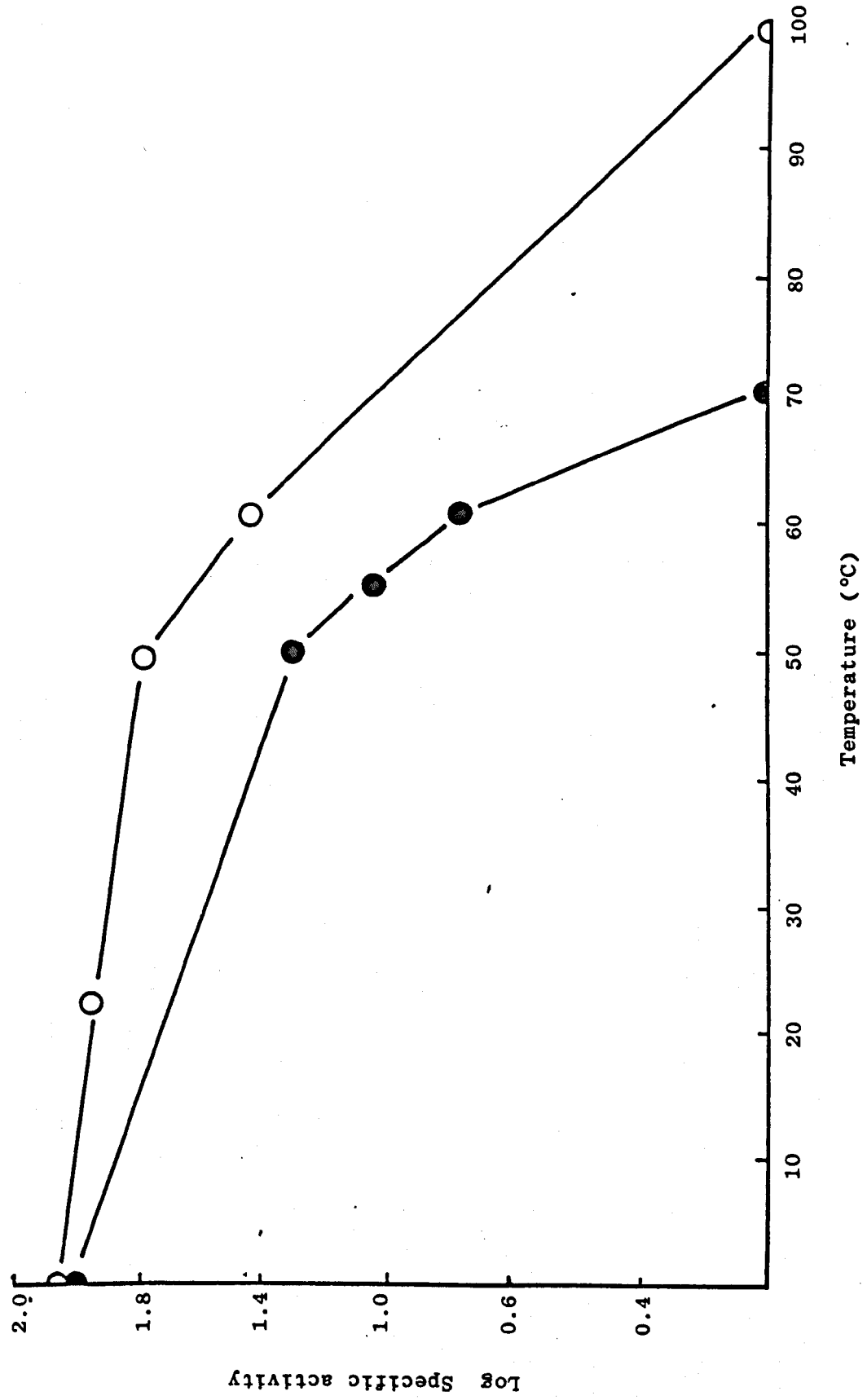

Log Specific activity
2.0
1.8
1.4
1.0
0.6
0.4
10
20
30
40
50
60
70
80
90
100
Temperature (°C)

Fig. 2.

Table 4. Results of a typical purification of phosphoketolase from Candida famata NCYC 33 grown on xylose as sole carbon source

Fraction	Volume (ml)	Total units (nmol min^{-1})	Total protein (mg)	Specific activity nmol min^{-1} (mg protein)$^{-1}$	Ratio of specific activity with Xu5P as substrate to specific activity with Ru5P as substrate	Purification factor	Yield %
Crude	23	10788	402.5	27.0	0.87	1	100
		(12317)		(31.0)		(1)	(100)
50-75% $(NH_4)_2SO_4$	3.2	7720	193.3	41.0	0.73	1.5	72
		(10979)		(56.0)		(1.86)	(89)
Pooled Sepharose 6B eluate	68.0	7018	36.8	191	1.13	7.12	65
		(6245)		(170)		(5.5)	(50)
Heat treatment at 50°C for 15 minutes	65.0	5910	30.0	197	1.67	7.3	55
		(3540)		(118)		(4.4)	(28.7)
Pooled DEAE cellulose eluate	51.0	5842	14.8	395	4.16	14.6	54
		(1406)		(95.0)		(3.5)	(11.4)
70-100% $(NH_4)_2SO_4$	53.0	5601	9.5	591	3.89	22	52.0
		(1444)		(152)		(5.6)	(13.0)

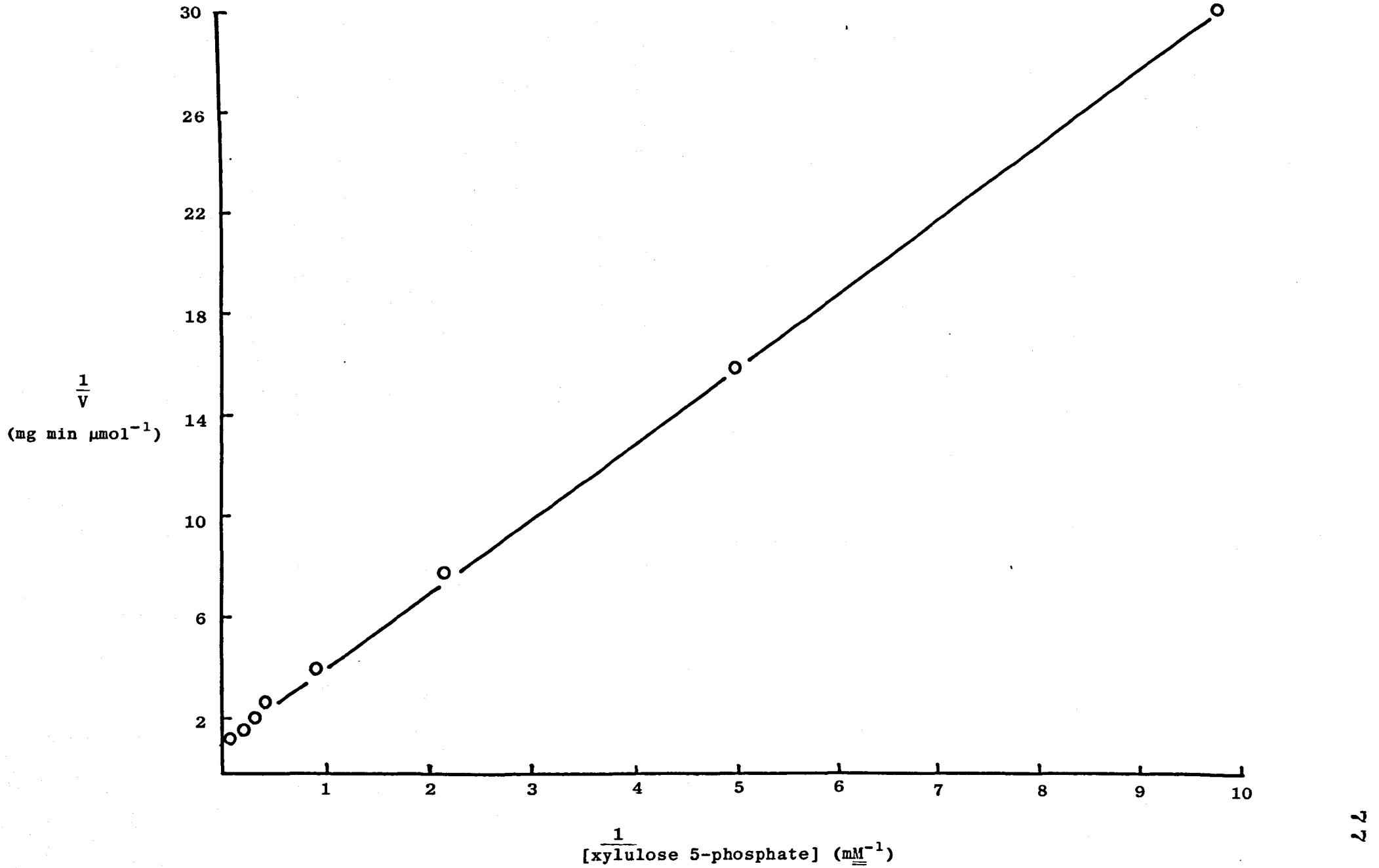
Values in Roman type = xylulose 5-phosphate as assay substrate

Values in parentheses = ribulose 5-phosphate as assay substrate

FIGURE 3

Lineweaver-Burk plot showing the variation in initial velocity of phosphoketolase from *Candida famata* NCYC 33 with varying concentration of xylulose 5-phosphate.

Fig. 3.


30
26
22
18
14
10
6
2
1
2
3
4
5
6
7
8
9
10
1/V
(mg min μmol⁻¹)
1/[xylulose 5-phosphate] (mM⁻¹)

1.5. Variation in the initial velocity of phosphoketolase from Candida famata with substrate concentration

Maximum activity was observed between pH 7.0-7.6 (Figure 4), and pH 7.0 was judged suitable for subsequent assays.

1.6. Inhibition of phosphoketolase from Candida famata NCYC 33 by citrate

Citrate was tested in the standard assay to see if it was capable of modulating the activity of phosphoketolase from C. famata. It was found to have an inhibitory effect only at high concentrations (> 20 m$\underline{\underline{M}}$) with about 70 m$\underline{\underline{M}}$ needed to reduce the enzyme activity by half (Figure 5).

The enzyme was not affected by citrate at concentrations (< 10 m$\underline{\underline{M}}$) which were likely to prevail intracellularly (Evans and Ratledge, 1983). The inhibition observed with higher concentrations of citrate (Ki approximately 70 m$\underline{\underline{M}}$) was unlikely to be of physiological significance and was attributed to the chelating properties of citrate for magnesium which was a co-factor in the enzyme assay.

1.7. The response of phosphoketolase from Candida famata NCYC 33 to acetyl-CoA

Acetyl-CoA was also tested to see if it could modulate the activity of phosphoketolase from C. famata. The enzyme was found to be slightly activated (~20-30%) at concentrations of acetyl-CoA above 0.1 m$\underline{\underline{M}}$ (Figure 6). However, such concentrations were much higher than those found normally in yeasts (~10 $\mu\underline{\underline{M}}$) and it was concluded that this effect was probably insignificant in vivo.

FIGURE 4

Variation in specific activity of phosphoketolase from Candida famata NCYC 33 with pH.

The standard assay mixture (Goldberg, 1966, Method A) was used with KH_2PO_4/Na_2HPO_4 buffer at a final concentration of 30 mM for pHs up to pH 7.5. Tris/HCl buffer at a final concentration of 30 mM was used for assays at pH 8.0 and 8.5.

Fig. 4.

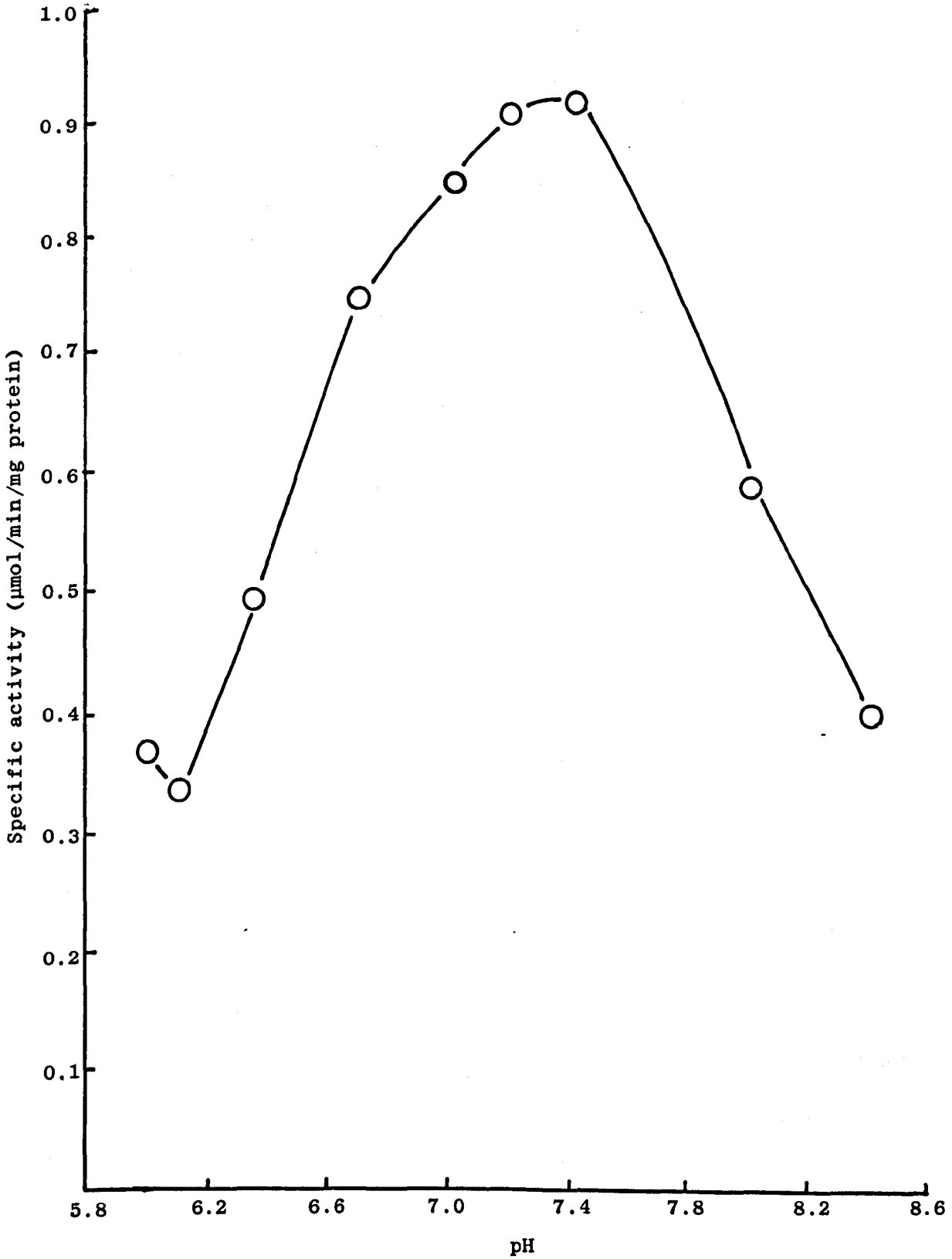

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Specific activity (μmol/min/mg protein)
5.8
6.2
6.6
7.0
7.4
7.8
8.2
8.6
pH

FIGURE 5

Variation in the initial velocity of phosphoketolase from *Candida famata* NCYC 33 with citrate concentration.

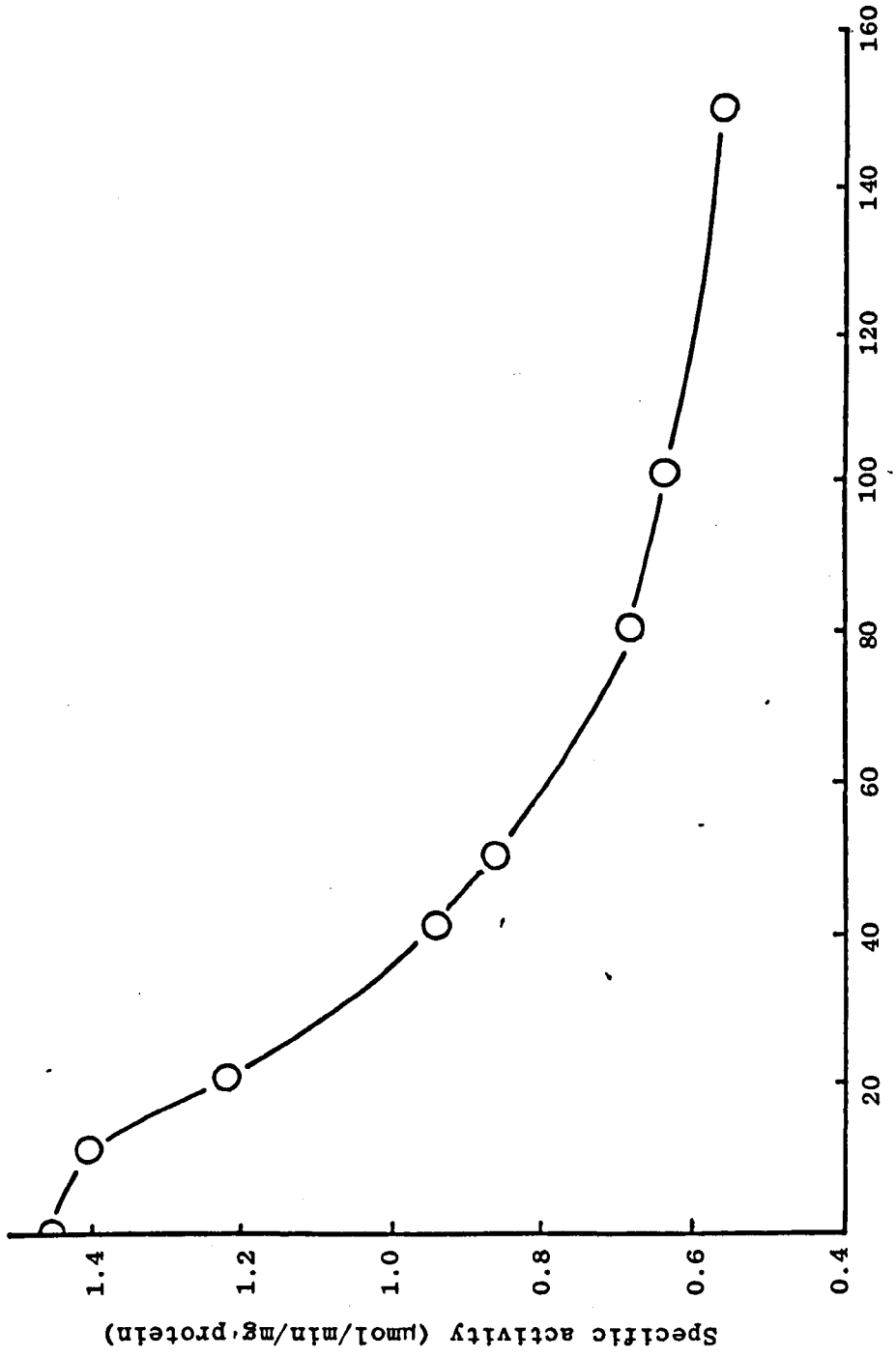
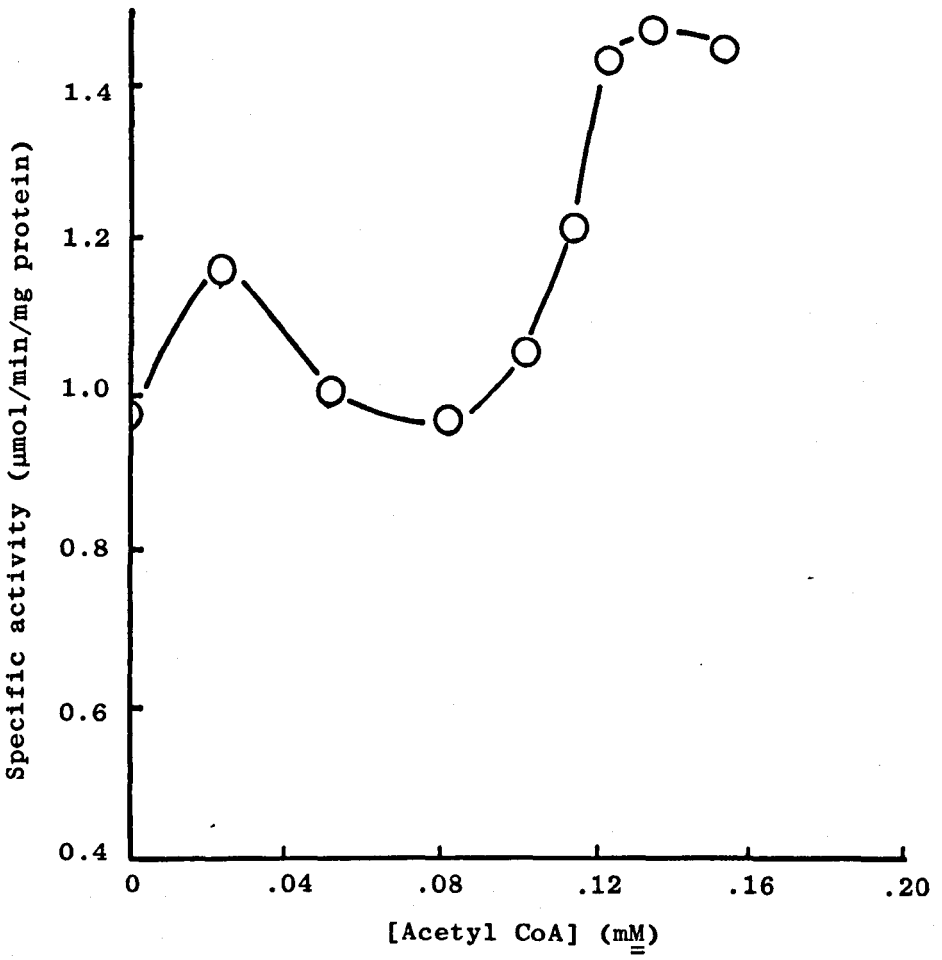

Specific activity (μmol/min/mg protein)
0.4
0.6
0.8
1.0
1.2
1.4
20
40
60
80
100
120
140
160
[Citrate] (mM)

Fig. 5

FIGURE 6

Variation in the initial velocity of phosphoketolase from Candida famata NCYC 33 with concentration of acetyl-CoA

Fig. 6.


Specific activity (µmol/min/mg protein)
1.4
1.2
1.0
0.8
0.6
0.4
0
.04
.08
.12
.16
.20
[Acetyl CoA] (mM)

1.8. Determination of the molecular weight of phosphoketolase from Candida famata NCYC 33.

The molecular weight of phosphoketolase from C. famata NCYC 33 was determined by passing a sample down a column of Sepharose-6B and comparing the elution volume with the elution volumes of proteins of known molecular weight (Methods Section 5.6.). The elution profile of the gel filtration on Sepharose 6B is shown in Figure 7. Phosphoketolase from C. famata NCYC 33 was shown to have a molecular weight of 495,000 (±5000) (Figure 8).

Since a homogenous preparation of phosphoketolase was not available, it was not possible to investigate whether or not the enzyme was composed of subunits.

No alternative method of determining the molecular weight could be attempted (e.g. gel electrophoresis) due to the high cost of assay substrates, and the limited budget allowed for this work (approximately £1,500).

1.9. Determination of the effect of the presence of phosphoketolase on lipid content and ATP:citrate lyase activity in oleaginous yeasts

These present studies and those of Evans and Ratledge (1984) have shown that oleaginous yeasts grown on glucose and xylose exhibit a phosphoketolase activity when grown on the latter, which was absent, or insignificant when grown on the former. As one of the products of phosphoketolase is a C_2 unit which is converted to acetyl-phosphate, ATP:citrate lyase activities in glucose and xylose-grown yeasts were compared with their lipid levels to see if the additional acetyl-CoA

FIGURE 7

Gel filtration pattern of phosphoketolase from Candida famata NCYC 33 on sepharose 6B.

● protein concentration (A_{280})

○ enzyme activity with Xu5P substrate

□ enzyme activity with Ru5P substrate.

Fig. 7.

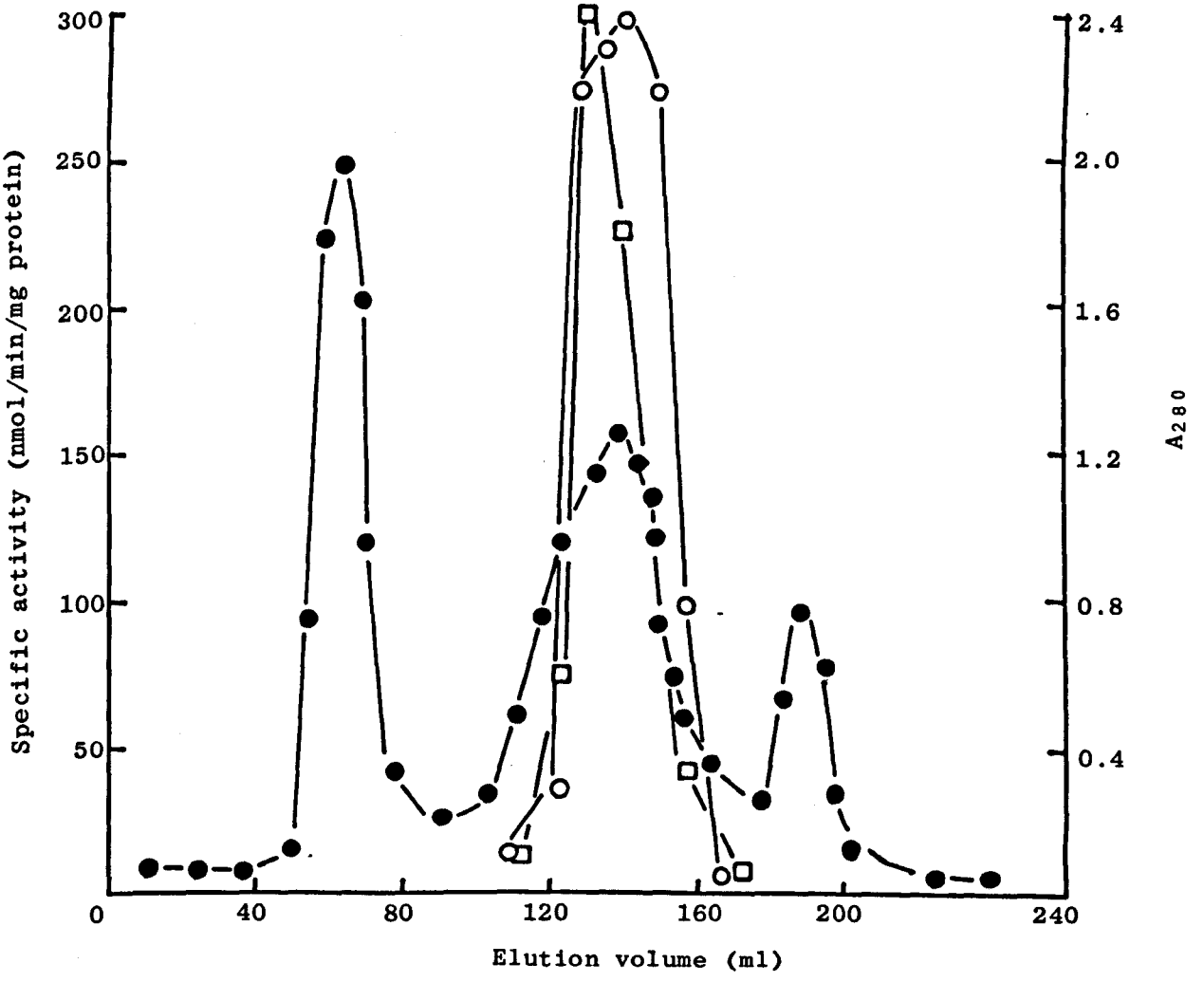
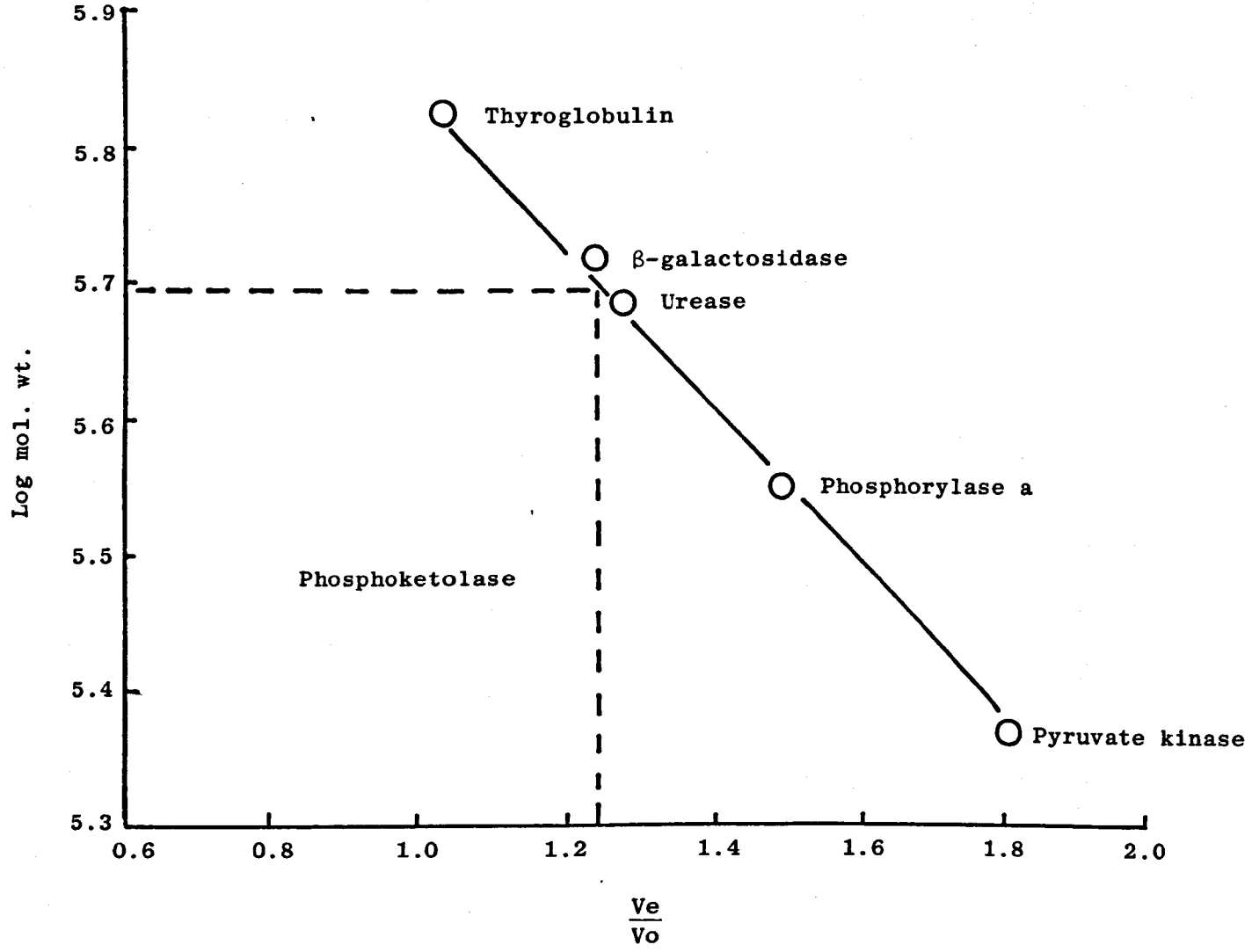

Specific activity (nmol/min/mg protein)
300
250
200
150
100
50
0
40
80
120
160
200
240
Elution volume (ml)
2.4
2.0
1.6
1.2
0.8
0.4
A280

FIGURE 8

Determination of the molecular weight of phosphoketolase from Candida famata NCYC 33 by column chromatography on Sepharose 6B.

The column was calibrated using the marker proteins as indicated (described in Methods 5.6.). Purified phosphoketolase (2.5 ml, specific activity 10 μmol.min^{-1} (mg protein)$^{-1}$) was loaded on to the column and eluted in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.0. Elution volume of peak activity of phosphoketolase and marker enzymes = Ve and void volume of column = Vo.

Fig. 8.


5.9
5.8
5.7
5.6
5.5
5.4
5.3
Log mol. wt.
Thyroglobulin
β-galactosidase
Urease
Phosphorylase a
Phosphoketolase
Pyruvate kinase
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
Ve/Vo

formed from acetyl-phosphate exerted any control over ATP: citrate lyase activity and hence lipid levels.

Nine oleaginous yeasts, all of which exhibited Xu5P phosphoketolase activity when grown on xylose, were surveyed. The results are shown in Table 5.

Contrary to the findings of Evans and Ratledge (1984) whose studies were confined to the yeast C. curvata D, the lipid levels in all yeasts examined were not greatly increased when grown on xylose. The only yeast examined that did show this effect was L. starkeyi CBS 1809, in which the lipid levels increased from 28% to 37% (w/w) when grown on xylose.

The ATP:citrate lyase activities in all yeasts grown on xylose did not significantly differ from those when grown on glucose.

Table 5. Survey of the occurrence of ATP:citrate lyase in oleaginous yeasts grown on glucose or xylose as sole carbon source, compared to their lipid content

YEAST STRAIN	GLUCOSE GROWN CELLS		XYLOSE GROWN CELLS	
	Specific activity of ATP:citrate lyase (nmol/mg/min)	lipid content (% w/w)	Specific acitvity of ATP:citrate lyase (nmol/mg/min)	lipid content (% w/w)
Candida curvata 'D'	9	30	15	29
Candida curvata 'R'	5	23	12	17
Lipomyces lipofer NCYC 165	0	2	0	2
Lipomyces starkeyi 2512	27	28	24	25
Liopmyces starkeyi CBS 1809	80	28	100	37
Rhodotorula glutinis NCYC 59	21	13	17	9
Rhodosporidium toruloides CBS 14	31	22	26	24
Rhodosporidium toruloides CBS 2370	13	13	14	7
Rhodosporidium toruloides 26217	25	8	18	5

Yeasts were grown in vortex aerated vessels on nitrogen-limited media (Methods 1.2) at 30°C. Assays for ATP:citrate lyase were performed according to Srere (1953) after 46 hours growth and lipid analysis was performed by the method of Folch et al (1957) after 5 days growth.

Chapter 2: Lipid turnover studies

Lipid accumulates in oleaginous micro-organisms in the form of discrete droplets, the quantity accumulated depending on the cultural conditions. The prime requisite for lipid accumulation is for the culture medium to contain an excess of carbon over some other nutrient. Thus in batch culture there would be a period of cell proliferation followed by a fattening stage when lipid levels would increase, this being initiated by the exhaustion of the limiting nutrient. During the fattening stage, the excess carbon would continue to be transported into the cell and converted to lipid. One aim of this thesis was to answer the question "Could this lipid be reconverted into an energy source in a carbon-free environment once the previous nutrient limitation was lifted?". Three strategies were employed to resolve this question:

(a) Two-stage batch culture experiments where a yeast was grown until replete with lipid, then further nutrient medium lacking a carbon source was added.

(b) Two-stage continuous culture studies wherein high lipid yeasts were generated in the first vessel then passed into a second vessel without further carbon nutrients being added.

(c) Transition single-stage continuous culture wherein the medium was changed abruptly from high C:N ratio which had induced lipid accumulation to low or nil C:N ratio.

Before attempting such studies a theoretical maximum yield of biomass from lipid was evaluated, in order that the efficiency of lipolysis could be calculated.

Darlington (1964) reported that a typical microbial composition is:

	%	Divided by	Molecular weight		
carbon	47		12	=	4
hydrogen	6.5		1	=	6.5
nitrogen	7.5		14	=	0.5
oxygen	31		16	=	2
ash	8		-		

The empirical formula is therefore $C_4 \cdot H_{6.5} \cdot N_{0.5} \cdot O_2$. Assuming complete carbon conservation and representing lipid as hydrocarbon:

$$4CH_2 \longrightarrow C_4H_{6.5}N_{0.5}O_2$$

$$56 \text{ g} \longrightarrow 93.5 + 8\% \text{ ash} = 101.6 \text{ g}$$

$$\therefore \quad 100 \text{ g lipid} \longrightarrow 181.4 \text{ g biomass}$$

Therefore the maximum yield of biomass from a hydrocarbon, assuming complete carbon conservation is:

$$\frac{181}{100} = 1.8 \text{ g biomass formed per g lipid metabolized,}$$

$$= \sim 2 \text{ g biomass formed per g lipid metabolized.}$$

2.1. Batch Culture Studies

Nine yeasts were grown in two-stage batch culture as described in Methods Section 6.1.1, such that lipid accumulated in stage one and carbon starvation conditions prevailed in stage two. A sample was removed from the second stage immediately after transfer of culture from the first vessel so that the initial biomass, lipid, NH_4^+ and glucose concentrations could be determined accurately. Such measurements

were also made at the final time of sampling for vessels one and two.

The results are shown in Tables 5 and 6. With the exception of the three Lipomyces species, all yeasts were able to utilize their storage lipid for cell proliferation. Values for the increase in biomass per decrease in lipid ranged from 0.53 to 1.21 g biomass per g lipid. These figures lie well below the calculated maximum yield and were of the order expected.

The fact that all three Lipomyces species examined failed to utilise their storage lipid for cell proliferation, was a very interesting observation. The reason for their inability to do this was not clear, but it was possibly due to the lack of a lipase. This hypothesis was examined later and is reported in a subsequent section of this Chapter (Results 2.4.1.).

Subsequent electron microscopy studies, which are documented in Results Chapter 4, revealed that these oleaginous yeasts could also accumulate another storage product, namely glycogen.

Glycogen analysis was performed on three oleaginous yeasts grown in two stage batch culture as documented in Methods 6.11. Samples were then analysed for glucose and hence, glycogen content as described in Methods 1.6.5. The results are shown in Table 7.

All yeasts examined accumulated glycogen as well as lipid under nitrogen limited conditions. The yeast Lipomyces starkeyi CBS 1809 accumulated a large percentage (22.7% of the cell dry weight). Both C. curvata D and T. cutaneum CBS 40 degraded

Table 5. The effect of transition from nitrogen limitation to carbon starvation on the biomass and lipid content of oleaginous yeasts

Yeast	STAGE 1 NITROGEN LIMITATION			STAGE 2 CARBON STARVATION					
	Dry weight (g/l)	% lipid (w/w)	volume of media transferred to Stage 2 (ml)	initial glucose concentration (g/l)	final glucose concentration (g/l)	initial NH_4^+ concentration (g/l)	final NH_4^+ concentration (g/l)	Dry weight (g/l)	% lipid (w/w)
Candida curvata D	9.2	34	240	0.6	0.025	1.5	0.76	4.48	8
Candida curvata R	8.24	28.1	215	0.045	0.03	1.78	0.92	3.28	11
Lipomyces lipofer NCYC 165	2.64	1.26	214	0.03	0.022	1.37	1.35	0.96	1.2
Lipomyces starkeyi CBS 2512	10.8	31.3	230	0.054	0.022	1.23	1.06	3.95	18.8
Lipomyces starkeyi CBS 1809	10.3	34	222	0.072	0.027	1.6	1.32	3.7	28
Rhodosporidium toruloides CBS 14	9.1	24	209	0.043	0.033	1.2	0.8	3.86	2.8
Rhodosporidium toruloides ML 2590	7.54	7.1	223	0.47	0.039	1.88	0.87	3.14	0.43
Rhodosporidium toruloides ATCC 26217	9.4	22.4	201	0.25	0	1.41	0.9	3.7	1.68
Trichosporon cutaneum CBS 40	11.92	23	233	0.27	0.035	1.37	0.94	4.91	5.91

NB Yeasts were grown for five days on nitrogen limited media (700 ml) in 1 l vortex aerated vessels at 30°C. A recorded volume of culture was then aseptically transferred to a similar vessel containing carbon-free medium (400 ml) and grown for a further 36 hours.

Table 6. The increase in biomass and decrease in lipid content of oleaginous yeasts brought about by transition from nitrogen limitation to carbon starvation

Yeast	Biomass transferred from Stage 1 to Stage 2 (g)	Biomass in Stage 2 after 36 hours (g)	Increase in biomass (g)	Lipid transferred from Stage 1 to Stage 2 (g)	Lipid in Stage 2 after 36 hours (g)	Decrease in lipid (g)	Increase in biomass per decrease in lipid (g/g)	Increase in biomass per decrease in lipid values corrected for residual glucose in transfer inoculum (g/g)
Candida curvata D	2.2	2.87	0.67	0.748	0.23	0.518	1.29	0.93
Candida curvata R	1.77	2.02	0.25	0.498	0.222	0.276	0.91	0.91
Lipomyces lipofer NCYC 165	0.56	0.59	0.029	0.007	0.007	0	0	0
Lipomyces starkeyi CBS 2512	2.48	2.49	0.01	0.78	0.47	0.31	0.03	0
Lipomyces starkeyi CBS 1809	2.29	2.3	0.01	0.78	0.64	0.14	0.07	0
Rhodosporidium toruloides CBS 514	1.9	2.35	0.45	0.46	0.07	0.39	1.15	1.15
Rhodosporidium toruloides ML 2590	1.68	1.96	0.28	0.12	0.084	0.11	2.5	1.21
Rhodosporidium toruloides ATCC 26217	1.89	2.22	0.33	0.42	0.037	0.383	0.36	0.67
Trichosporon cutaneum CBS 40	2.78	3.11	0.33	0.64	0.18	0.46	0.72	0.53

Values for amounts of biomass and lipid transferred and for increase in biomass and decrease in lipid were calculated from the values in Table 4. Values for increase in biomass per decrease in lipid (g/g) were corrected for the small amount of glucose that was transferred from vessel one, assuming that 1 g glucose would give rise to 0.5 g biomass.

Table 7. Glycogen content of oleaginous yeasts grown in two stage batch culture

Yeast	STAGE 1 NITROGEN LIMITATION		STAGE 2 CARBON STARVATION		
	Dry weight (gl^{-1})	% glycogen (w/w)	% glycogen (w/w) 2 hours after transfer	7 hours after transfer	24 hours after transfer
Candida curvata D	14.2	10.2	5.5	5.2	3.2
Trichosporon cutaneum CBS 40	10.3	26.0	4.9	4.3	4.0
Lipomyces starkeyi CBS 1809	9.1	22.7	21.2	17.1	12.7

Yeasts were grown in stage one for five days under nitrogen limitation such that lipid and glycogen accumulated. Culture was then transferred to a second vessel in which carbon starvation conditions prevailed. Glycogen content of the yeasts was monitored at various times after transfer.

most of the glycogen within 2 hours of transfer to carbon starvation conditions, the yeast L. starkeyi CBS 1809, was much slower to degrade the glycogen and 12.7% remained even after 24 hours in carbon starvation medium.

Therefore, it was obvious that the yields of biomass increase per decrease in lipid (g/g) were incorrect due to glycogen degradation occurring simultaneously. Unfortunately the fact that glycogen also accumulated in these yeasts was not discovered until the end of this period of study, and hence it was not possible to re-evaluate the yields of biomass increase per decrease in lipid. In all the lipid turnover studies, glycogen accumulation would have occurred during growth under nitrogen limited conditions; because of this the yields of increase in biomass per decrease in lipid (g/g) are over estimates.

2.2. Two-stage continuous culture studies

Oleaginous yeasts grown in continuous culture can attain lipid concentrations equal to those seen in batch cultures providing a dilution rate of 0.03 to 0.15 h^{-1} is used and the medium has a high C:N ratio. Under these conditions lipid accumulation proceeds because cell proliferation is restricted by nitrogen limitation, and the residence time in the growth vessel is such that the yeast can convert excess carbon in the medium to lipid.

A two-stage chemostat presents ideal conditions for examination of turnover or utilization of the accumulated lipid in a starved yeast, due to the fact that a high degree of control is available and hence the conditions are reproducible.

Cells with a high lipid content can be grown in the first stage (as described above) and passed into a second vessel in which all other nutrients except carbon are provided. Cell proliferation should take place if lipid mobilization occurs. Candida curvata D was grown in two-stage continuous culture as described in Methods 6.1.2. A diagramatic representation of the apparatus is shown in Figure 9.

Steady state conditions in vessel 1 were maintained without change during the entire course of the experiment. After a change in the steady state conditions in vessel 2, at least five changes of medium occurred before samples were taken on two successive days. If the biomass values did not agree, further samples were taken until they did.

The increase in biomass (or decrease in lipid) per litre of medium in vessel 2 was calculated as:

$$x' - \frac{xf}{f'}$$

where x and x' are the biomass (or lipid) concentrations (grams per litre) entering and leaving vessel 2 at flow rates f and f', respectively. The growth rate of the yeast in the second stage was calculated as:

$$\mu' = \frac{f'x' - fx}{v_2 x'}$$

where v_2 is the volume of medium in vessel 2.

The dilution rate in vessel 2 was varied by altering the flow rate of fresh carbon-free medium into stage 2. The concentration of glucose in stage 2 remained at about 0.025 gl^{-1} and that leaving stage 1 was at a steady state concentration of 2 gl^{-1}. The results are shown in Table 8.

FIGURE 9

Diagrammatic representation of the two-stage continuous culture apparatus.

The dilution rate in Vessel 2 was varied by altering the flow rate of fresh carbon-free medium into Stage 2.

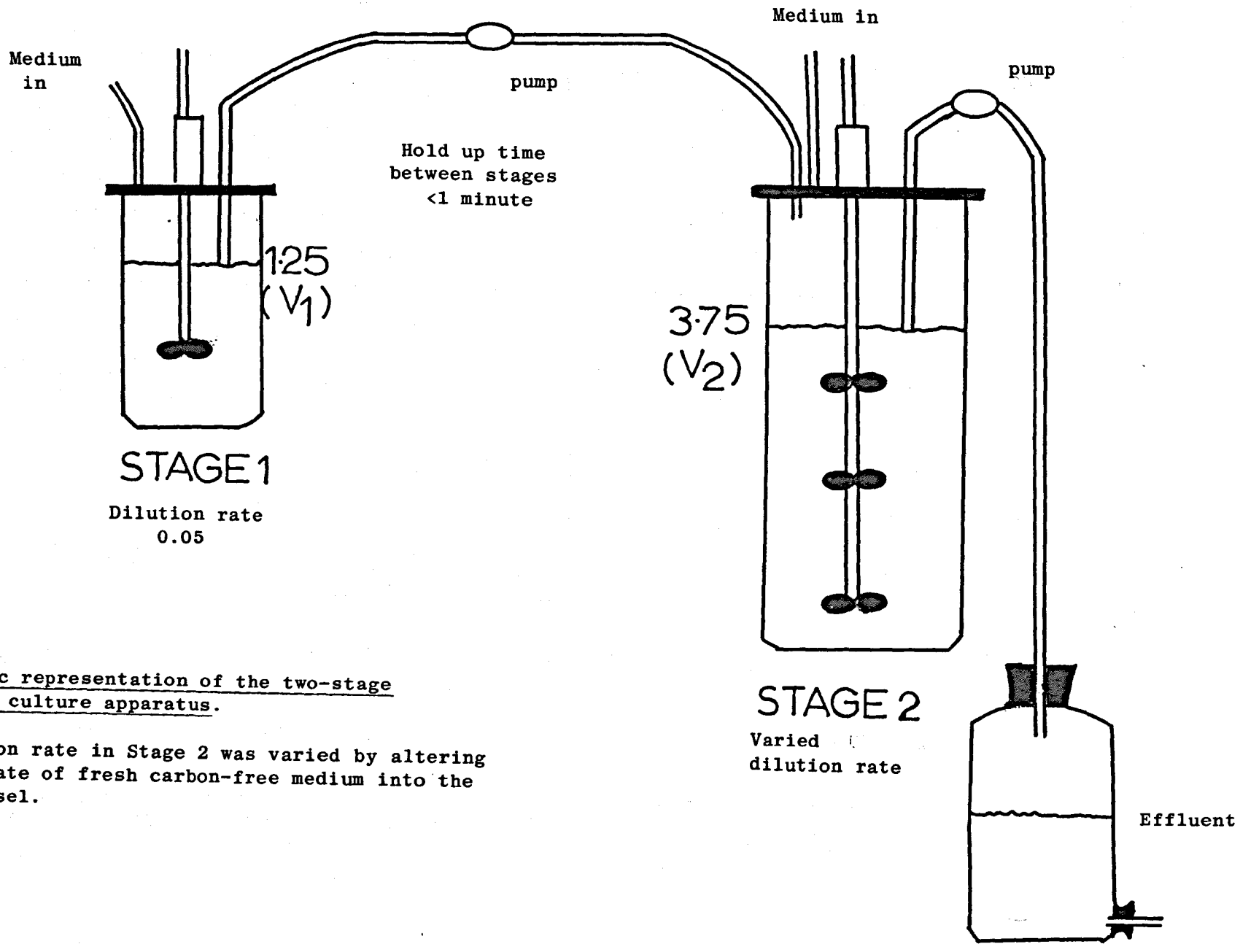


Figure 9.

Diagramatic representation of the two-stage continuous culture apparatus.

The dilution rate in Stage 2 was varied by altering the flow rate of fresh carbon-free medium into the second vessel.

Table 8. Effect of residence time on lipid degradation in Candida curvata D growing in two-stage continuous culture

Vessel	Dil'n rate (h^{-1})	Residence time (h)	Specific growth rate (h^{-1})	Biomass (gl^{-1})	$\frac{f}{f'}$ [b]	Biomass increase (gl^{-1})	% Lipid in biomass (w/w)	Lipid (gl^{-1})	Actual decrease in lipid (gl^{-1})	Increase in biomass per decrease in lipid (g cells/ g lipid)
1[a]	0.05	20	0.05	13.1	-	-	29.5	3.86	-	-
2	0.036	27.8	0.005	7.34	0.48	1.43	4.1	0.3	1.56	0.92 (.34)
2	0.043	23.3	0.01	7.06	0.39	1.69	4.25	0.3	1.04	1.63 (.76)
2	0.053	28.9	0.013	5.95	0.32	1.74	3.0	0.18	1.06	1.64 (.79)
2	0.063	16.1	0.016	5.04	0.27	1.45	4.3	0.22	0.84	1.73 (.65)
2	0.072	13.9	0.021	4.44	0.24	1.25	5.9	0.26	0.68	1.84 (.51)
2	0.077	13.0	0.024	4.01	0.21	1.21	6.1	0.25	0.61	1.98 (.51)

[a] Average values pertaining in vessel 1, acting as a feed vessel over course of run; biomass $\pm 0.7\ gl^{-1}$; lipid per cent $\pm$ 1.5.

[b] $\frac{f}{f'}$ = Dilution factor, where f and f' are the flow rates of culture entering and leaving vessel 2, respectively.

Numbers in brackets (final column) represent values for increase in biomass per decrease in lipid (g cells/ g lipid) corrected for biomass increase due to glucose entering vessel 2 from vessel 1.

It was seen that in going from nitrogen limitation to carbon starvation, C. curvata D was capable of utilising its storage lipid for proliferation.

The total lipid concentration in Vessel 2 was variable with dilution rate and varied from 0.3 gl^{-1} to 0.18 gl^{-1}. There was an increase in biomass due to proliferation at every dilution rate, this being greatest at dilution rate of 0.053 (0.79 gl^{-1}).

The decrease in lipid concentration in Vessel 2 increased with decreasing dilution rate. It appeared that new biomass was made from the lipid which was degraded. Values for the increase in biomass per decrease in lipid concentration are shown, these varied with dilution rate. It should be noted however that the concentration of glucose entering Vessel 2 was 2 gl^{-1} and that leaving was approximately 0.025 gl^{-1}. Therefore approximately 2 gl^{-1} of glucose was utilised in Vessel 2 for cell proliferation. In Vessel 1, glucose was supplied at a concentration of 30 gl^{-1} and the outgoing medium from Vessel 1 had a glucose concentration of 2 gl^{-1}. Therefore approximately 28 gl^{-1} was utilised. As this amount of glucose yielded 13.1 gl^{-1} of biomass, it would follow that 1 gl^{-1} of glucose would yield approximately 0.45 gl^{-1} of biomass.

If values for biomass increase are corrected for increase in biomass due to utilization of residual glucose (i.e. subtract 0.9 gl^{-1}), new values for increase in biomass per decrease in lipid (g cells/g lipid) can be calculated. These are shown in brackets in Table 8.

The highest actual increase in biomass per decrease in lipid (g cells/g lipid) was at a dilution rate of 0.053 h^{-1}; at dilution rates above or below this value there was a lower yield. This is illustrated in Figure 10.

It should be noted that these values did not take into account the degradation of glycogen as described in Section 2.1, and hence actual yield values are probably overestimates.

2.3. Single stage chemostat transition studies

Boulton (1983) noted that in previous studies of the behaviour of oleaginous yeasts grown in continuous culture (Gill et al., 1977; Hall and Ratledge, 1977; Ratledge and Hall, 1979) the organisms had only been investigated after the establishment of a steady state. He suggested that it might be a more fruitful approach to examine the physiological changes that occurred when chemostat cultures underwent transitions from carbon limitation to nitrogen limitation.

This approach was adopted to study the reverse of this transition, i.e. from nitrogen limitation to carbon starvation with the intention of examining a variety of metabolic parameters during transitions and establishing a sequence of events culminating in lipid degradation for cell proliferation and possibly identifying a "trigger" event.

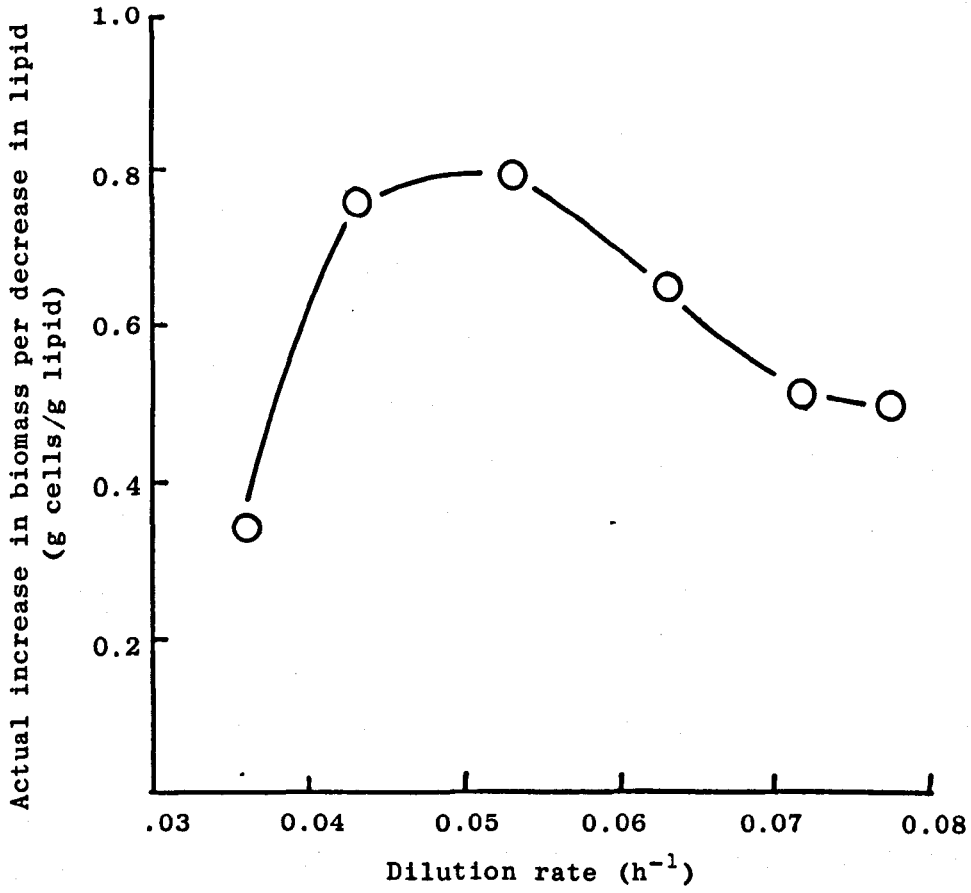
2.3.1. Non-radioactive transition experiment with Candida curvata D

Culture conditions which were used were as described in Methods (6.1.3.). The chemostat was operated at a dilution rate of 0.05 h^{-1}. This was chosen as it gave reasonable biomass and lipid levels. The yeast was originally

FIGURE 10

Variation in the increase in biomass per decrease in lipid (g cells/g lipid) with dilution rate for *Candida curvata D* grown in two-stage continuous culture.

Fig. 10.


Actual increase in biomass per decrease in lipid
(g cells/g lipid)
1.0
0.8
0.6
0.4
0.2
.03
0.04
0.05
0.06
0.07
0.08
Dilution rate (h^{-1})

grown on a nitrogen limited medium and then switched to a carbon starvation medium (Methods 1.2), samples were removed at regular time intervals and analysed for lipid content. The results are shown in Table 9 and Figures 11 and 12.

It was seen that the lipid content of the yeast fell rapidly over the first four hours, going from a value of 30% (w/w) to 21% (w/w); this was followed by a fairly steady drop from 21% to 10% over the next 12 hours, the lipid content then appeared to remain constant at a value of 10%.

The biomass of the yeast fell slowly over the first 1.5 hours compared with the decrease in lipid level. This was followed by a gradual decrease from a value of 12.6 gl^{-1} to 2.7 gl^{-1} over the next 32 hours.

The glucose concentration in the medium fell immediately after transition from nitrogen limitation to carbon starvation to a negligible value. The NH_4^+ concentration took approximately 24 hours to reach a steady state value of 1.5 gl^{-1}.

If a dye were to be injected into a sterile chemostat operating at a dilution rate of Dh^{-1}, and samples were taken at intervals for up to four changes of pot volume ($\frac{4}{D}$ h), then the loss of dye from the chemostat would follow an exponential decline. This curve could also be produced theoretically by plotting e^{-Dt} against t where (D = dilution rate and e^{-Dt}, the fraction of the dye which remained after t hours). If expressed as a semi-logarithmic plot (the natural logarithm of the dye concentration being taken i.e. $\ln(e^{-Dt}) = -Dt$, then a straight line with a slope equal to minus the dilution rate (-D) would be produced.

Table 9. The effect of transition from nitrogen limitation to carbon starvation on Candida curvata D grown in single stage continuous culture

Time after transition (h)	Concentration of glucose in chemostat (gl^{-1})	Concentration of NH_4^+ in chemostat (gl^{-1})	Biomass (gl^{-1})	% Lipid (w/w)
0	1.68	0.0015	13.15	30.5
1.5	0.027	0.00125	12.6	23.6
4.25	0.058	0.18	10.04	21.3
7.33	0.029	0.39	8.41	20.0
9.0	0.023	0.50	7.95	17.3
11.7	0.024	0.71	7.15	14.4
14.83	0.022	0.91	5.95	11.0
17.33	0.021	1.1	5.15	9.6
24.0	0.021	1.44	3.83	10.2
33.0	0.025	1.5	2.66	9.7

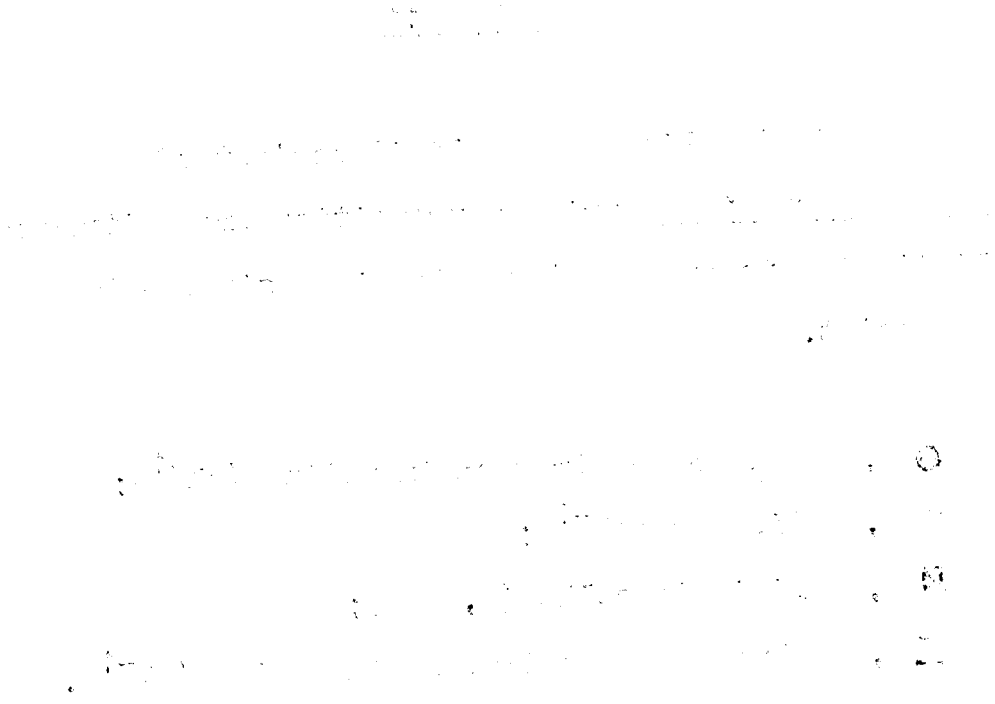


FIGURE 11

Changes in the biomass and lipid content of Candida curvata D during a transition from nitrogen limitation to carbon starvation in a single-stage chemostat.

● , NH_4^+ concentration in medium (gl^{-1});

○ , Biomass (gl^{-1});

■ , Lipid in cells (%, w/w);

□ , Glucose concentration in medium (gl^{-1}).

Fig. 11.

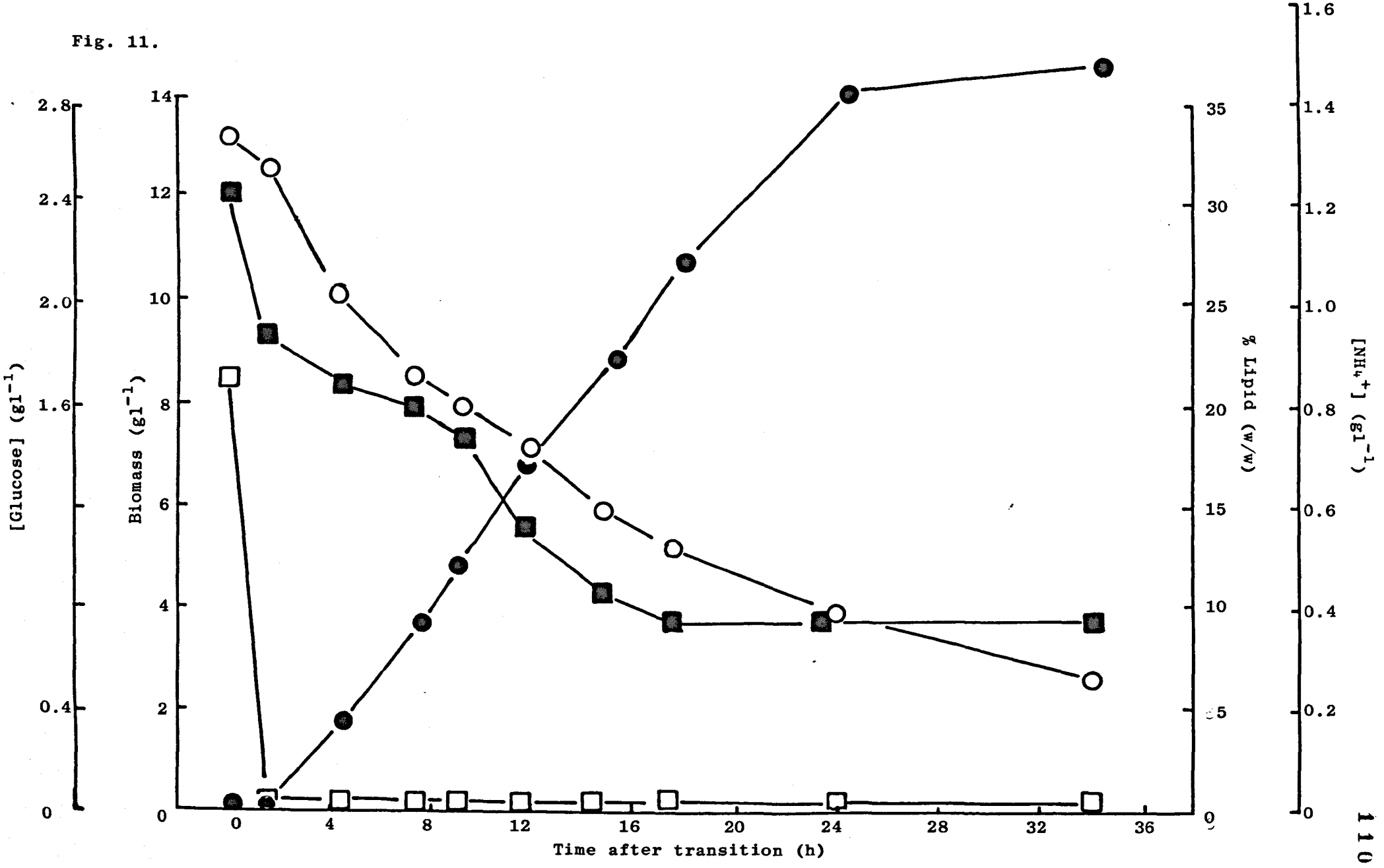

[Glucose] (gl^{-1})
Biomass (gl^{-1})
% Lipid (w/w)
[NH_4^+] (gl^{-1})
Time after transition (h)
0
4
8
12
16
20
24
28
32
36
0
0.4
1.6
2.0
2.4
2.8
2
6
10
14
15
25
30
35
0.2
0.6
0.8
1.0
1.2
1.4
1.6

FIGURE 12

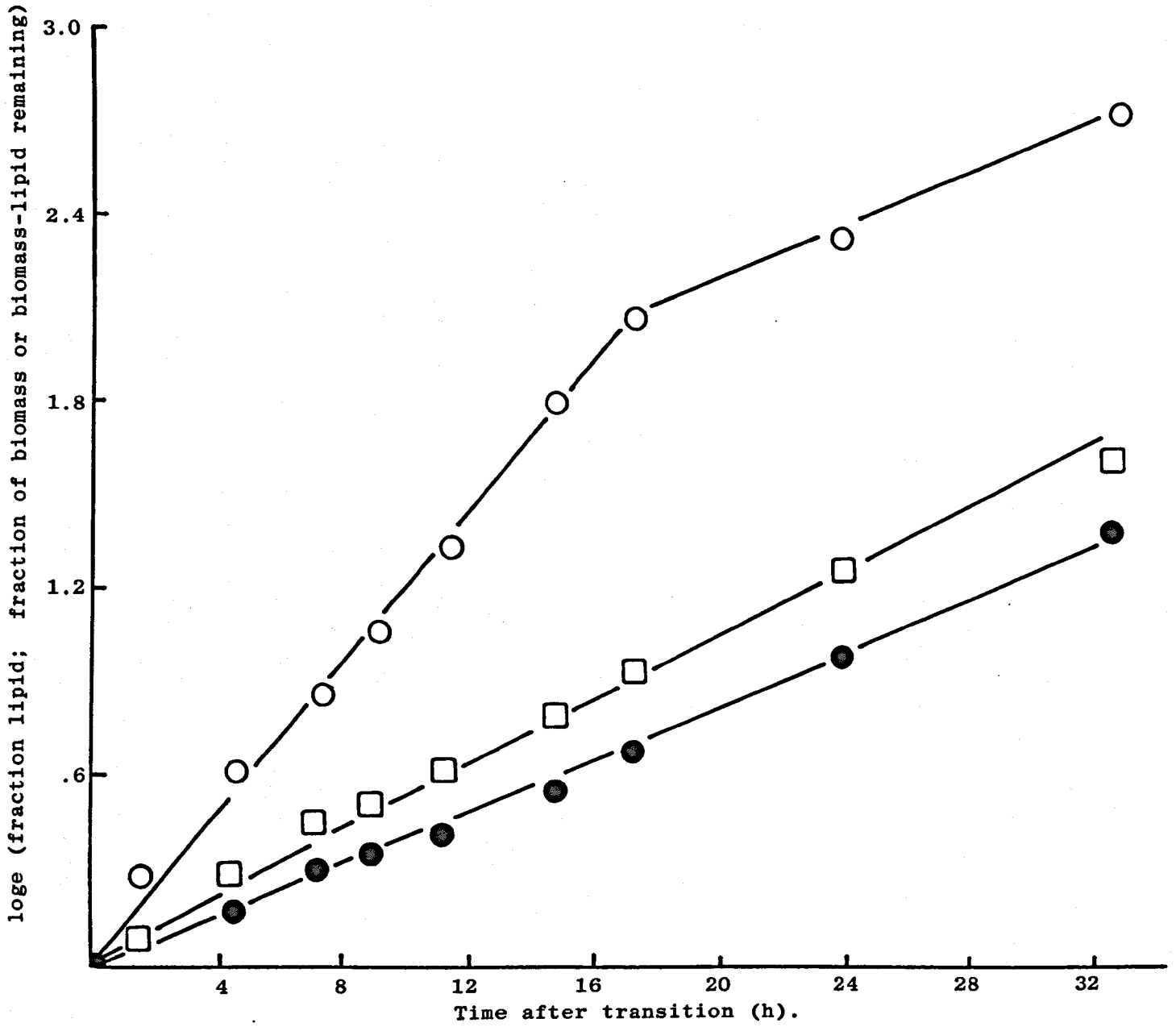
The loss of biomass, lipid and lipid-free biomass from a culture of Candida curvata D grown in a chemostat at time intervals after transition from nitrogen limitation to carbon starvation conditions.

○ , Lipid, slopes, 0·042 and 0.12;

□ , Biomass, slope, 0.048;

● , Lipid-free biomass, slope 0.04.

Fig. 12.


loge (fraction lipid; fraction of biomass or biomass-lipid remaining)
3.0
2.4
1.8
1.2
.6
4
8
12
16
20
24
28
32
Time after transition (h).

Similarly therefore, the wash-out rate of cells within a chemostat could be predicted as above. This would also show an exponential decline and give a straight line semi-logarithmic plot of natural logarithm of fraction of biomass remaining, against time with slope equal to the dilution rate.

Figure 12 shows a semi-logarithmic plot of the natural logarithm of fraction of total biomass [$\ln(\frac{xt}{xo})$ where x_o = total biomass at time = 0 and xt = total biomass at time t] remaining in the chemostat, and fraction of lipid [$\ln(\frac{lt}{l_o})$, where l_o = lipid concentration gl^{-1}) at time = 0, lt = lipid concentration at time t] against time.

Also plotted is the natural logarithm of the fraction of biomass excluding lipid $\ln(\frac{xt - lt}{xo - lt})$ remaining at any time.

It was seen that the lipid plot was biphasic, the slope of the first phase being 0.12, the second being 0.041. The dilution rate (D) of the chemostat was 0.05 h^{-1}. Therefore, over the first 17 hours lipid disappeared from the chemostat much faster than would be effected by wash-out.

The total biomass plot showed a slope of 0.048 which was very close to the theoretical value of wash-out of 0.05, however, when the fraction of biomass (excluding lipid) remaining at each time was plotted, it was seen that the slope was 0.04, much slower than theoretical wash-out. Therefore it would appear that C. curvata D was degrading its storage lipid and converting it to biomass during the transition from nitrogen limitation to carbon starvation.

2.3.2. Radioactive transition experiment

The aim of this experiment was to confirm the findings of the non-radioactive transition experiment; namely, that lipid reserves which are utilised under carbon starvation and high nitrogen conditions are used for cell proliferation and not simply oxidised to CO_2 and ATP.

The study was carried out as detailed in Methods 6.1.3.2. [1-^{14}C]Acetate was injected into the chemostat 45 minutes prior to switching from nitrogen limitation to carbon starvation media. The radioactivity in the lipid, total biomass and supernatant samples were determined as detailed in Methods 6.2.

The results are shown in Figure 13. The loss of lipid, biomass and glucose and increase in NH_4^+ concentration throughout the transition showed profiles similar to those obtained in the non-radioactive study, and hence were not presented.

Botham (1978) showed that the injection of carrier-free [1-^{14}C]sodium acetate into a steady state yeast culture, resulted in the uptake and assimilation of the acetate to a metabolic end-product without disturbance of the steady state. He also demonstrated that there was exponential loss of this end-product (lipid) if no metabolism occurred.

Therefore in these studies, it would be expected that the acetate would enter the various lipid pools and simply be lost from the chemostat exponentially if no lipid turnover occurred, or it would be lost more rapidly if lipid turnover for cell proliferation occurred.

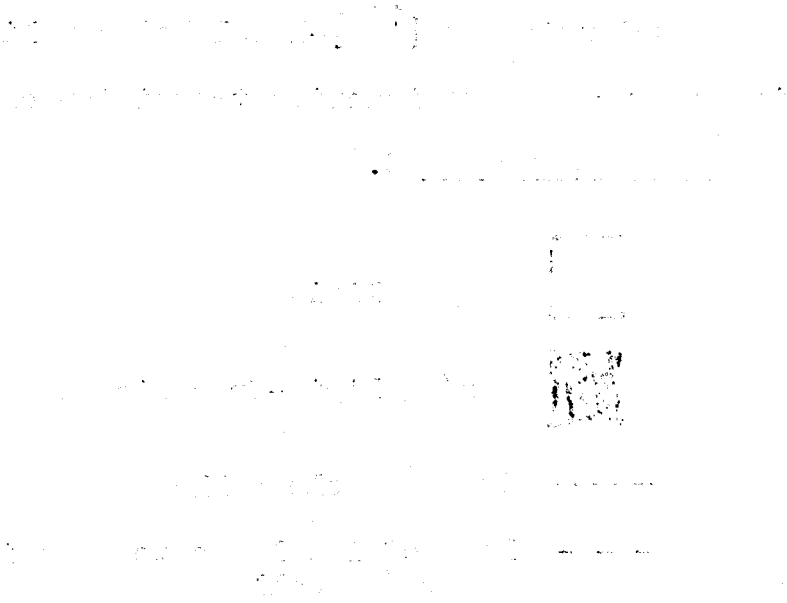


FIGURE 13

The distribution of [^{14}C]-label between lipid and biomass during a radioactive transition experiment with Candida curvata D.

dpm/mg lipid

dpm/mg lipid-free biomass

——— dpm/mg whole cells

- - - loss of label due to sampling (dpm/mg cells)

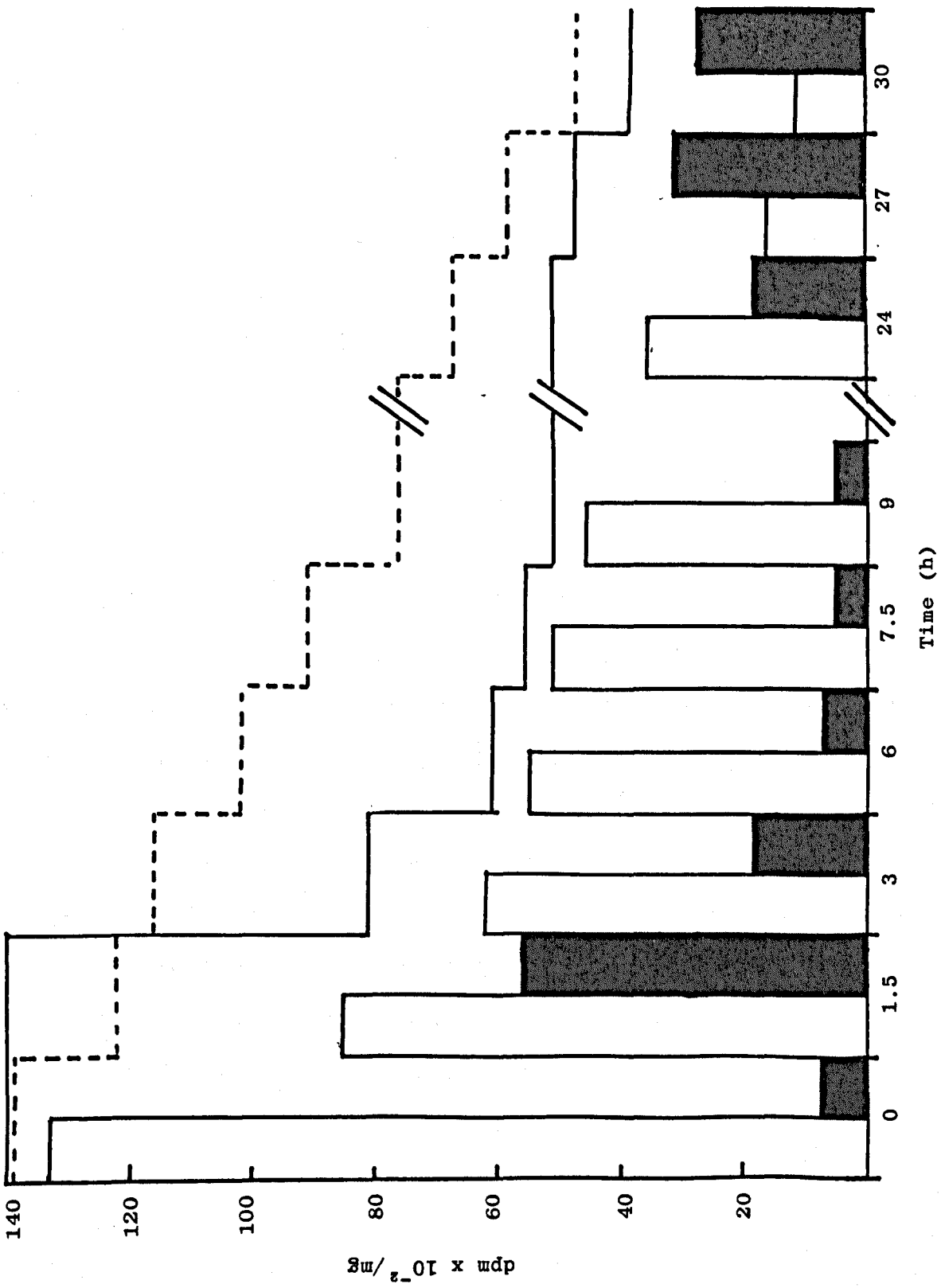


Fig. 13

Figure 13 illustrates that 96% of the presented [1-^{14}C]acetate was incorporated into the lipid during the 45 minute incubation period prior to the experiment starting with the switching of the media. After 1.5 hours (i.e. the first sample) the lipid appeared to have been degraded and only 60% of the total ^{14}C remained in the lipid fraction, the other 40% having been incorporated into biomass. This confirmed that during lipid turnover, the lipid was metabolised and its end-products (e.g. acetate) were incorporated into cell biomass. However, after 1.5 hours, the label in the biomass gradually decreased at a far faster rate than could be accounted for by wash-out. The reason for this was not known, but one possibility is that the labelled lipid was converted to a carbon intermediate which was metabolised as an energy source with the [^{14}C]-label being lost as $^{14}CO_2$. This would seem likely as the total dpm/mg cells in the chemostat was less than the expected dpm/mg (which took into account the loss of label due to sampling) at all times after 1.5 hours. Therefore label was lost from the chemostat in some other way, which could only have occurred as $^{14}CO_2$.

Twenty-four hours after transition, a much greater percentage of the label had been incorporated into the biomass with the final distribution of label being 74% in the total biomass and only 26% in the lipid.

Therefore, although the intermediate fate of the label between 9 and 24 hours was not clear, it was concluded that lipid degradation leading to cell proliferation had occurred.

2.4 Examination of key enzymes in lipid metabolism during transition from lipid accumulating conditions to lipid turnover conditions.

The previous transition studies have demonstrated that lipid turnover is a rapid process occurring within 1.5 hours of switch from nitrogen limitation to carbon starvation. Therefore it would be expected that enzyme activities involved in the conversion of lipid to biomass would be highly controlled within the cell, and that significant changes would occur upon transition. It would therefore be expected that enzymes involved in lipid biosynthesis would be rapidly inhibited and their synthesis repressed whereas those involved in lipid degradation and gluconeogenesis would be activated and, if necessary, have their synthesis derepressed.

Seven enzymes were studied in five oleaginous yeasts growing in two-stage batch culture and two-stage chemostat culture (Methods 6.1.1. and 6.1.2.). In the first stage, lipid was allowed to accumulate and in the second stage carbon starvation conditions prevailed such that endogenous lipid utilisation occurred.

In parallel with these studies, the same enzyme activities were measured in yeasts grown on the exogenous lipid, triolein (glycerol trioleate), as sole carbon source in batch culture. This was an attempt to see if there were any significant differences between growth on an external (exogenous) lipid source and growth on an internal (endogenous) lipid source.

2.4.1. Batch culture studies

The results of the batch culture studies are shown in Table 10. Yeasts were grown for 48 hours in Stage 1 and for 24 hours in Stage 2. Yeasts grown on triolein media were grown for 48 hours. It was not possible to examine Lipomyces starkeyi in this study as it was not able to grow on either its endogenous lipid or on the exogenous lipid source (triolein).

(1) ATP:citrate lyase. This enzyme catalyses the ATP-dependent cleavage of citrate to oxaloacetate and acetyl-CoA. It is considered to occupy a key role in lipogenesis by furnishing acetyl-CoA for fatty acid biosynthesis. From the results, it was clearly seen that in all species of yeast examined there was a definite decrease in activity when grown on either endogenous or exogenous lipid. This result was as expected, as Boulton (1982) has previously reported that fatty acyl CoA esters (FACES) are potent inhibitors of ATP:citrate lyase in L. starkeyi CBS 1809.

In order to ascertain whether or not the decrease in activity was due to inhibition by the FACES or similar regulatory signals, or due to repression of the enzyme, the yeasts were grown on nitrogen-limited media and on trolein media and aliquots of the extracts were mixed and re-assayed for the enzyme.

On mixing equal volumes of the extracts, the activity was found to be half that observed in the nitrogen-limited extract alone. This indicated that the enzyme is probably repressed rather than inhibited by the FACES, as, if inhibition

Table 10. Enzyme Activities in yeasts cultivated on nitrogen limited, carbon starvation and triolein media in batch culture

1(a) *Candida curvata* D

Growth media	ATP: citrate lyase	Carnitine acetyl-transferase	Catalase	$NADP^+$-dependent isocitrate dehydrogenase	Isocitrate lyase	Malic enzyme
Nitrogen limitation (lipid accumulating)	17	239	51×10^3	31	4	111
Carbon starvation (endogenous lipid)	2	189	59×10^3	49	16	85
Triolein (exogenous lipid)	1	722	84×10^3	41	122	0

N.B. All activities are in nmol/min/mg protein.

Table 10 continued...

1(b) Trichosporon cutaneum CBS 40

Growth media	ATP: citrate lyase	Carnitine acetyl-transferase	Catalase	$NADP^+$-dependent isocitrate dehydrogenase	Isocitrate lyase	Malic enzyme
Nitrogen limitation (lipid accumulating)	30	56	51×10^3	71	4	34
Carbon starvation (endogenous lipid)	7	85	64×10^3	95	29	35
Triolein (exogenous lipid)	0	255	70×10^3	103	77	12

N.B. All activities are in nmol/min/mg protein

Table 10 continued ...

1(c) Rhodosporidium toruloides CBS 14

Growth media	ATP: citrate lyase	Carnitine acetyl-transferase	Catalase	$NADP^+$-dependent isocitrate dehydrogenase	Isocitrate lyase	Malic enzyme
Nitrogen limitation (lipid accumulating)	65	95	4×10^3	45	7	37
Carbon starvation (endogenous lipid)	11	92	2×10^3	40	62	20
Triolein (exogenous lipid)	8	469	2×10^3	64	137	21

N.B. All activities are in nmol/min/mg protein

Table 10 continued...

1(d) Rhodosporidium toruloides ATCC 26217

Growth media	ATP: citrate lyase	Carnitine acetyl-transferase	Catalase	$NADP^+$-dependent isocitrate dehydrogenase	Isocitrate lyase	Malic enzyme
Nitrogen limitation (lipid accumulating)	74	96	4×10^3	32	4	18
Carbon starvation (endogenous lipid)	13	101	2×10^3	43	65	25
Triolein (exogenous lipid)	3	785	1×10^3	49	144	0

N.B. All activities are in nmol/min/mg protein

had occurred, less than half or no activity would have been observed.

(2) Malic enzyme. This enzyme catalyses the reversible oxidative deacrboxylation of L-malate to pyruvate and CO_2. It is cytosolic and its putative role in lipogenesis is that it can provide reducing equivalents in the form of NADPH, which can be used for fatty acid biosynthesis.

The results documented in Table 10, show that when the yeasts were grown on endogenous lipid there were small changes in activity (up to a 45% decrease). However when grown on exogenous lipid (triolein), there was a clear decrease in the enzyme activity, and it was undetected in two of the yeasts when grown on triolein.

(3) Carnitine acetyltransferase. The end-product of β-oxidation of fatty acyl-CoA esters is either acetyl-CoA or propionyl-CoA plus acetyl-CoA depending on whether the fatty acyl group has an even or odd number of carbon atoms. Neither compound nor co-enzyme A itself can be transported across the membrane of an organelle, whether it is a peroxisome or a mitochondrion. The presence of carnitine acetyltransferase has been recognised for many years in the mitochondrion of advanced eukaryotic cells whereby the acetyl group is transferred from co-enzyme A to carnitine and the acetylcarnitine is then transported across the membrane.

Carnitine acetyltransferase therefore catalyses the reaction:

Acetyl-CoA + carnitine $\longrightarrow$ Acetyl carnitine + CoA.

As the transport of acetyl carnitine can be in either direction across the mitochondrial membrane, it would be expected that activities of this enzyme would be high during lipolysis i.e. when growing on triolein or endogenous lipid reserves, and low when accumulating lipids, i.e. under nitrogen limitation. Recent work by Ratledge and Gilbert (1985) has shown that the activity of this enzyme can be increased up to 30-fold by growth of yeasts on triacylglycerol.

The results are shown in Table 10 and it can be seen that the activities are low during lipid accumulation and very high during growth on triolein. However, during the utilisation of endogenous reserves, the activities remain low.

(4) Isocitrate lyase. This is one of the key enzymes of the glyoxylate cycle. It catalyses the cleavage of isocitrate to succinate and glyoxylate. It is known to be principally located in the peroxisomes of yeasts (Fukui and Tanaka, 1979a,b) and is an inducible enzyme. It is thought that this enzyme controls or regulates the simultaneous operation of the TCA and glyoxylate cycles.

Activities of the enzyme were examined in the four yeasts. The results are shown in Table 10. There was clear induction of the enzyme in every case during growth on exogenous lipid (triolein) or endogenous lipid (carbon starvation media). The activity of the enzyme was negligible during lipid accumulation. This result indicated the presence of peroxisomes during growth on lipid, whether exogenous or endogenous.

(5) NADP$^+$-Dependent isocitrate dehydrogenase. This enzyme catalyses the reaction:

$$\text{isocitrate} + NADP^+ \longrightarrow \alpha\text{-ketoglutarate} + NADPH + CO_2$$

It is reported by Fukui and Tanaka (1979a,b) to be present in the peroxisomes. They reported a two-fold increase in the activity of the enzyme during growth on alkanes when compared to growth on glucose as sole carbon and energy source.

The activity of this enzyme was studied in the four different yeasts grown in batch culture on glucose, endogenous and exogenous lipid, the results are shown in Table 10.

There was a slight increase in activity during growth on exogenous lipid. No increase in activity was observed during growth on endogenous lipid.

(6) Catalase. This enzyme catalyses the reaction:

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

It is regarded as a marker enzyme for peroxisomes (Fukui and Tanaka, 1979a,b) and, by studying it, it was hoped to establish whether or not these microbodies are formed in oleaginous yeasts utilising endogenous lipid reserves.

The results are shown in Table 10. There was no significant increase in the activity of the enzyme during growth on exogenous or endogenous lipid over that observed during lipid accumulation.

This may indicate that peroxisomes are always present in these yeast cells.

(7) Triacylglycerol lipase. Attempts to study this enzyme in yeast cells during lipid accumulation, growth on endogenous

lipid and growth on exogenous lipid were made. The assay was carried out as detailed in Methods 8, and was performed using cell-free extracts, whole sphaeroplasts, lysed sphaeroplasts and sphaeroplasts prepared in the presence of protease inhibitors (leupeptin 5 mg/l; p-aminobenzamidine, 137 mg/l; and αα-dipyridyl, 10 mM in the sphaeroplasting buffer). The assay was also attempted in the presence of CoA, (1 mM); $MgCl_2$, (15 mM); ATP, (15 mM), and β-mercaptoethanol, (8 mM) but no activity could be observed in any case. The viability of the assay system was tested using a commercial lipase (Sigma type L-1754) and was shown to be fully operational. Therefore no data regarding lipase activity in these yeasts could be generated.

2.4.2. Continuous culture studies

Two-stage continuous culture study of the enzymes examined in batch culture was carried out using the oleaginous yeast C. curvata D. The only enzyme not examined in this system was triacylglycerol lipase.

Enzyme activities were studied at two different dilution rates in the second stage. The results are shown in Table 11.

Observations were similar to those made in two-stage batch culture. ATP:citrate lyase was clearly either repressed or inhibited during lipid utilisation. Carnitine acetyltransferase and isocitrate lyase were both stimulated to a significant extent during lipolysis. Changes in activity of malic enzyme, catalase and $NADP^+$:dependent isocitrate dehydrogenase were similar to those observed in two-stage batch culture in that there was no significant change in their activities during endogenous lipid utilisation.

Table 11. Enzyme activities in Candida curvata D during growth on nitrogen limited media (lipid accumulation) and carbon starvation media (endogenous lipid degradation) in a two-stage chemostat

Growth media and stage	Dilution rate (h^{-1})	ATP: citrate lyase	Carnitine acetyl transferase	Catalase	$NADP^+$-dependent isocitrate dehydrogenase	Isocitrate lyase	Malic enzyme
Stage 1 NLM	0.05	29	50	21×10^3	102	1	24
Stage 2 CSM	0.05	1	220	30×10^3	81	143	20
Stage 2 CSM	0.06	2	99	39×10^3	137	100	36

All activities are in nmol/h/mg protein.

NLM = Nitrogen limited media (steady state);

CSM = Carbon starvation media (steady state).

Chapter 3: Studies of triacylglycerol biosynthesis

The biosynthesis of lipids in oleaginous yeasts, i.e. species possessing ATP:citrate lyase activity and thus producing more than 25% of biomass as lipids, has been studied by Boulton (1982) and Evans (1983). That work concentrated on the events surrounding the production of acetyl-CoA in these organisms.

Attention has now turned to the terminus of lipid biosynthesis and a consideration of how triacylglycerols are synthesized and stored. They are the principal form in which lipid is accumulated in yeasts and so far their biosynthesis in these organisms has only been studied in non-oleaginous yeasts (Christiansen, 1978; Schlossman and Bell, 1978; Belov and Davidova, 1982). Lipid particles are reported to be the site of biosynthesis in Saccharomyces cerevisiae (Christiansen, 1978).

An attempt to study triacylglycerol biosynthesis in two oleaginous yeasts, namely, Candida curvata D and Lipomyces starkeyi CBS 1809 was therefore carried out. The former was selected due to the fact that much work has already been performed on this yeast in our laboratories and elsewhere. The latter was chosen because in earlier studies (Results, Chapter 2) it was noted that this yeast could not degrade its own storage lipid for cell proliferation during carbon starvation conditions, hence it was thought that it may lack a lipase and that the lipid globules may be more stable and thus suitable for triacylglycerol biosynthesis study.

3.1. Preliminary studies

Biosynthesis of triacylglycerol in the lipid particles of S. cerevisiae proceeds via the α-glycerol phosphate pathway (Scheme 3) according to Christiansen (1978).

Initial study of triacylglycerol biosynthesis in oleaginous yeasts followed the work of Christiansen (1978). Yeasts were cultivated as detailed in Methods 7.1 and extracts were prepared by sphaeroplast disruption (Methods, 2.2). Assay methods were as described in Methods 7.4. The work of Christiansen (1978) was performed with the intention of locating the site of triacylglycerol biosynthesis in S. cerevisiae and therefore subcellular fractions were used as protein source in assays. However as no previous study of triacylglycerol biosynthesis had been performed using oleaginous yeasts, much of the preliminary work in this study was performed using crude cell-free extracts prepared by sphaeroplast disruption in order to standardise assay conditions prior to characterizing the system.

3.1.1. Sphaeroplast preparation

Sphaeroplasts were prepared as detailed in Methods 2.2.2. and 2.2.3. using Novozym 234 and Zymolyase 20T. Sphaeroplasts were lysed and centrifuged prior to use in assays as described in Methods 2.2.3. Assays were performed for acyl-CoA synthetase and triacylglycerol biosynthesis by oleate esterification and glycerol 3-phosphate acylation (Methods 7.4). Typical results are shown in Tables 12, 13 and 14.

Table 12. Acyl-CoA synthetase assays

Assays were carried out for 4 minutes and stopped by the addition of 1 ml Dole reagent, 0.35 ml H_2O and 0.6 ml heptane.

Yeast	Acyl-CoA synthetase activity (nmol/min/mg protein)	
	Novozym extract	Zymolyase extract
Candida curvata D	3	7
Lipomyces starkeyi CBS 1809	2	2

Table 13. Oleate esterification studies

(i) With added glycerol 3-phosphate as acyl acceptor

Yeast	Labelled products of assay	nmol [1-^{14}C]oleate incorporated/ h/mg protein	
		Novozym 234 extract	Zymolyase 20T extract
Candida curvata D	Acyl-CoA	122	251
	Phosphatidic acid	49	92
	Diacylglycerol	23	50
	Triacylglycerol	15	15
Lipomyces starkeyi CBS 1809	Acyl-CoA	30	228
	Phosphatidic acid	8	71
	Diacylglycerol	7	0
	Triacylglycerol	33	18

(ii) Without added glycerol 3-phosphate as acyl acceptor

Yeast	Labelled products of assay	nmol [1-^{14}C]oleate incorporated/ h/mg protein	
		Novozym 234 extract	Zymolyase 20T extract
Candida curvata D	Acyl-CoA	120	240
	Phosphatidic acid	14	10
	Diacylglycerol	1	0
	Triacylglycerol	7	20
Lipomyces starkeyi CBS 1809	Acyl-CoA	51	210
	Phosphatidic acid	2	8
	Diacylglycerol	1	0
	Triacylglycerol	2	5

Table 14. Glycerol 3-phosphate acylation studies

Yeast	nmol [2-^{3}H] Glycerol 3-phosphate incorporated/h/mg protein into:			
	Phosphatidic acid		Triacylglycerol	
	Novozym 234 extract	Zymolyase 20T extract	Novozym 234 extract	Zymolyase 20T extract
Candida curvata D	4	9	2	0
Lipomyces starkeyi CBS 1809	4	5	0	0

The Table shows the labelled products of the [2-^{3}H] glycerol 3-phosphate acylation assay as determined by thin layer chromatography on silica gel G plates by comparison with known standards.

Activities determined in lipid particles of S. cerevisiae (Christiansen, 1978) were as detailed in Table 15. Therefore it can be seen that the results obtained for C. curvata D and L. starkeyi CBS 1809 are much lower by comparison.

Both sphaeroplasting enzymes produced sphaeroplasts from the yeasts, however a lower percentage were produced using Zymolyase 20T than using Novozym 234. Triacylglycerol biosynthesis activities recorded were roughly equal for both sphaeroplasting enzymes with the exception of incorporation of [1-^{14}C]oleic acid into acyl-CoA using the oleate esterification method. In this case Zymolyase 20T extracts showed greater incorporation of label than did Novozym 234 extracts. Therefore it was decided to use Novozym 234 in all subsequent preparations.

In order to establish if the activities of triacylglycerol biosynthesis being measured in this experiment were less than expected a theoretical rate for triacylglycerol biosynthesis was calculated. This was done on the basis of previous observations.

Boulton (1982) observed that the specific rate of lipid synthesis in the oleaginous yeast Lipomyces starkeyi CBS 1809 when growing in a chemostat at a dilution rate of 0.15 h^{-1} was: 0.036 g lipid/g fat-free yeast/h. Assuming that all lipid was triacylglycerol (TAG) and that the molecular weight of the TAG was 884.5, then:

$$\text{Specific rate of lipid synthesis} = 40.7\ \mu\text{mole TAG/g/h}$$

Table 15. Triacylglycerol biosynthetic enzyme activities in lipid particles of Saccharomyces cerevisiae (Christiansen, 1978).

Acyl-CoA synthetase

142 nmol [1-^{14}C]oleate incorporated into acyl-CoA/minute/mg protein

Oleate esterification assays (with added acyl acceptor)

Lipid fraction	nmol [1-^{14}C]oleate incorporated/h/mg protein
Phosphatidic acid	200
Diacylglycerol	290
Triacylglycerol	380

Glycerol 3-phosphate acylation

Lipid fraction	nmol [2-^{3}H]Glycerol 3-P incorporated/h/mg protein
Phosphatidic acid	630
Diacylglycerol	150
Triacylglycerol	62

As the lipid content of the yeast was 19.4% (w/w) then:

1 g fat-free yeast = 80.6% of total weight of yeast.

Therefore, for every 1 g fat-free yeast, there will be 1.24 g yeast including lipid.

The total protein content of a yeast is approximately 20% (w/w) of the total cell dry weight, therefore:

Protein in 1.25 g yeast = 0.248 g

$\therefore$ 40.7 µmole TAG/0.248 g protein/h

= 164 nmol TAG synthesized/h/mg protein

= 492 nmol [1-^{14}C] oleic acid incorporated/h/mg protein.

Therefore it was obvious that the enzyme activities detected in the oleaginous yeasts were not sufficient to account for the specific rate of lipid synthesis. The rate of incorporation of [1-^{14}C] oleate into triacylglycerol was only 7% of the expected value.

The activities observed by Christiansen (1978) were in lipid particle sub-cellular fractions of S. cerevisiae and were expressed as nmoles/h/mg protein. The activities recorded for C. curvata D and L. starkeyi CBS 1809 were in crude cell-free extracts, containing protein from every cell component, and were also expressed in nmoles/h/mg protein. Therefore it was thought that expression of activities as specific activity may account for differences seen and that performing assays using subcellular fractions may provide more comparable results.

3.1.2. Sub-cellular fractionation

Sub-cellular fractionations of both C. curvata D and L. starkeyi CBS 1809 were performed as detailed in Methods 3.

However, the pellet (2000 g x 5 minutes) which contained whole cells and debris was discarded and not used in the assays. The fractionation was performed on the crude supernatant. Results were as shown in Tables 16, 17 and 18.

Activities were still low when compared with the known specific rate of lipid synthesis. No incorporation of [2-^{3}H]glycerol 3-phosphate into triacylglycerol occurred in any fraction with either yeast. However incorporation of [1-^{14}C]oleic acid into triacylglycerol was slightly increased in most fractions. Triacylglycerol biosynthesis did not occur exclusively in any fraction. The major subcellular site of acyl-CoA synthetase activity appeared to be the intermediate fraction in L. starkeyi CBS 1809. In C. curvata D very low activity of this enzyme was observed and it was solely in the lipid and microsomal fractions.

Glycerol 3-phosphate acylation was maximal in the lipid fractions of both L. starkeyi CBS 1809 and C. curvata D.

The overall conclusion was that these observed rates of triacylglycerol biosynthesis were not sufficient to account for the known specific rates of lipid synthesis.

3.1.3. Use of yeasts grown under carbon limitation or in complete media in the assay for triacylglycerol biosynthesis

All previous work on triacylglycerol biosynthesis had employed extracts prepared from C. curvata D and L. starkeyi CBS 1809 grown on nitrogen limited media. As little activity had been detected in these extracts, assays were performed using extracts from the yeasts cultivated on carbon-limited and complete media (Methods, 1.2) in an attempt to see if observed activities could be improved.

Table 16. Acyl-CoA synthetase assays

Subcellular fraction	Acyl-CoA synthetase activity (nmol/h/mg protein)	
	Lipomyces starkeyi CBS 1809	*Candida Curvata* D
Intermediate	30	0
Lipid	3	1
Microsomal	3	2
Mitochondrial	2	0

Table 17. Glycerol 3-phosphate acylation studies

Subcellular fraction	nmol [2-^{3}H] Glycerol 3-phosphate, incorporated/h/mg protein into phosphatidic acid	
	Lipomyces starkeyi CBS 1809	*Candida curvata* D
Intermediate	0	9
Lipid	73	12
Microsomal	13	10
Mitochondrial	0	7

Table 18. Oleate esterification studies

(i) With added glycerol 3-phosphate as acyl acceptor

Yeast	Subcellular fraction	nmol [1-^{14}C]oleate incorporated/h/mg protein into:		
		Acyl-CoA and Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	Intermediate	22	10	53
	Lipid	5	2	18
	Microsomal	12	3	21
	Mitochondrial	1	6	12
Lipomyces starkeyi CBS 1809	Intermediate	21	7	20
	Lipid	10	16	42
	Microsomal	31	32	0
	Mitochondrial	4	35	2

(ii) Without added glycerol 3-phosphate as acyl acceptor

Yeast	Subcellular fraction	nmol [1-^{14}C]oleate incorporated/h/mg protein into:		
		Acyl-CoA and Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	Intermediate	19	10	41
	Lipid	4	2	13
	Microsomal	8	4	20
	Mitochondrial	11	2	11
Lipomyces starkeyi CBS 1809	Intermediate	8	7	21
	Lipid	26	9	31
	Microsomal	17	6	18
	Mitochondrial	3	6	6

There was no increase in incorporation of [1-^{14}C]oleate into any class of lipid over that observed when yeasts were grown under nitrogen-limited conditions. Therefore cells grown under nitrogen limitation were used in all subsequent experiments.

3.1.4. Effect of acyl-acceptors other than α-glycerol 3-phosphate on lipid synthesis

The effect of acyl acceptors other than α-glycerol 3-phosphate on the incorporation of [1-^{14}C]oleic acid into phosphatidic acid, diacylglycerol and triacylglycerol was examined using the assay system detailed in Methods 7.4.4. β-Glycerol 3-phosphate, dihydroxyacetone phosphate, 2,3-diphosphoglyceric acid, 3-phosphoglyceric acid and glycerol were all tested at a final concentration of 5 m$\underline{M}$ in the assay mixture. The results are shown in Table 19.

Most acyl acceptors examined showed incorporation of the order seen for α-glycerol 3-phosphate, for this reason α-glycerol 3-phosphate was used as acyl acceptor in all subsequent experiments.

3.1.5. Effect of added triacylglycerols and partial acylglycerols on lipid synthesis by cell-free extracts

A possible reason for the low triacylglycerol synthesizing activity by extracts was that a more hydrophobic environment was necessary for enzyme activity. The effect of adding triacylglycerol (10 m$\underline{M}$), and diacylglycerols (diolein and 1,3-diolein) 2 m$\underline{M}$, was studied with crude cell-free extracts from C. curvata D and L. starkeyi CBS 1809.

Prior to addition to the reaction mixture, the triolein or partial acylglycerols were diluted with bovine serum albumin

Table 19. The effect of substrates other than α-glycerol phosphate on [1-^{14}C]oleate incorporation into phosphatidic acid, diacylglycerol and triacylglycerol by crude cell-free extracts from Candida curvata D (CcD) and Lipomyces starkeyi CBS 1809 (Ls)

Yeast	Substrate	nmol [1-^{14}C]oleate incorporated/h/mg protein into:			
		Acyl-CoA	Phosphatidic acid	Diacylglycerol	Triacylglycerol
CcD	αGP	122	49	23	15
CcD	βGP	76	51	3	18
CcD	DHAP	118	30	0	10
CcD	PGA	0	11	0	7
CcD	2,3 DPGA	137	2	0	22
CcD	G	24	10	1	13
Ls	αGP	30	8	7	33
Ls	βGP	47	12	2	34
Ls	DHAP	10	12	0	22
Ls	PGA	15	13	0	40
Ls'	2,3 DPGA	22	20	10	31
Ls	G	6	14	14	17

Key: αGP = α-glycerophosphate; DHAP = dihydroxyacetone phosphate; G = glycerol; βGP = β-glycerophosphate; 2,3 DPGA = 2,4-diphosphoglyceric acid; PGA = Phosphoglyceric acid

(7% w/v) and dispersed by sonication on ice for 4 x 1 minute with 1 minute intervals of cooling. The results are shown in Table 20.

Addition of triolein gave an apparent depression of oleate incorporation into all classes of lipid in both yeasts. Addition of diolein and 1,3-diolein gave an apparent stimulation of oleate incorporation into diacylglycerol and phosphatidic acid in C. curvata D and an apparent decrease in incorporation into triacylglycerol.

Addition of 1,3-diolein to L. starkeyi CBS 1809 showed a stimulation of incorporation into phosphatidic acid and acyl-CoA but no increase in incorporation into triacylglycerol or diacylglycerol.

Observed activities were, however, still insufficient to account for the known specific rate of lipid synthesis.

3.1.6. Effect of the use of cyclodextrins as emulsifiers for oleate

A recent problem when measuring enzyme activities towards water-insoluble substrates is the choice of emulsifier. In all previous experiments bovine serum albumin stabilized oleate emulsions have been used. Owing to the low activities observed when using these emulsions other emulsifiers were tested to see if activities could be enhanced.

α- and β- cyclodextrins are composed of cyclohexamylose and cycloheptamylose, respectively. α-Cyclodextrin has been shown to bind lipid derivatives below the critical micellular concentration (Yabusaki and Ballou, 1978) and it is thought that the lipid is included within the hydrophobic space of the helical chain.

Table 20. Effect of triacylglycerol and diacylglycerol addition on lipid synthesis by crude cell-free extracts of Candida curvata D and Lipomyces starkeyi CBS 1809

Yeast	Addition to incubation	nmol [1-^{14}C]oleate incorporated/ h/ mg protein into:			
		Acyl-CoA	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	None	122	49	30	15
	Triolein	3	4	0	0
	Diolein	22	57	64	1
	1,3-Diolein	8	111	84	0
Lipomyces starkeyi CBS 1809	None	30	8	7	43
	Triolein	43	0	0	0
	Diolein	197	53	0	43
	1,3-Diolein	263	0	0	33

The use of cyclodextrins as emulsifiers for oleate was examined in the hope that this may provide a more hydrophobic environment for lipid synthesis. The results are shown in Table 21.

The use of both α- and β-cyclodextrins as emulsifiers for oleate showed a considerable stimulation in the incorporation of label into acyl-CoA and a slight stimulation of incorporation into phosphatidic acid in C. curvata D. There was no stimulation of incorporation into diacylglycerol or triacylglycerol.

No stimulation of incorporation of label into any class of lipid was seen for L. starkeyi CBS 1809.

3.1.7. Use of protease inhibitors

The degradation of proteins by endogenous proteases during isolation and purification of enzymes is a common problem, and it was postulated that this was the reason for the inability to reflect the in vivo levels of the enzymes of triacylglycerol biosynthesis in these yeasts.

Protease inhibitors were freshly added to both washing and sphaeroplasting buffers in the following concentrations: leupeptin (5 mg/l), p-aminobenzamidine (137 mg/l) and αα-dipyridyl (10 mM) in isopropanol. Assays for triacylglycerol biosynthesis by oleate esterification were then carried out in the usual manner.

It was seen that although the use of protease inhibitors showed slight enhancement of incorporation of label into acyl-CoA and phospholipids, no such effect was seen for incorporation of [1-^{14}C]oleate into diacylglycerol and triacylglycerol.

Table 21. Effect of the use of cyclodextrins as emulsifiers for oleate on lipid synthesis in crude cell-free extracts of Candida curvata D and Lipomyces starkeyi CBS 1809

Yeast	Emulsifier	nmol [1-^{14}C] oleate incorporated/ h/mg protein into:			
		Acyl-CoA	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	Bovine serum albumin	122	49	30	15
	α-Cyclodextrin	735	97	0	1
	β-Cyclodextrin	499	158	6	1
Lipomyces starkeyi CBS 1809	Bovine serum albumin	30	8	7	43
	α-Cyclodextrin	2	6	4	50
	β-Cyclodextrin	0	4	0	40

Therefore, it was concluded that proteolysis was not the cause of failure of the enzyme assays.

3.1.8. Use of whole sphaeroplasts as protein source in triacylglycerol biosynthesis assays

Sphaeroplasts were prepared as detailed in Methods 2.2.2. However, unlike previous experiments, they were not lysed prior to use in assays. The results are shown in Table 22.

Activities of acyl-CoA synthetase were sufficient not to limit the flow of carbon through the rest of the pathway.

The rates of incorporation of label into the intermediates of the pathway of triacylglycerol metabolism were much greater than those observed when using cell-free extracts. Lysis of the sphaeroplasts would occur when they were added to the assay mixture due to osmotic shock. Therefore the increase in activity was not due to the retention of intact cells. It was postulated that there could be an association between the heavier cell components and the nucleus such that even when extracts were spun at 2000xg for 5 minutes, these were sedimented leading to loss of activity.

It would be expected that the rate of incorporation of [U-^{14}C]glycerol 3-phosphate into lysophatidic acid would be the same as the rate of incorporation of [1-^{14}C]oleate incorporation; for phosphatidic acid and diacylglycerol it would be expected to be halved and for triacylglycerol only 33%, due to the fact that 3 molecules of fatty acid would combine with one molecule of glycerol 3-phosphate to produce triacylglycerol.

Comparison of Tables 22(b) and 22 (c) shows that within the limits of experimental error, the results comply with this stoichiometry.

Table 22. Assay for the enzymes of triacylglycerol biosynthesis in whole sphaeroplast preparations of Candida curvata D and Lipomyces starkeyi CBS 1809 under conditions of lipid accumulation

(a) Fatty acyl-CoA synthetase assay

Yeast	nmol [1-^{14}C]oleate incorporated into acyl-CoA/h/mg protein
Candida curvata D	510
Lipomyces starkeyi CBS 1809	246

(b) Oleate esterification studies

Yeast	Plus or minus added acyl acceptor	nmol [1-^{14}C]oleate incorporated/h/mg protein into:				
		Acyl-CoA	Lysophosphatidic acid	Phosphatidic acid	Diacyl-glycerol	Triacyl-glycerol
Candida curvata D	+	505	295	339	148	117
	-	630	39	23	0	37
Lipomyces starkeyi CBS 1809	+	63	12	25	7	68
	-	19	16	0	0	10

(c) Glycerol 3-phosphate acylation studies

Yeast	nmol [U-^{14}C]glycerol 3-phosphate incorporated/h/mg protein into:			
	Lysophosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	120	121	74	61
Lipomyces starkeyi CBS 1809	10	18	2	21

Having established a system for assaying the enzymes of triacylglycerol biosynthesis it was possible to examine the characteristics of the system.

3.2. Characteristics of triacylglycerol biosynthesis in oleaginous yeasts

3.2.1. Use of whole sphaeroplasts and lysed sphaeroplasts to establish a time scale for the incorporation of [1-^{14}C]-oleate into the intermediates of triacylglycerol biosynthesis

Assays were carried out for time intervals of 10, 20, 30 and 45 minutes using both whole sphaeroplasts and sphaeroplasts that had been lysed prior to addition to the assay mix (Methods 2.2.3.). Incorporation of [1-^{14}C]oleate into the intermediates triacylglycerol biosynthesis using added acyl acceptor was recorded for both C. curvata D and L. starkeyi CBS 1809. The results are shown in Figures 14 and 15.

C. curvata D showed a linear incorporation of label into both diacylglycerol and triacylglycerol over 45 minutes using whole and lysed sphaeroplasts. Incorporation of label into acyl-CoA and phosphatidic acid was not linear, but it occurred over a much faster time scale such that it did not limit flow of label through the pathway. A small and almost constant amount of label was incorporated into lysophosphatidic acid. No significant difference was seen between use of whole sphaeroplasts and lysed sphaeroplasts, confirming that centrifugation at 2000xg for five minutes was sedimenting a cell component necessary for triacylglycerol biosynthesis and that intact cells were not necessary for the biosynthetic pathway to proceed.

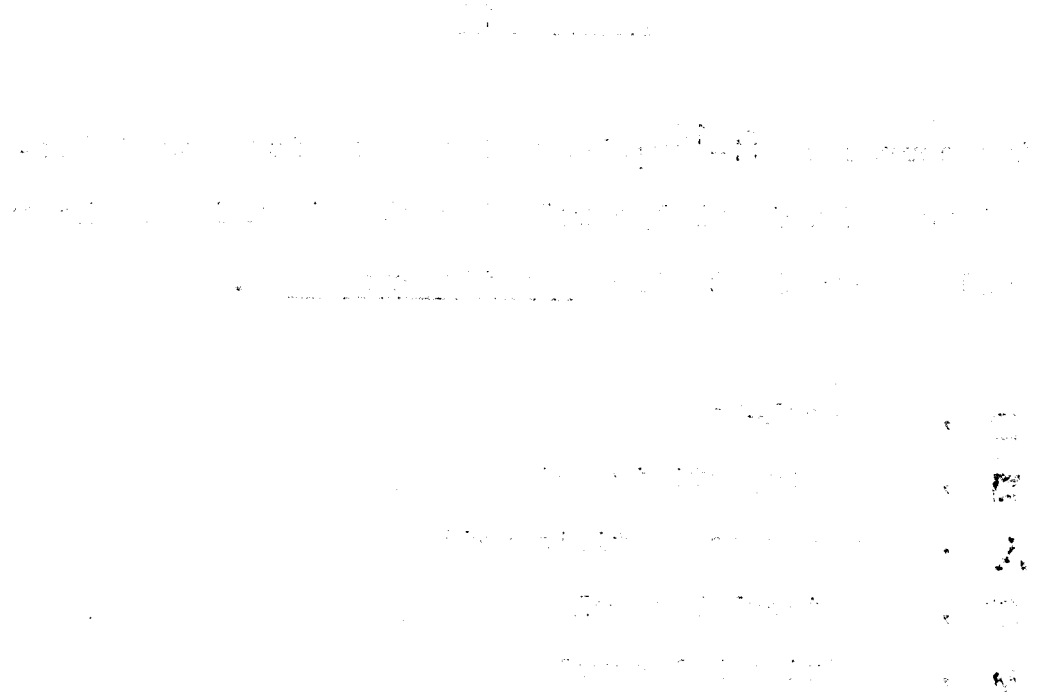


FIGURE 14

Time course of [1-^{14}C]oleate incorporation into intermediates of triacylglycerol biosynthesis using whole and lysed sphaeroplasts from Candida curvata D.

□ , Acyl-CoA

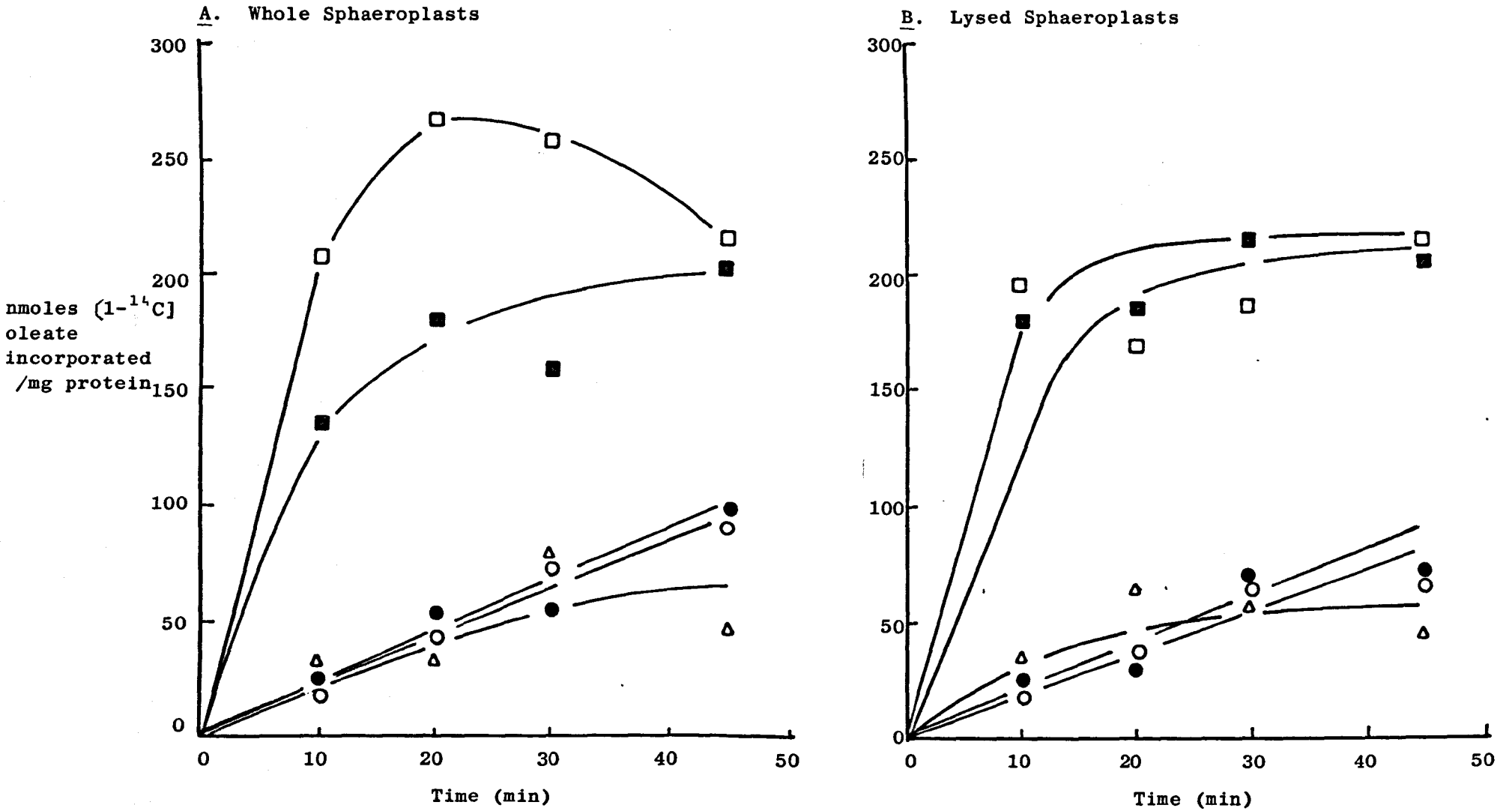
■ , Phosphatidic Acid

△ , Lysophosphatidic acid

○ , Diacylglycerol

● , Triacylglycerol

Fig. 14.


A. Whole Sphaeroplasts
B. Lysed Sphaeroplasts
nmoles [1-14C] oleate incorporated /mg protein
300
250
200
150
100
50
0
0
10
20
30
40
50
Time (min)

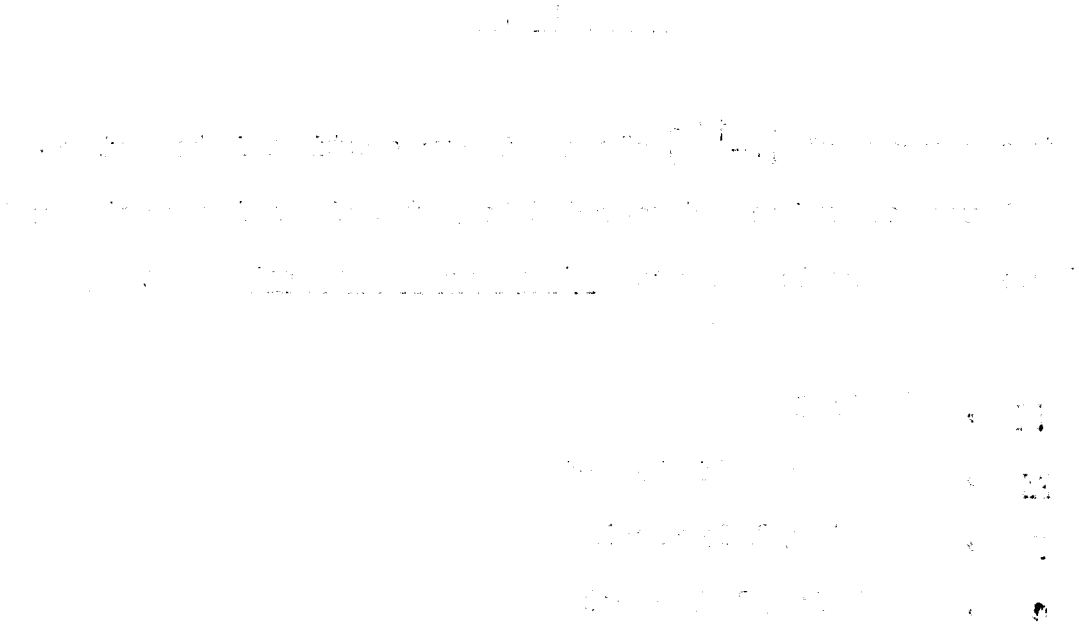


FIGURE 15

Time course of [1-^{14}C] oleate incorporation into intermediates of triacylglycerol biosynthesis using whole and lysed sphaeroplasts from Lipomyces starkeyi CBS 1809

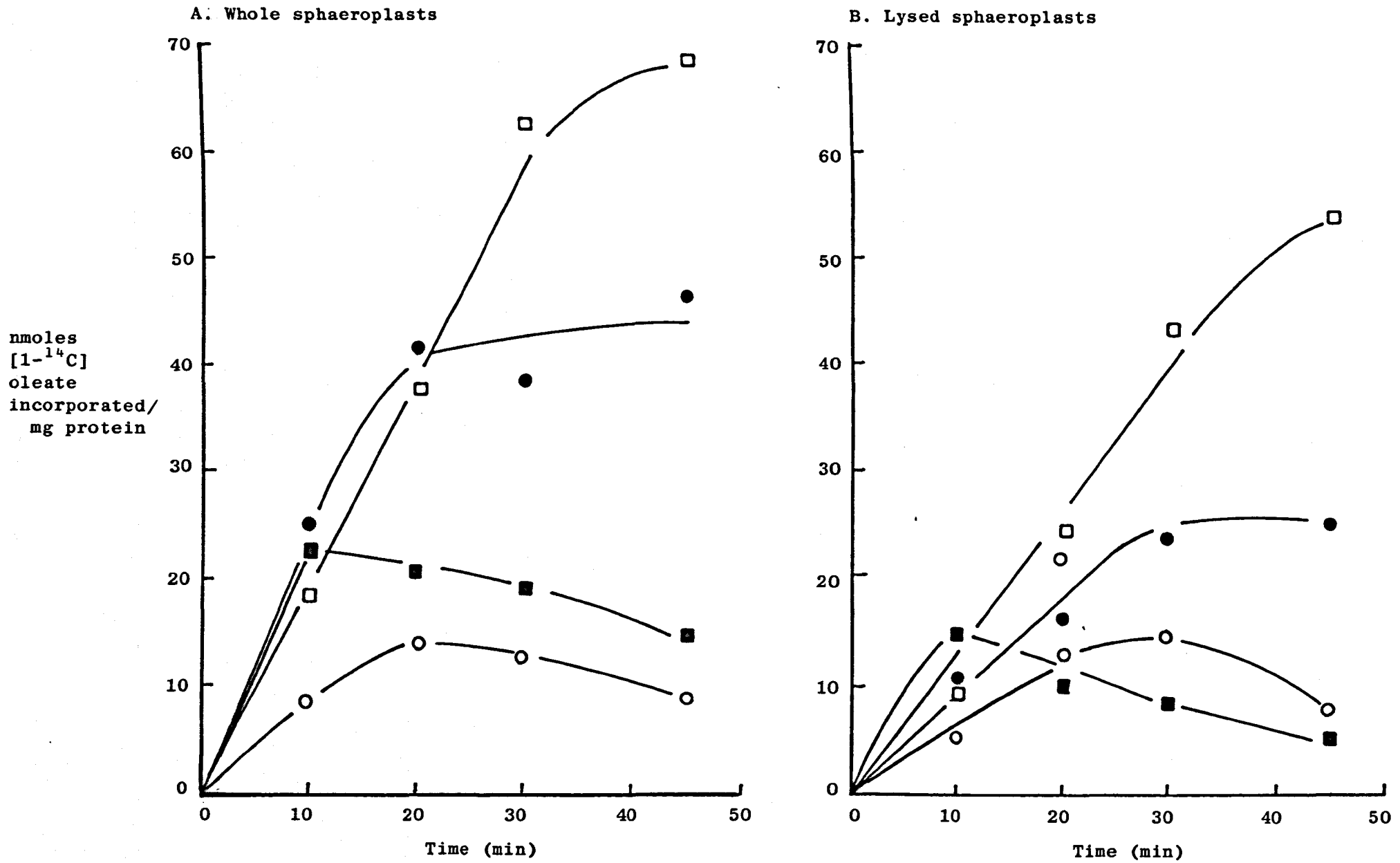
□ , Acyl-CoA

■ , Phosphatidic acid

○ , Diacylglycerol

● , Triacylglycerol

Fig. 15.


A. Whole sphaeroplasts
B. Lysed sphaeroplasts
nmoles [1-^{14}C] oleate incorporated/ mg protein
0
10
20
30
40
50
60
70
Time (min)

L. starkeyi CBS 1809 also showed linear incorporation of label into both diacylglycerol and triacylglycerol over a time interval of 30 minutes. The rate of incorporation of [1-^{14}C]oleate was much slower than that observed for C. curvata D, and again incorporation of label into phosphatidic acid and acyl-CoA did not appear to limit the process.

Therefore a time interval of 30 minutes was selected for all subsequent assays for triacylglycerol biosynthesizing enzymes using either yeast.

3.2.2. Assay of pellet and cell-free extracts of C. curvata D and L. starkeyi in an attempt to determine the location of triacylglycerol biosynthetic activity

It was postulated that there may be a co-operative effect between constituents of pellet and extract fractions, such that both are necessary for triacylglycerol biosynthesis to occur. Therefore enzymes of the biosynthetic pathway were assayed in cell-free extracts, pellets, whole sphaeroplasts and mixtures of cell-free extract and pellet (containing 0.1 mg protein from each fraction). All assays were performed using added glycerol 3-phosphate as acyl acceptor. The results are shown in Table 23.

C. curvata D showed most activity in the pellet fraction. However cell-free extracts showed greater incorporation of label into acyl-CoA than the pellet fractions did. Mixtures of equal amounts of protein from cell-free extracts and pellets showed activities approximately equal to sum of the individual activities, thus demonstrating that there was no enhancement of rate due to co-operation between the two fractions.

Table 23. Demonstration of triacylglycerol biosynthesis activity in pellet and cell-free extracts of Candida curvata D and Lipomyces starkeyi CBS 1809

Sample	nmol [1-^{14}C]oleate incorporated/ h/mg protein into:				
	Acyl-CoA	Lyso phosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D					
cell-free extract (CFE)	518	57	146	57	30
pellet	391	41	176	303	151
whole sphaeroplasts	350	38	130	63	112
pellet + CFE	357	37	358	68	81
Lipomyces starkeyi CBS 1809					
cell-free extract (CFE)	116	30	22	5	64
pellet	53	35	20	4	25
whole sphaeroplasts	63	12	25	7	68
pellet + CFE	72	31	58	14	42

Whole sphaeroplasts were prepared as detailed in Methods 2.2, cell-free extracts and pellet fractions were the supernatant and pellet, respectively, resulting from a 2000xg centrifugation of lysed sphaeroplasts. Assays employing both pellet and CFE as protein source contained 0.1 mg protein from each fraction.

L. starkeyi CBS 1809 showed similar results to those of C. curvata D. However, greater activity was exhibited in the cell-free extract than in the pellet. Again no co-operative effect was seen between pellet and extract.

3.2.3. Use of gentle homogenisation of sphaeroplasts to produce extracts with high triacylglycerol biosynthesizing activity

In an attempt to produce a cell-free extract with triacylglycerol biosynthesizing activity, gentle separation of the cell components was brought about by the use of a Potter homogeniser prior to sedimentation of nuclei and whole cells by centrifugation at 2000xg for 5 minutes (Methods 3.1.). Assays were performed on extracts which had been treated with 10 strokes of the homogeniser and with 20 strokes using both endogenous and added (exogenous) glycerol 3-phosphate as acyl acceptor. Pellet, extract and whole sphaeroplasts were assayed. The results are shown in Table 24.

Ten strokes of the Potter homogeniser resulted in approximately equal amounts of activity in pellet and cell-free extract for both yeasts. However 20 strokes of the homogeniser resulted in loss of incorporation of label into triacylglycerol and diacylglycerol in the extract of both yeasts and also in the pellet in the case of L. starkeyi CBS 1809. One possible explanation was that the greater number of strokes resulted in actual homogenisation of the cells rather than gentle separation of the subcellular components which might have been affected by the lower number of strokes. In all subsequent work, ten strokes of the Potter homogeniser were used to prepare cell-free extracts from the yeasts.

Table 24. Use of gentle homogenisation of sphaeroplasts to produce cell-free extracts with triacylglycerol biosynthesizing activity

(a) Using 10 strokes of the homogeniser

Yeast	Sample	nmol [1-^{14}C]oleate incorporated/h/mg protein into:				
		Acyl-CoA	Lysophosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	CFE + G3P	5	0	137	100	66
	CFE - G3P	242	15	9	24	38
	Pellet + G3P	0	28	101	70	49
	Pellet - G3P	160	14	4	48	64
Lipomyces starkeyi CBS 1809	CFE + G3P	0	3	8	32	114
	CFE - G3P	7	6	0	41	46
	Pellet + G3P	0	0	5	64	107
	Pellet - G3P	0	7	0	41	71

Continued ...

Table 24 Continued

(b) Using 20 strokes of the homogeniser

Yeast	Sample	nmol [1-^{14}C]oleate incorporated/h/mg protein into:				
		Acyl-CoA	Lysophosphatidic acid	Phosphatidic acid	Diacyl-glycerol	Triacyl-glycerol
Candida curvata D	CFE + G3P	219	32	5	12	7
	CFE - G3P	102	19	2	2	2
	Pellet + G3P	52	20	35	156	88
	Pellet - G3P	176	49	0	2	18
Lipomyces starkeyi CBS 1809	CFE + G3P	10	0	0	0	17
	CFE - G3P	79	2	0	5	5
	Pellet + G3P	35	1	5	0	0
	Pellet - G3P	64	5	1	0	2

3.2.4. Assay for triacylglycerol biosynthesizing activity in strains of Saccharomyces cerevisiae

This experiment was performed in order to generate some data relating to a non-oleaginous yeast. All the work of Christiansen (1978) was performed using subcellular fractions (Table 16) and hence could not be directly compared with the results obtained for oleaginous yeasts in the previous experiments.

Triacylglycerol biosynthesizing enzymes were examined in three strains of S. cerevisiae. Yeasts were grown as detailed in Methods 7.1. and sphaeroplasts were prepared (Methods 2.2.1.). Whole sphaeroplasts were used as protein source in all assays. Assays were performed with and without added glycerol 3-phosphate (G3P) as acyl acceptor. The results are shown in Table 25.

The results were an average of two separate determinations, and there appeared to be a significant difference between the three species examined.

The activities observed using S. cerevisiae NCYC 24860 were similar to those obtained using C. curvata D (Table 22); those with S. cerevisiae NCYC 87 were similar to the results with L. starkeyi CBS 1809. The reason for the apparent lack of activity in S. cerevisiae NCYC 1006 was not known, however it did underline the fact that there were significant variations even between strains of the same species.

Activities of acyl-CoA synthetase showed a similar variation between strains - the highest activity being observed in S. cerevisiae NCYC 24860, the lowest in S. cerevisiae NCYC 1006.

Table 25. Assay for the enzymes of triacylglycerol biosynthesis by the incorporation of [1-^{14}C]oleic acid using whole sphaeroplasts prepared from three strains of S. cerevisiae

Fatty acyl-CoA synthetase assays

Yeast	nmol [1-^{14}C]oleic acid incorporated into acyl-CoA/min/mg protein
S. cerevisiae NCYC 87	29
S. cerevisiae NCYC 1006	5
S. cerevisiae NCYC 24860	87

Oleate esterification assays

Yeast	Plus or minus added acyl acceptor	nmol [1-^{14}C]oleic acid incorporated/h/mg protein into:				
		Acyl-CoA	Lysophosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
S. cerevisiae NCYC 87	+	206	5	55	16	13
	-	447	9	0	10	5
S. cerevisiae NCYC 1006	+	80	1	2	0	0
	-	91	0	0	0	0
S. cerevisiae NCYC 24860	+	144	3	756	78	208
	-	593	0	28	0	34

3.2.5. Use of various '1-^{14}C labelled' fatty acids in the assay for triacylglycerol biosynthesis in Candida curvata D and Lipomyces starkeyi CBS 1809

[1-^{14}C] Labelled linoleic, palmitic, stearic and oleic acids were used in assays for fatty acyl-CoA synthetase and triacylglycerol biosynthesis. Each fatty acid was bound to bovine serum albumin (fraction V, Sigma) by sonication (4 x 10 seconds) prior to use in assays, thus rendering them more dispersed. Assays were performed using lysed sphaeroplasts with and without added glycerol 3-phosphate as acyl receptor using the oleate esterification assay (Methods 7.4.4.). The results are shown in Tables 26 and 27.

C. curvata D showed similar rates of incorporation of oleic, palmitic and linoleic acids into acyl-CoA and into all intermediates of triacylglycerol biosynthesis. However, stearic acid showed a very low rate of incorporation into stearyl-CoA and hence into all other intermediates of the pathway. One possible reason for this was that due to the insoluble nature of stearic acid it was not readily available to the fatty acyl-CoA synthetase, however the same reasoning could be applied to palmitic acid which showed good activity. Another possibility is that the yeast may lack a fatty acyl-CoA synthetase capable of reacting with stearic acid due to the fact that this fatty acid is normally presented to it in the form of steacyl-CoA by the action of the enzymes of fatty acid oxidation.

L. starkeyi CBS 1809 showed similar results to those of C. curvata D.

Table 26. The use of various [1-^{14}C] labelled fatty acids in the assay for fatty acyl-CoA synthetase in sphaeroplast preparations of Candida curvata D and Lipomyces starkeyi CBS 1809

Yeast	Labelled fatty acid	nmol [1-^{14}C] labelled fatty acid incorporated into acyl-CoA/h/mg protein
Candida curvata D	Linoleic	720
	Oleic	510
	Palmitic	750
	Stearic	90
Lipomyces starkeyi CBS 1809	Linoleic	600
	Oleic	246
	Palmitic	312
	Stearic	0

Table 27. The use of various [1-^{14}C] labelled fatty acids in the assay for triacylglycerol biosynthesis by incorporation of label into intermediates of the phosphatidic acid pathway in sphaeroplast preparations of Candida curvata D and Lipomyces starkeyi CBS 1809

Yeast	Plus or minus added acyl acceptor	Fatty acid	nmol [1-^{14}C] labelled fatty acid incorporated/h/mg protein into:				
			Acyl-CoA	Lysophosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	+	linoleic	120	79	214	16	90
	-	linoleic	47	41	11	22	15
	+	oleic	51	50	218	59	70
	-	oleic	297	97	7	5	11
	+	palmitic	154	82	178	4	70
	-	palmitic	391	56	34	7	38
	+	stearic	23	8	0	5	11
	-	stearic	5	0	1	10	2
Lipomyces starkeyi CBS 1809	+	linoleic	43	7	1	5	31
	-	linoleic	34	1	4	5	22
	+	oleic	52	5	4	17	27
	-	oleic	64	2	3	6	25
	+	palmitic	37	4	10	24	13
	-	palmitic	53	1	7	14	4
	+	stearic	4	3	0	20	17
	-	stearic	5	0	0	12	13

3.2.6. Use of steaoyl-CoA and oleoyl-CoA in the assay for triacylglycerol biosynthesis using [U-^{14}C]glycerol 3-phosphate

Assays were performed using lysed sphaeroplasts from C. curvata D and L. starkeyi CBS 1809 according to Methods 7.4.3.1. The results are shown in Table 28.

C. curvata D did not show a marked increase in activity when using steaoyl-CoA instead of stearic acid and no incorporation of label into triacylglycerol was observed. Similar rates of incorporation of oleic acid and oleoyl-CoA into intermediates of the triacylglycerol pathway were observed for C. curvata D. However incorporation of label into triacylglycerol was lower when using the latter.

L. starkeyi CBS 1809 showed similar results for incorporation of label into lysophosphatidic acid, phosphatidic acid and triacylglycerol using either fatty acid or fatty acyl-CoA. However, incorporation of label into diacylglycerol when using steaoyl-CoA or oleoyl-CoA instead of their respective fatty acids was markedly increased.

3.2.7. Assay for the enzymes of triacylglycerol biosynthesis in sphaeroplast preparations from Candida curvata D and Lipomyces starkeyi CBS 1809 under carbon starvation conditions

Yeasts were starved for 24 hours following growth on nitrogen-limited media prior to preparation of sphaeroplasts for use in assays.

Assays were carried out with and without added glycerol 3-phosphate as acyl acceptor and using [1-^{14}C] esterification as a measure of activity. The results are shown in Table 29.

The activity of fatty acyl-CoA synthetase was much higher in

Table 28. Use of steacyl-CoA and oleoyl-CoA in the assay for triacylglycerol biosynthesis by measuring incorporation of [U-^{14}C]glycerol-3-phosphate in C. curvata D and L. starkeyi CBS 1809

Yeast	Fatty acid or fatty acyl-CoA	nmol [U-^{14}C]glycerol 3-phosphate incorporated/h/mg protein into:			
		Lyso-phosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	Oleic acid	72	225	46	61
	Oleoyl-CoA	13	366	83	15
	Stearic acid	43	24	8	0
	Steacyl-CoA	42	70	4	0
Lipomyces starkeyi CBS 1809	Oleic acid	1	18	0	8
	Oleoyl-CoA	22	15	328	2
	Stearic acid	17	0	0	0
	Steacyl-CoA	30	7	260	0

Table 29. Assay for the enzymes of triacylglycerol biosynthesis in sphaeroplast preparations from Candida curvata D and Lipomyces starkeyi CBS 1809 under carbon starvation conditions

(a) Fatty acyl-CoA synthetase assays

Yeast	nmol [1-^{14}C]oleate incorporated into acyl-CoA/h/mg protein
Candida curvata D	20880
Lipomyces starkeyi CBS 1809	1560

(b) Oleate esterification studies

Yeast	Plus or minus added acyl acceptor	nmol [1-^{14}C]oleate incorporated/h/mg protein into:				
		Acyl-CoA	Lysophosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Candida curvata D	+	76	19	0	0	0
	-	21	18	5	0	8
Lipomyces starkeyi CBS 1809	+	0	0	2	0	11
	-	26	1	3	0	11

both yeasts than those observed during lipid accumulation. However, rates of incorporation of label into the intermediates of triacylglycerol biosynthesis were much slower than those observed in preparations grown under nitrogen limitation and hence accumulating lipid.

3.2.8. Use of subcellular fractions of Candida curvata D in the assay for enzymes of triacylglycerol biosynthesis

Subcellular fractionation of C. curvata D was carried out as detailed in Methods (3). Each fraction was used immediately in assays for fatty acyl-CoA synthetase and the enzymes of triacylglycerol biosynthesis by the incorporation of [1-^{14}C]oleate (oleate esterification) or [U-^{14}C] glycerol 3-phosphate (glycerol 3-phosphate acylation) into the intermediates of the phosphatidic acid pathway. The results are shown in Tables 30, 31 and 32. Figure 16 shows the distribution of protein and units of incorporation of [1-^{14}C]oleate into triacylglycerol between fractions.

The results indicate that the highest rates of incorporation of label into acyl-CoA and phosphatidic acid pathway intermediates were in the pellet fraction which would have contained whole sphaeroplasts and whole cells.

Fatty acyl-CoA synthetase also showed high activity in the lipid fraction and much lower rates in all other fractions.

Assays utilizing [U-^{14}C]glycerol 3-phosphate as acyl acceptor showed the pellet fraction to be the main site of biosynthesis with a small amount of activity in all other fractions, in particular the mitochondrial and intermediate. Assays utilizing [1-^{14}C]oleate showed similar results and it was noted that only negligible activity was detected in the lipid fraction.

Table 30. Assay for fatty acyl-CoA synthetase in subcellular fractions of Candida curvata D

Subcellular fraction	nmol [1-^{14}C]oleate incorporated into acyl-CoA/h/mg protein
Pellet	8760
Mitochondrial	1830
Lipid	6300
Intermediate	1290
Microsomal	75
Soluble supernatant	240

Assay for mitochondrial and microsomal markers

Subcellular fraction	NADPH-cytochrome c reductase (nmol/min/mg prot)	Citrate synthase (nmol/min/mg prot)
Pellet	10	162
Mitochondrial	9	439
Lipid	8	9
Intermediate	9	20
Microsomal	24	24
Soluble supernatant	24	8

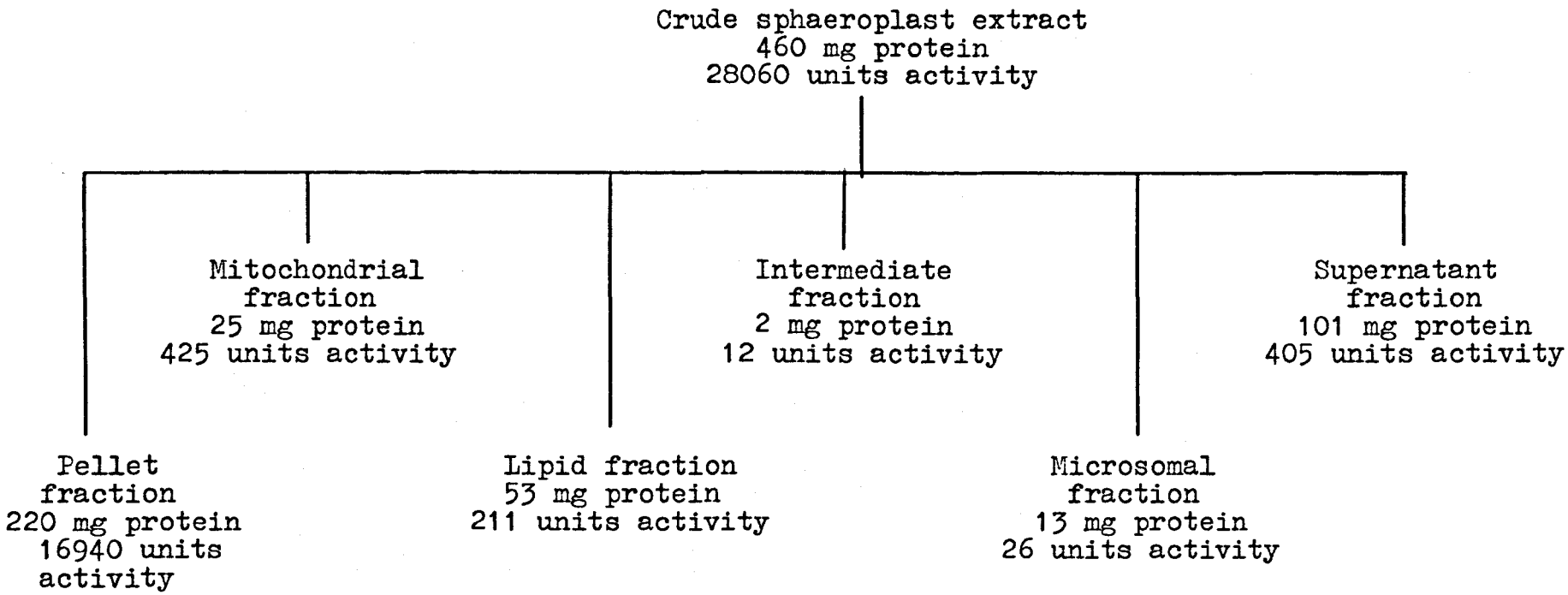
Table 31. Assay for the enzymes of triacylglycerol biosynthesis by the acylation of [U-^{14}C]glycerol 3-phosphate in subcellular fractions of Candida curvata D

Subcellular fraction	nmol [U-^{14}C]glycerol 3-phosphate incorporated/h/mg protein into:			
	Lysophosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Pellet	97	121	27	147
Mitochondrial	0	20	3	3
Lipid	9	0	4	0
Intermediate	13	46	6	2
Microsomal	30	0	0	0
Soluble supernatant	0	0	16	0

Table 32. Assay for the enzymes of triacylglycerol biosynthesis by the esterification of [1-^{14}C]oleate with and wihout added glycerol 3-phosphate as acyl acceptor in subcellular fractions of Candida curvata D

Subcellular fraction	Plus or minus added acyl acceptor	nmol [1-^{14}C]oleate incorporated/h/mg protein into:				
		Acyl-CoA	Lysophosphatidic acid	Phosphatidic acid	Diacylglycerol	Triacylglycerol
Pellet	+	96	359	134	121	77
	-	404	47	17	92	11
Mitochondrial	+	305	159	112	37	17
	-	259	29	5	11	12
Lipid	+	545	60	35	4	4
	-	238	38	6	0	4
Intermediate	+	27	54	0	30	8
	-	217	9	3	15	9
Microsomal	+	33	66	61	0	2
	-	33	5	6	27	3
Soluble supernatant	+	66	17	32	3	4
	-	80	16	35	17	2

Figure 16. Subcellular fractionation of Candida curvata D showing distribution of protein and units of triacylglycerol biosynthesizing activity



1 unit = 1 nmol 1-^{14}C oleate incorporated into triacylglycerol/h

NADPH-cytochrome c reductase was assayed as a marker enzyme for the microsomal fraction. The specific activity of this enzyme did not show an exclusive microsomal origin; however, most activity was detected in the microsomal and soluble supernatant fractions.

Citrate synthase was assayed as mitochondrial marker following sonication of the fractions (2 x 10 seconds). The highest specific activity of this enzyme was found in the mitochondrial fraction with additional high activity in the pellet fraction. Low activity of this enzyme was also detected in lipid, microsomal and soluble supernatant fractions.

Therefore it appeared that triacylglycerol biosynthesis would only occur maximally in extracts containing all cell components. One possibility was that there may be an association between two fractions of the cell, e.g. lipid globules and mitochondria, such that on fractionating the cell, the activity was lost.

This hypothesis was tested by recombining the fractions after fractionation. This resulted in the return of activity of the order seen in whole sphaeroplasts. Combination of two or more of the fractions in any other order did not result in enhancement of the rate. Therefore it was concluded that all fractions were necessary for triacylglycerol biosynthesis to occur. The highest activity was shown in the pellet fraction because this contained whole sphaeroplasts and hence all subcellular fractions.

Chapter 4: Structural Studies

Structural studies were undertaken in order to gain some information about the ultrastructural organisation of oleaginous yeasts. It was also hoped to confirm some of the biochemical observations, e.g. the proliferation of peroxisomes in yeasts utilising triacylglycerol as sole carbon source.

The studies were divided into two parts:

(a) Scanning electron microscopy: It was hoped that this would provide information about the surface properties of lipid globules from oleaginous yeasts and in conjunction with studies of chemical composition of the globules would confirm whether or not these are membrane-bound.

(b) Transmission electron microscopy: It was hoped that by thin sectioning and freeze etching the yeasts it would be possible to gain information about the ultrastructural organisation of the yeasts as a whole.

4.1. Lipid globule studies

Lipid globules were isolated from three oleaginous yeasts (*C. curvata* D; *T. cutaneum* CBS 40; *L. starkeyi* CBS 1809) and purified by flotation as described in Methods Section 3.

4.1.1. Scanning electron microscopy

Globules were fixed with osmium tetroxide (2.5% w/v) and prepared for scanning electron microscopy as described in Methods Section 9.2. The micrographs obtained are shown in the subsequent pages and all details are provided in the legends to each photograph.

The scanning electron microscopy was carried out at Cadbury-Schweppes plc, with the kind help of Jane Heathcock.

Legend to Plate 1

Lipid globules from Candida curvata D isolated after 3 days growth on nitrogen-limited medium (Methods, Section 1.2).

Magnification: x 18,750

Diameter of globule: 3.2 to 3.3 μm

The outer surface of the globules appeared to be rough and laminated in structure.

——— 1 μm.

Legend to Plate 2

Lipid globules from Candida curvata D isolated after 3 days growth on nitrogen-limited medium (Methods, Section 1.2).

Magnification: x 6,000

Diameter of globule: 2.3 to 4.2 μm.

Globules showed significant variation in diameter. The outer surface of globules was rough and laminated. Irregular shaped globules linked the more typical sphaerical lipid bodies.

— 1 μm.

Plate 1

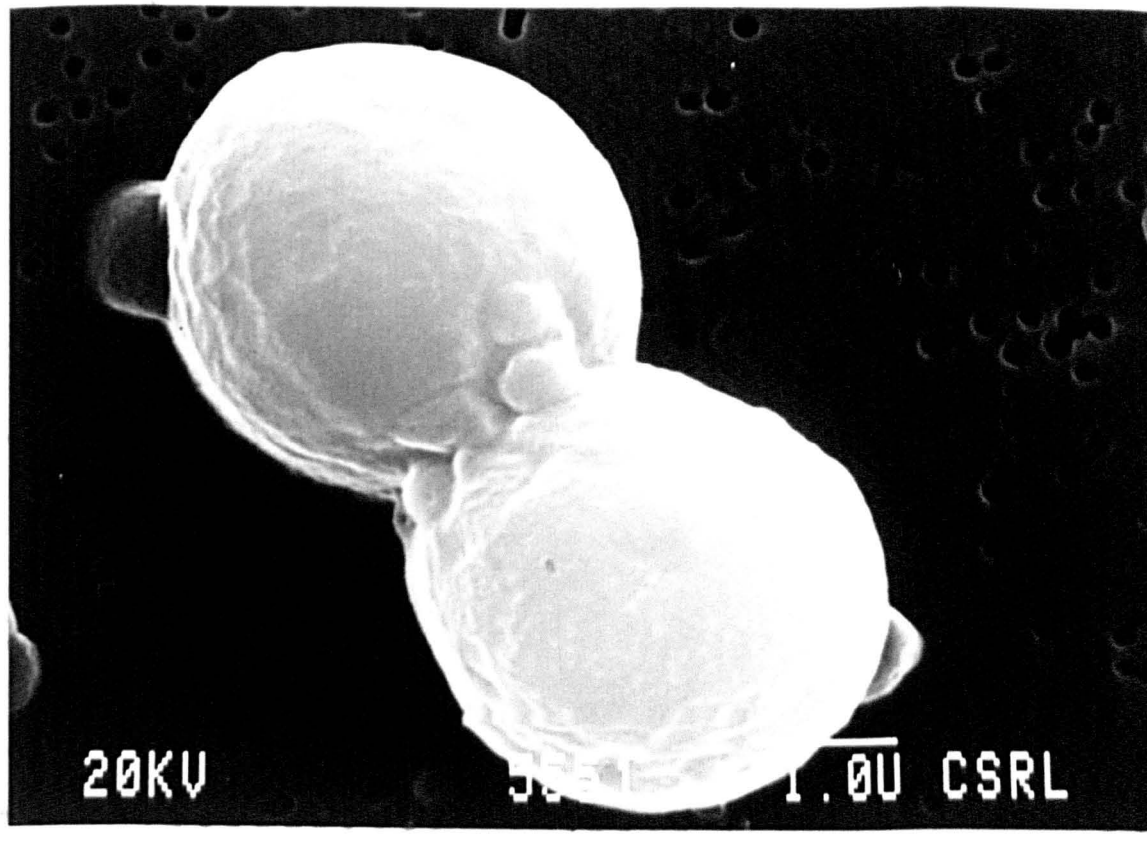
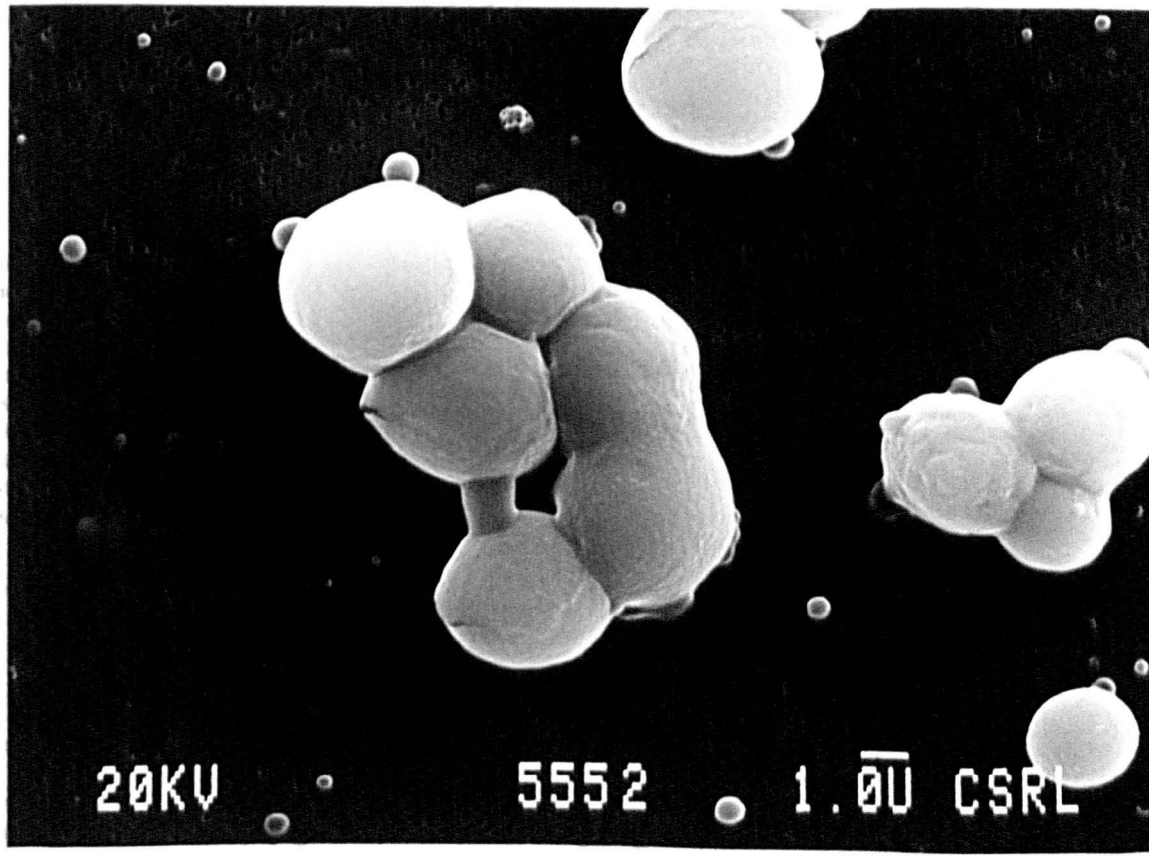


Plate 2



Legend to Plate 3

Lipid globules from Candida curvata D, isolated after 2 days growth on nitrogen-limited medium (Methods, Section 1.2).

Magnification: x 1250

Diameter of globules: 2 to 4 μm

Globules showed significant variation in size and aggregates of globules were also observed.

——— 10 μm.

Legend to Plate 4

Lipid globules from Trichosporon cutaneum CBS 40, isolated after 3 days growth on nitrogen-limited medium (Methods, Section 1.2).

Magnification: x 12,500

Diameter of globules: 3.5 to 5 μm.

Globules appeared to have a rough laminated outer structure similar to that of Candida curvata D.

——— 1 μm.

Plate 3

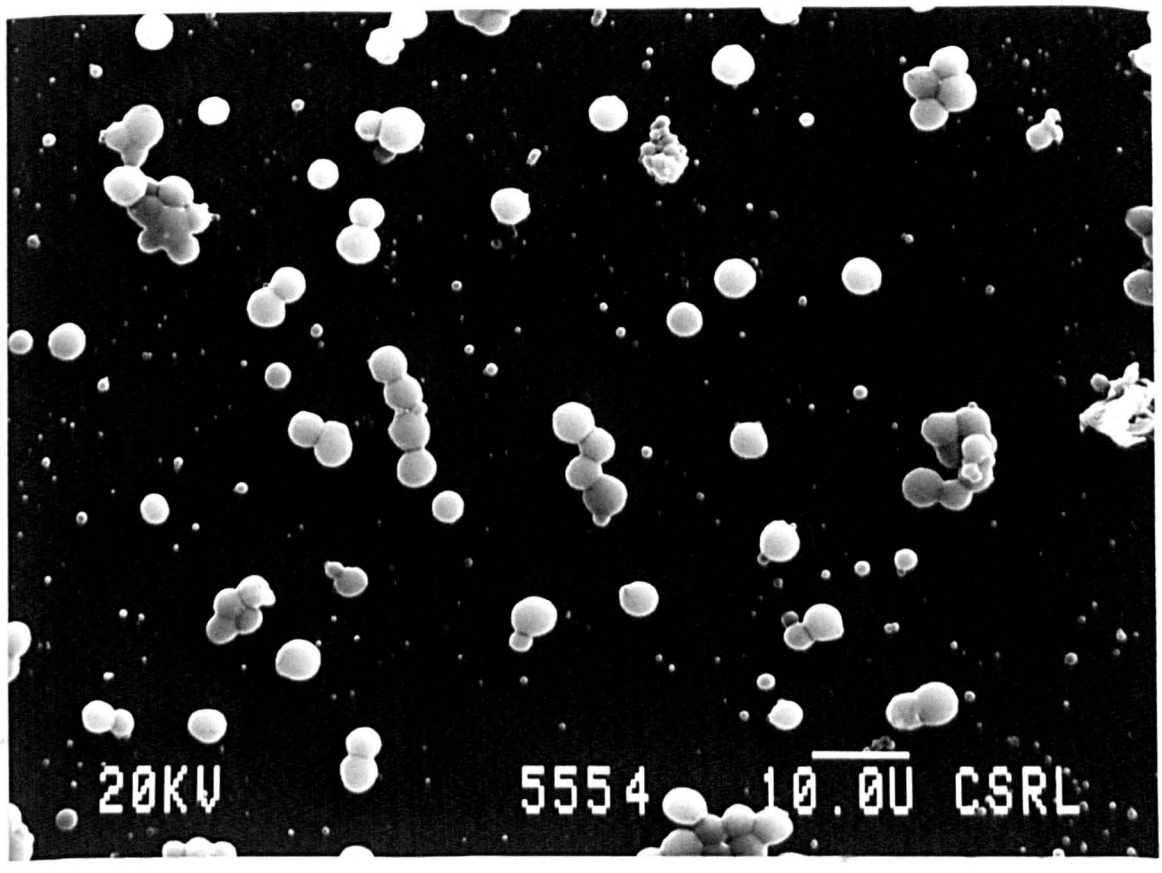
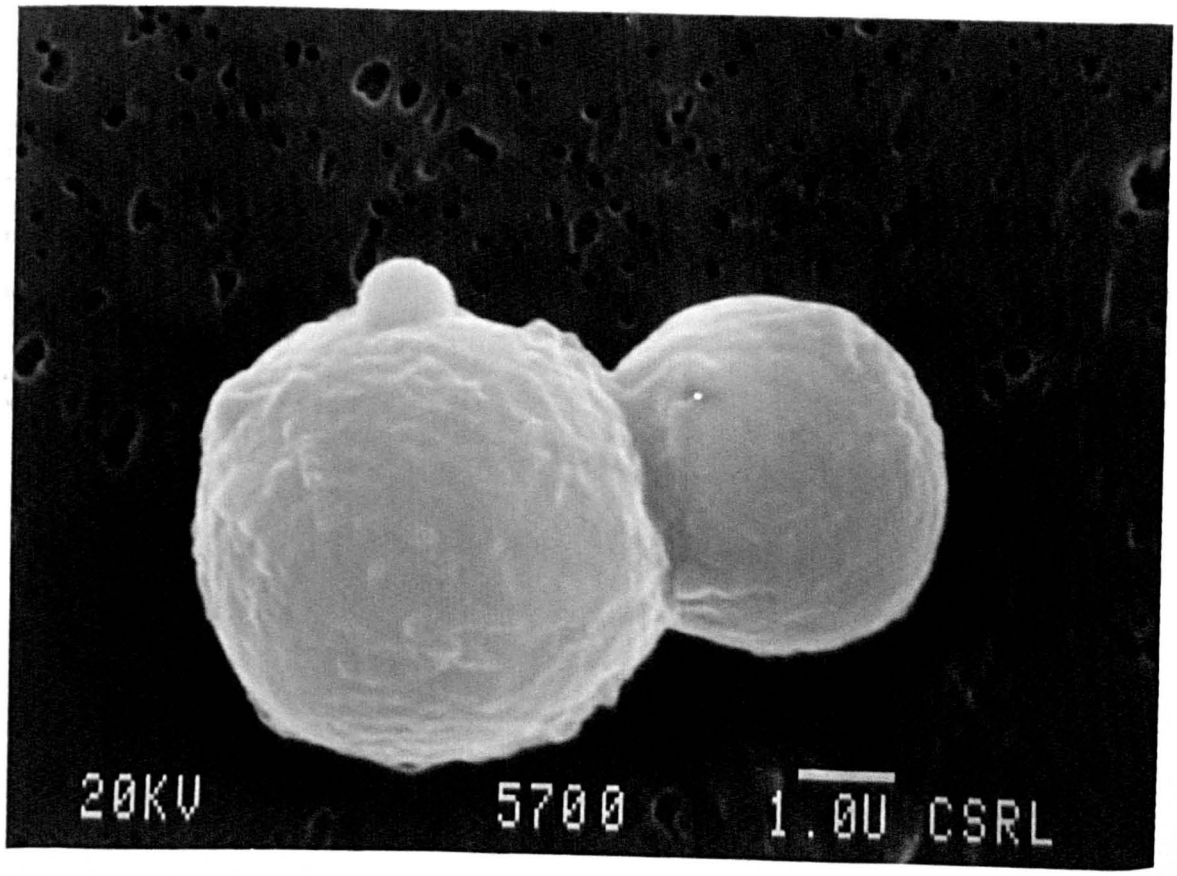


Plate 4



Legend to Plate 5

Lipid globules from Trichosporon cutaneum CBS 40 isolated after 2 days growth on nitrogen-limited medium (Methods Section 1.2).

Magnification: x 18,750

Diameter of globules: 2.6 to 4.1 μm

Globules had a rough laminated outer structure. Scars or tears were observed in this outer structure. Variation in globule size was similar to that observed for Candida curvata D.

——— 1 μm.

Legend to Plate 6

Lipid globules from Trichosporon cutaneum CBS 40 isolated after 4 days growth on nitrogen-limited medium (Methods, Section 1.2).

Magnification: x 7,500

Diameter of globules: 3.5 to 4.9 μm.

Globules had a laminated outer structure which was damaged in places and other material appeared to protrude through the surface opening.

—— 1 μm.

Plate 5

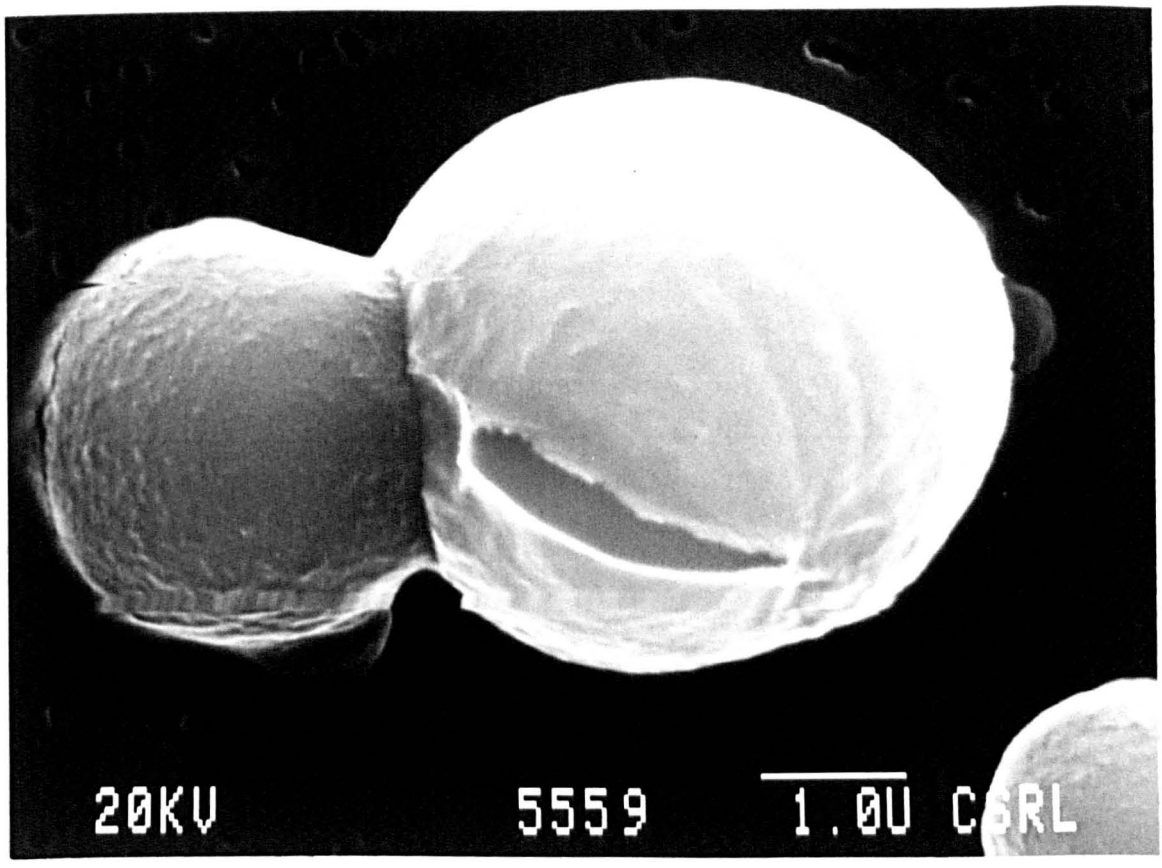
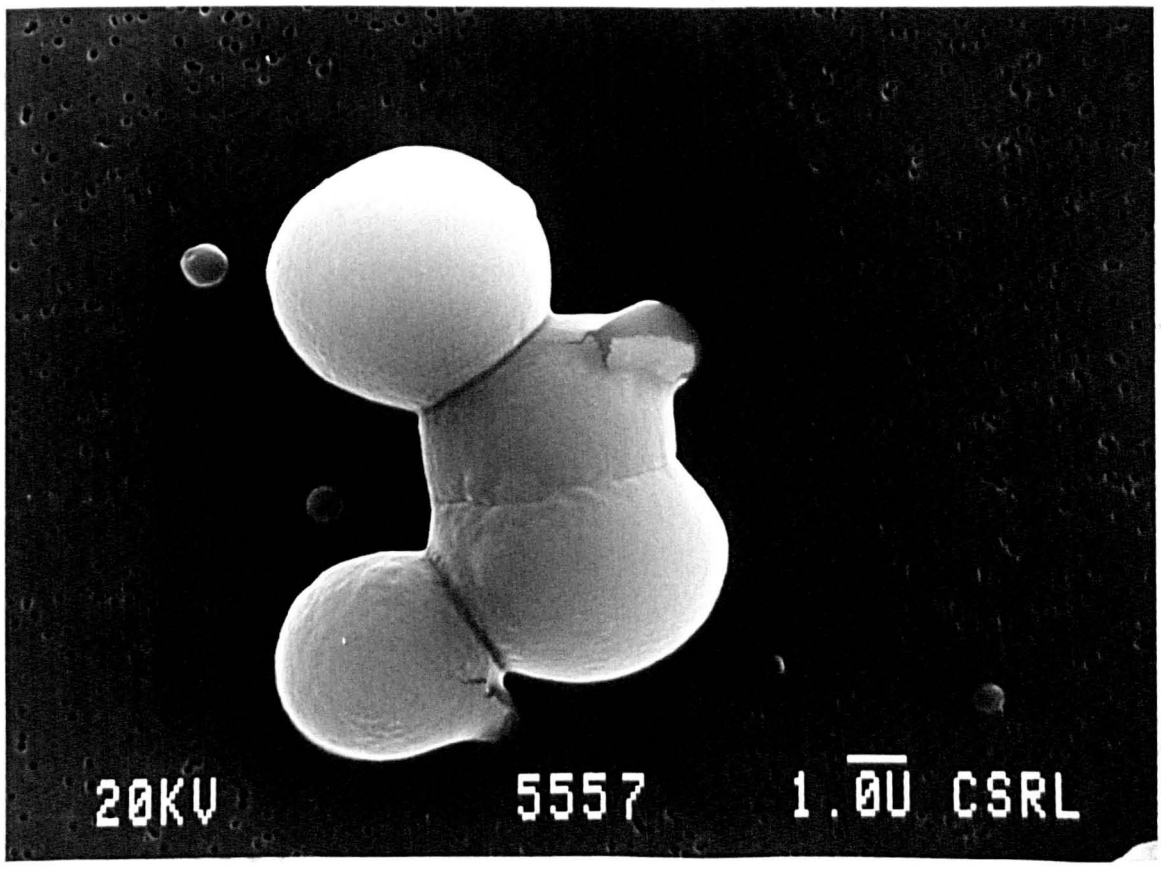


Plate 6



Legend to Plate 7

Lipid globules from Lipomyces starkeyi CBS 1809 isolated after 2 days growth on nitrogen-limited medium (Methods, Section 1.2).

Magnification: x 15,000.

Diameter of globules: 1.3 to 2.7 μm.

Globules had a rough laminar outer surface and were smaller than those isolated from C. curvata D and T. cutaneum CBS 40.

——— 1 μm.

Legend to Plate 8

Lipid globules from Lipomyces starkeyi CBS 1809 isolated after 4 days growth on nitrogen-limited medium (Methods, Section 1.2).

Magnification: x 12,500

Diameter of globules: 1.9 to 4.3 μm.

Globules had a very rough outer surface and the sample was slightly contaminated with non-lipid material.

——— 1 μm.

Plate 7

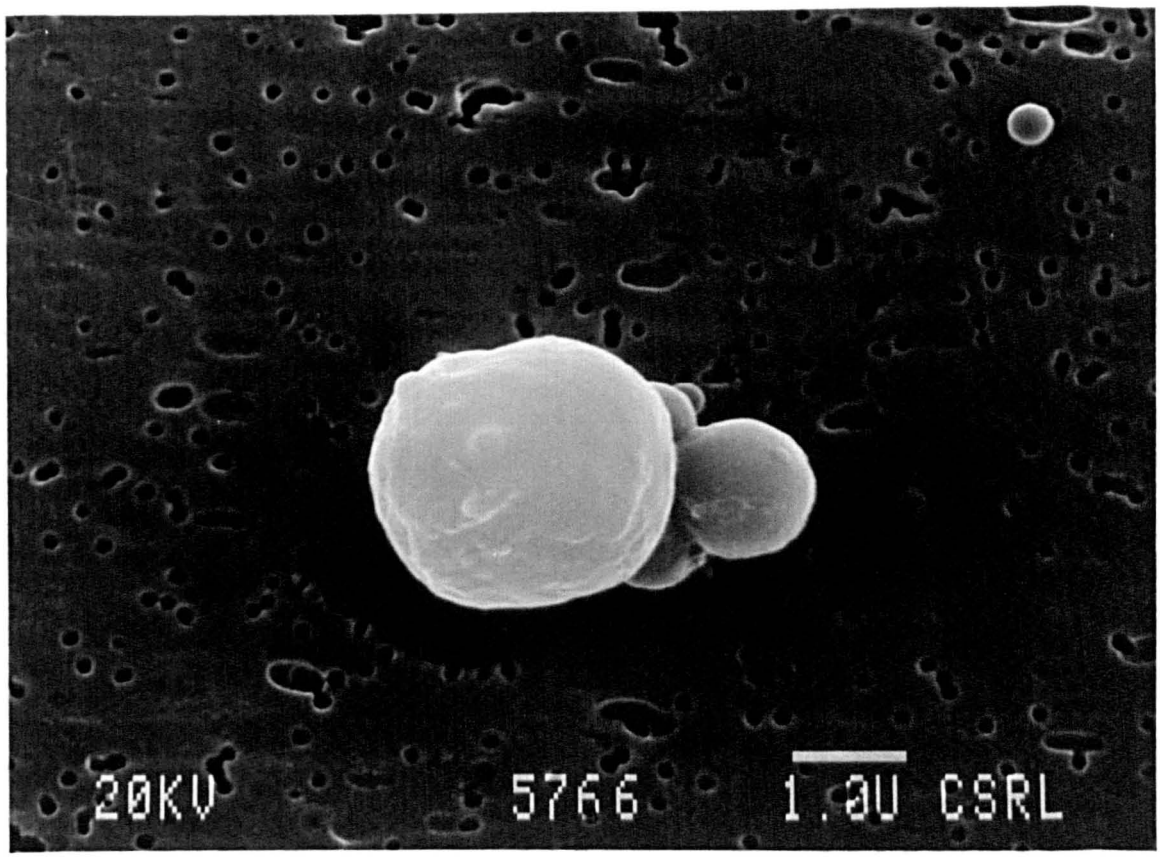
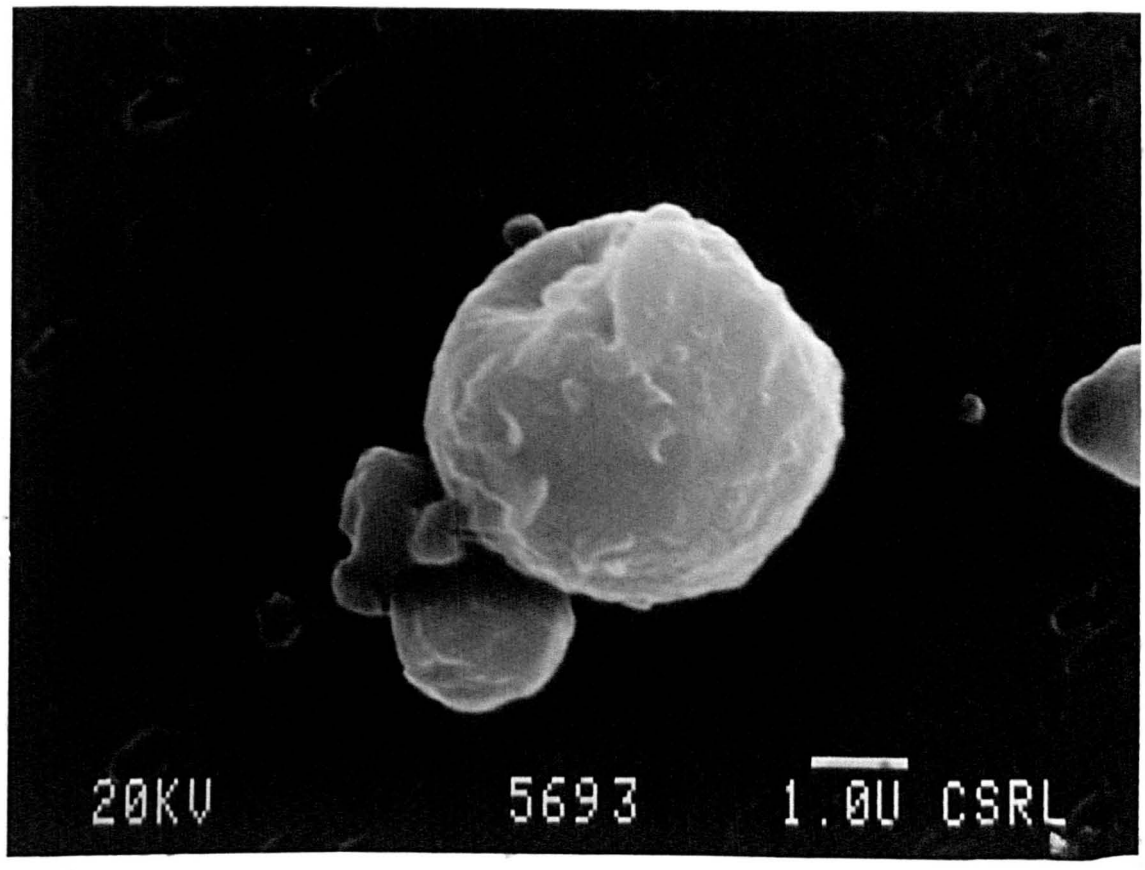


Plate 8



4.1.2. Chemical composition of oil globules

Lipid globules from the yeast Candida curvata D were labelled with [1-^{14}C]acetate, 45 minutes prior to being isolated and purified as described in Methods, Section 3. Lipid was extracted from the globules and analysed by thin-layer chromatography as described in Methods, Section 3.3.2. The results are shown in Table 33.

The major lipid of the oil globules was triacylglycerol with sterol, diacylglycerol and free fatty acid forming the other major components. The high percentages of diacylglycerol and free fatty acids may have resulted from breakdown of triacylglycerol during the extraction and separation.

The lipid globules contained a negligible percentage of polar lipids and this would make the possibility of the globules being surrounded by a bilayer or membrane extremely unlikely.

4.2. Transmission electron microscopy

Transmission electron microscopy was employed in order to gain some information about the ultrastructure of oleaginous yeasts. It was also hoped to identify any key differences between yeasts growing such that lipid accumulated and yeasts growing on endogenous or exogenous lipid as sole carbon source.

Two methods were used to observe the ultrastructure of these yeasts:

(1) Thin sectioning: A thin section was cut through the yeast sample, exposing sections through organelles.

(2) Freeze-etching: Cells were rapidly frozen at liquid nitrogen temperatures. The cells were then fractured by the

Table 33. Distribution of radioactivities among total lipids of oil globules isolated from Candida curvata D

Lipid class	Distribution of radioactivities (%)
Polar lipid (phospholipid)	0.102
Monoacylglycerol	0.274
Sterol	11.1
Diacylglycerol	5.25
Free fatty acid	3.1
Triacylglycerol	78.1
Sterol ester	2.1

Cells were grown in a vortex-aerated vessel at 30°C on nitrogen-limited medium (Methods, Section 1.2) for five days. 125 μCi [1-^{14}C]Acetate was injected into the growing culture 45 minutes prior to harvesting the cells and isolating the globules. Total lipids were extracted with chloroform:methanol (2:1) and separated on silica gel thin-layers with the solvent system of petroleum ether:diethyl ether:acetic acid (60:40:1). Chromatograms were scraped and the radioactivities were measured by a liquid scintillation counter and counts per minute were converted to percent of the total radioactivity recovered from chromatograms.

impact of a microtome knife. Cleavage usually occurred along a plane in the middle of a bilayer. The exposed regions were then shadowed with carbon and platinum and the replica was observed under the electron microscope.

4.2.1. Thin sectioning

Two fixation methods were used for cells. The first (Method A) involved staining with glutaraldehyde (2.5%, w/v) followed by osmium tetroxide (1% w/v). This method was used at PHLS, Porton Down and the work was kindly carried out by Barry Dowsett. The second (Method B) involved fixation with potassium permanganate (15% w/v) and was performed as University of Groningen, The Netherlands with the kind help of Marten Veenhuis. These fixation techniques are outlined in Methods, Section 9.1.

The micrographs obtained are shown in the subsequent pages and all details are provided in the legends to each photograph.

A summary of the ultrastructural observations is given in Table 34 (page 233).

Legend to Plate 9

Thin section of Candida curvata D grown for 3 days on nitrogen-limited medium (Methods, Section 1.2) and stained with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 51,000.

The yeast contained several oil globules of fairly regular sizes and glycogen deposits were also visible as black granules. The nucleus and nucleolus were clearly defined and surrounded by a cluster of mitochondria. Vacuoles were clearly visible in the section. Nothing that could clearly be defined as a peroxisome was visible in the cell.

L, lipid globule;

G, glycogen granules;

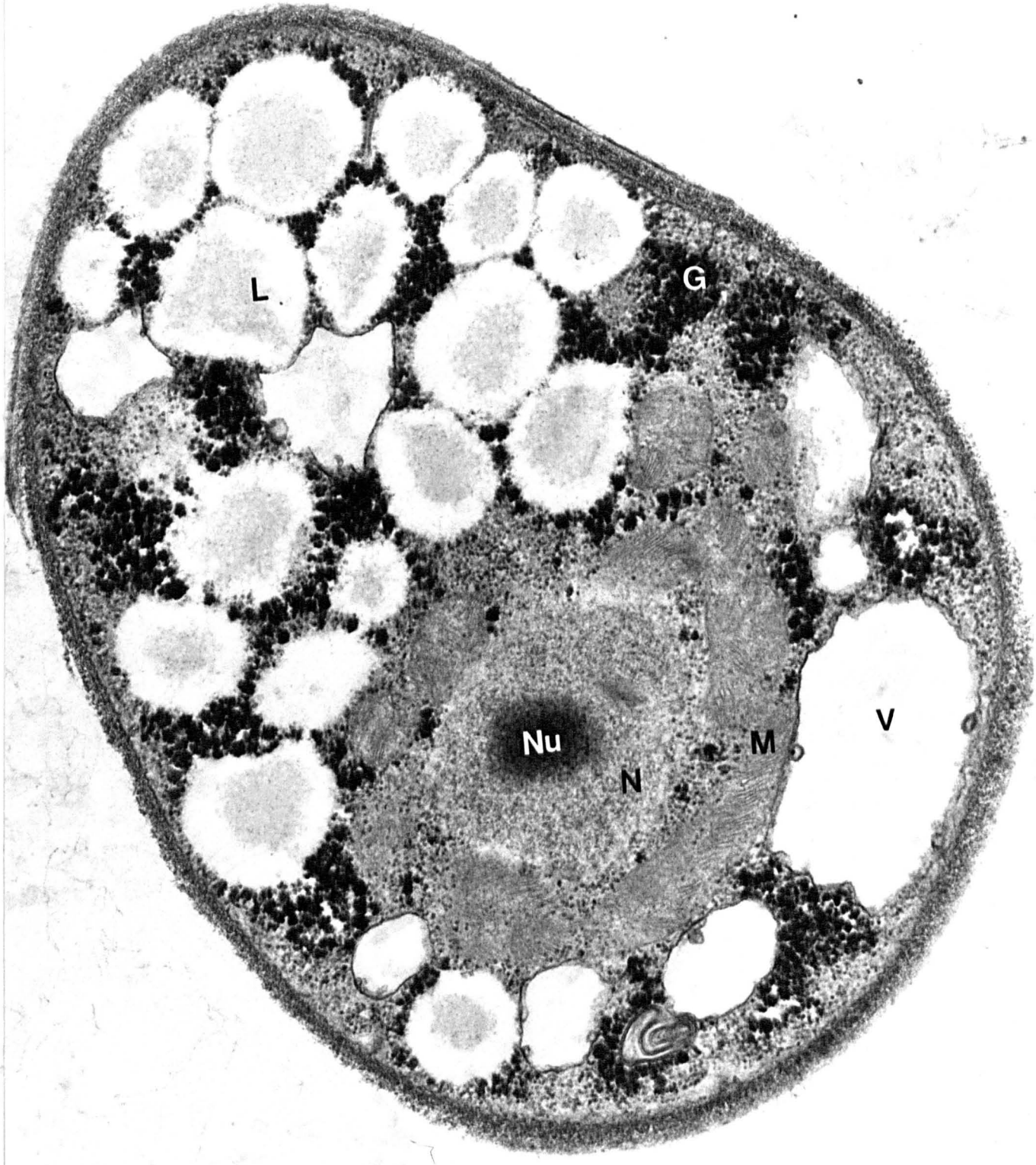
N, nucleus;

Nu, nucleolus;

M, mitochondrion;

V, vacuole

1 μm


L
G
Nu
N
M
V

Legend to Plate 10

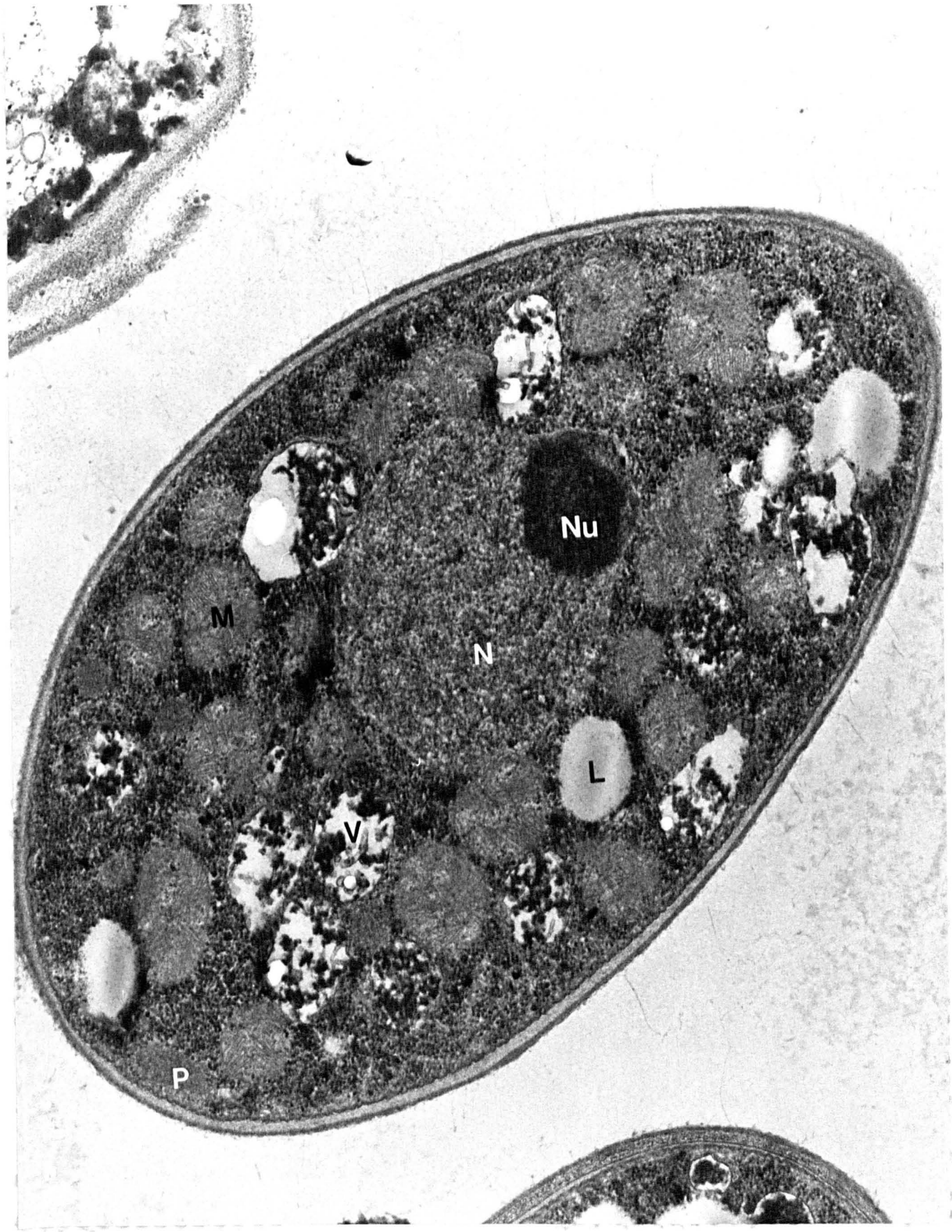
Thin section of Candida curvata D grown for 3 days on nitrogen-limited medium, then for 24 hours on carbon starvation medium (Methods, Section 1.2) so that the accumulated lipid was utilized. The yeast was stained with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 30,000

The lipid had now mostly disappeared from the cell and only a few small globules remained. Many mitochondria were visible in the cell and there was also a large number of vacuoles. The glycogen granules had completely disappeared. The nucleus and nucleolus were clearly visible. Some bodies which may have been peroxisomes were visible, but it was not possible to clearly discern them from mitochondria.

L, lipid globule;
N, nucleus;
Nu, nucleolus;
M, mitochondrion;
V, vacuole;
P, peroxisome (?)

1 μm


Nu
M
N
L
V
P

Legend to Plate 11

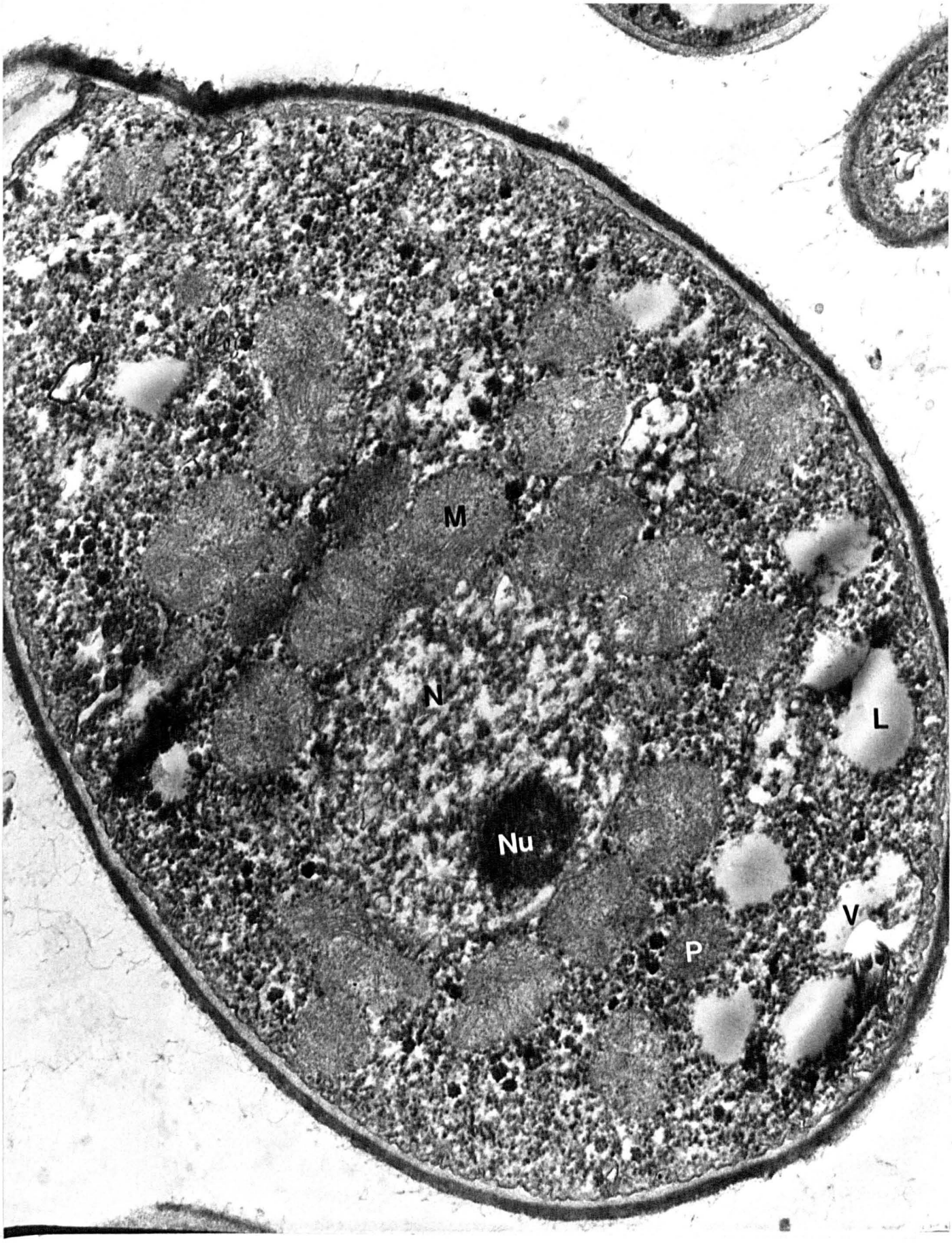
Thin section of Candida curvata D grown on triolein media (Methods, Section 1.2) for 2 days and fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 39,000.

Lipid globules were visible within the cell, but no glycogen granules were present. The cell contained many mitochondria and there were some bodies which may have been peroxisomes. However it was difficult to distinguish between these and mitochondria. The cell contained a few small vacuoles and the nucleus and nucleolus were clearly visible.

L, lipid globule;
N, nucleus;
Nu, nucleolus;
M, mitochondrion;
V, vacuole;
P, peroxisome (?)

1 μm


M
N
L
Nu
V
P

Legend to Plate 12

Thin section of Liopmyces starkeyi CBS 1809 grown for 2 days on nitrogen-limited medium (Methods, Section 1.2) and fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 51,000.

The yeast contained lipid in one large globule and glycogen also accumulated in granules within the cell. The nucleus and nucleolus were not visible in this section. The cell contained mitochondria and vacuoles.

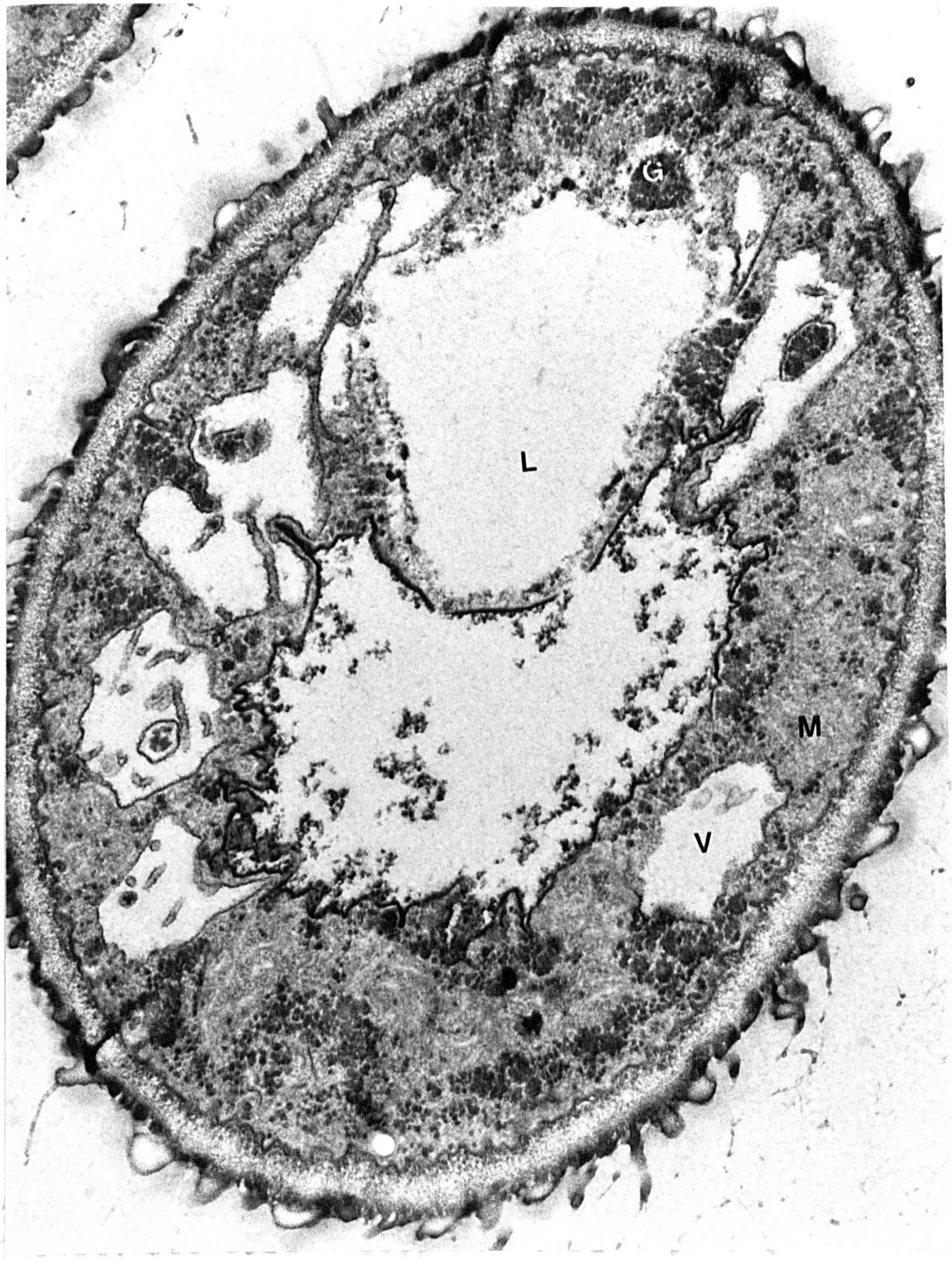
L, lipid globules;

M, mitochondrion;

V, vacuole;

G, glycogen.

1 μm


G
L
M
V

Legend to Plate 13

Thin section of Lipomyces starkeyi CBS 1809 after growth for 3 days such that lipid accumulated (nitrogen-limited medium) followed by growth for 24 hours on carbon starvation medium (Methods, Section 1.2). The cells were fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 30,000.

Lipid was still present within the cell in a large globule, however the glycogen granules were absent from the cell. Vacuoles were present within the cell as were mitochondria, but it was not possible to identify any microbodies. The nucleus and nucleolus were not present in this section.

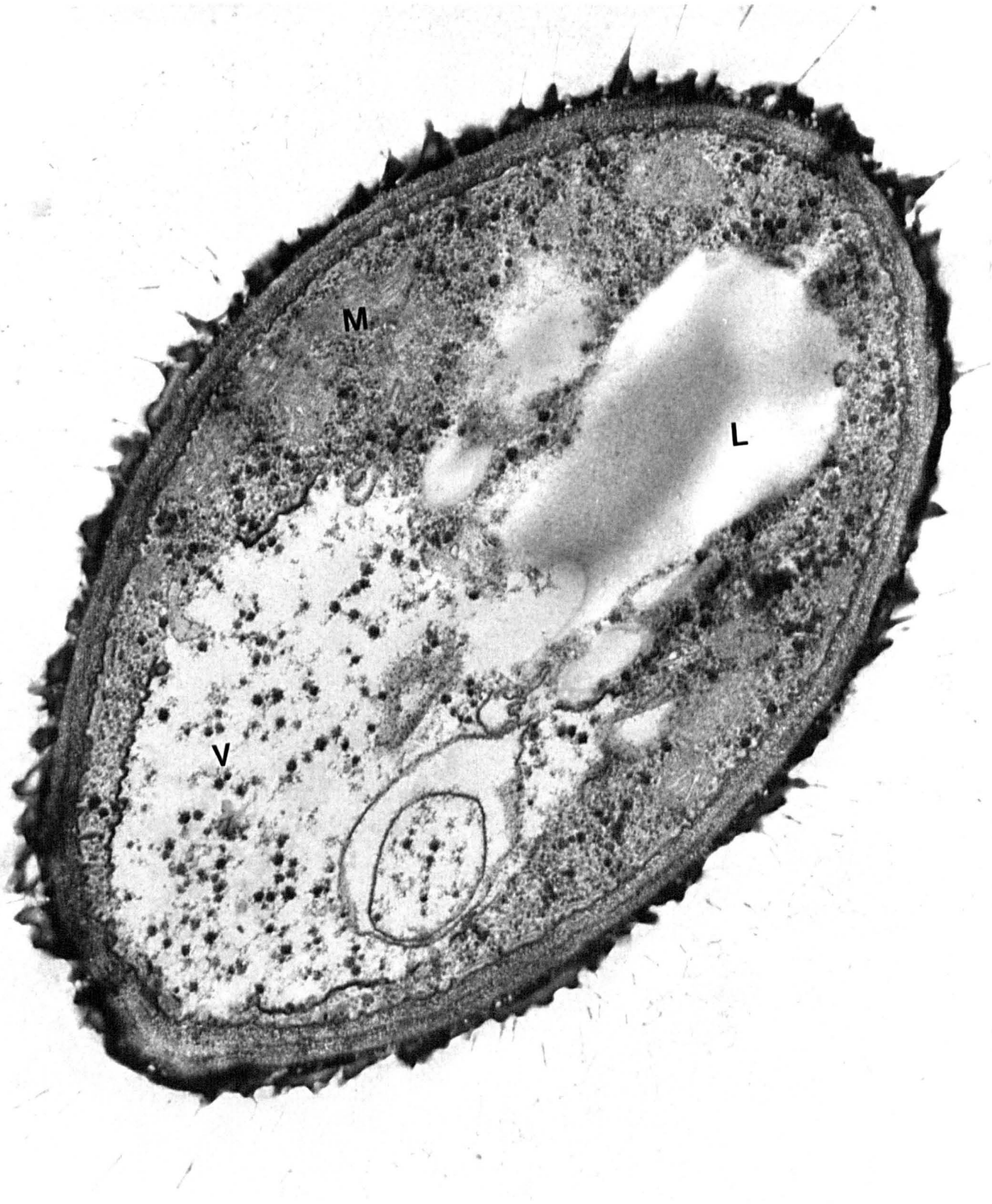
L, lipid globules;

M, mitochondrion;

V, vacuole.

1 μm ——————

Plate 13



Legend to Plate 14

Thin section of Trichosporon cutaneum CBS 40 grown for 3 days on nitrogen-limited medium (Methods, Section 1.2). The yeast was fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 51,000.

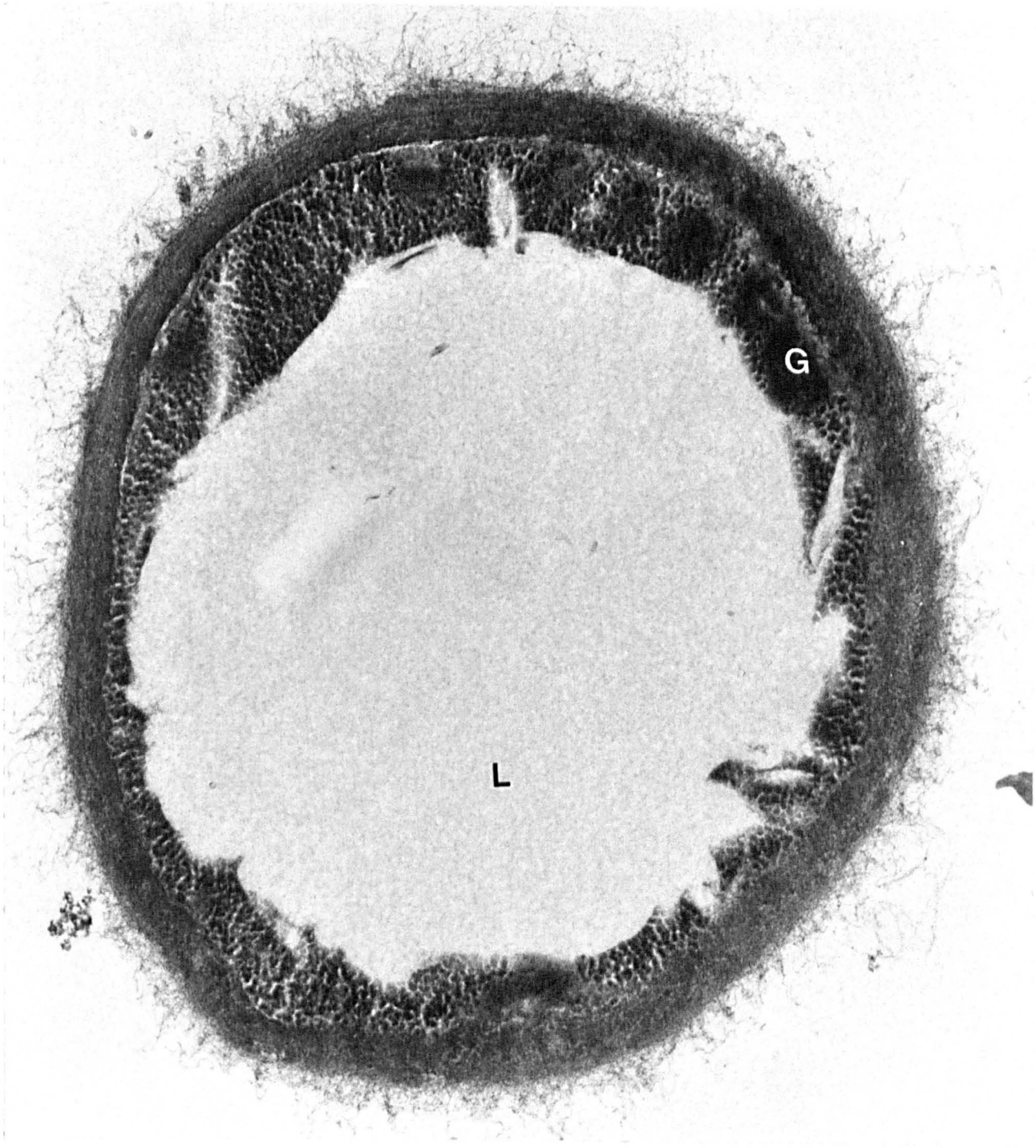
The yeast had an extremely thick cell wall when grown under these conditions and because of this poor internal fixation resulted. However the micrograph did illustrate that the yeast accumulated large amounts of lipid and probably glycogen as well.

L, lipid globule;

G, glycogen granules.

1 μm

Plate 14



Legend to Plate 15

Thin section of Trichosporon cutaneum CBS 40 grown for 3 days on nitrogen-limited medium then for 24 hours on carbon starvation medium (Methods, Section 1.2) so that the accumulated lipid was utilized. The cells were fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 39,000.

The cell wall was still extremely thick. However internal fixation was a little better than for lipid accumulating cells. The lipid and glycogen had disappeared from the cell. The nucleus was visible in this section as were vacuoles. Mitochondria were present, as were some bodies that may have been peroxisomes.

N, nucleus;

M, mitochondrion;

V, vacuole;

P, peroxisome (?)

1 μm

Plate 15

Legend to Plate 16

Thin section of Trichosporon cutaneum CBS 40 grown for 2 days on triolein medium (Methods, Section 1.2). The yeast was fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 30,000.

The yeast contained several small lipid globules but no glycogen. The nucleus and nucleolus were visible in the section and it contained several small vacuoles. Mitochondria were present in the section as were some microbodies which may have been peroxisomes. The wall of the cell was much thinner than that observed in cells accumulating lipid (Plate 14) or cells grown under carbon starvation conditions (Plate 15).

L, lipid globule;

N, nucleus;

Nu, nucleolus;

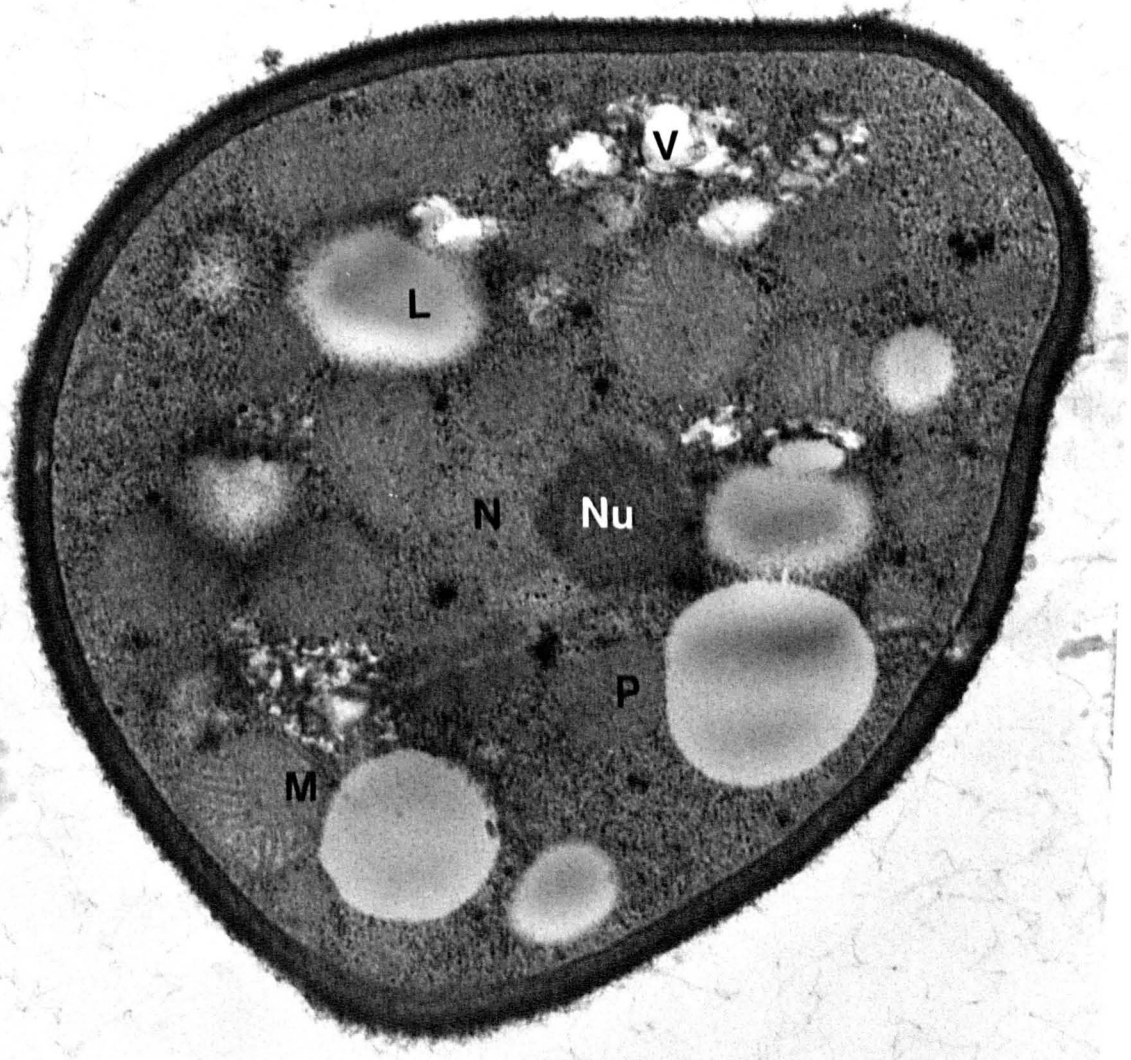
M, mitochondrion;

V, vacuole;

P, peroxisome (?)

1 μm

Plate 16



Legend to Plate 17

Thin section of Rhodosporidium toruloides CBS 14 grown on nitrogen-limited medium (Methods, Section 1.2) for 3 days. The yeast was fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 70,000.

The fixation in this yeast was poor, possibly due to the thickness of the cell wall. Lipid globules were present in the section, as were glycogen granules. The most interesting observation in this yeast was the presence of large quantities of endoplasmic reticulum which appeared to be closely associated with the lipid globules. It was not possible to distinguish any other subcellular components.

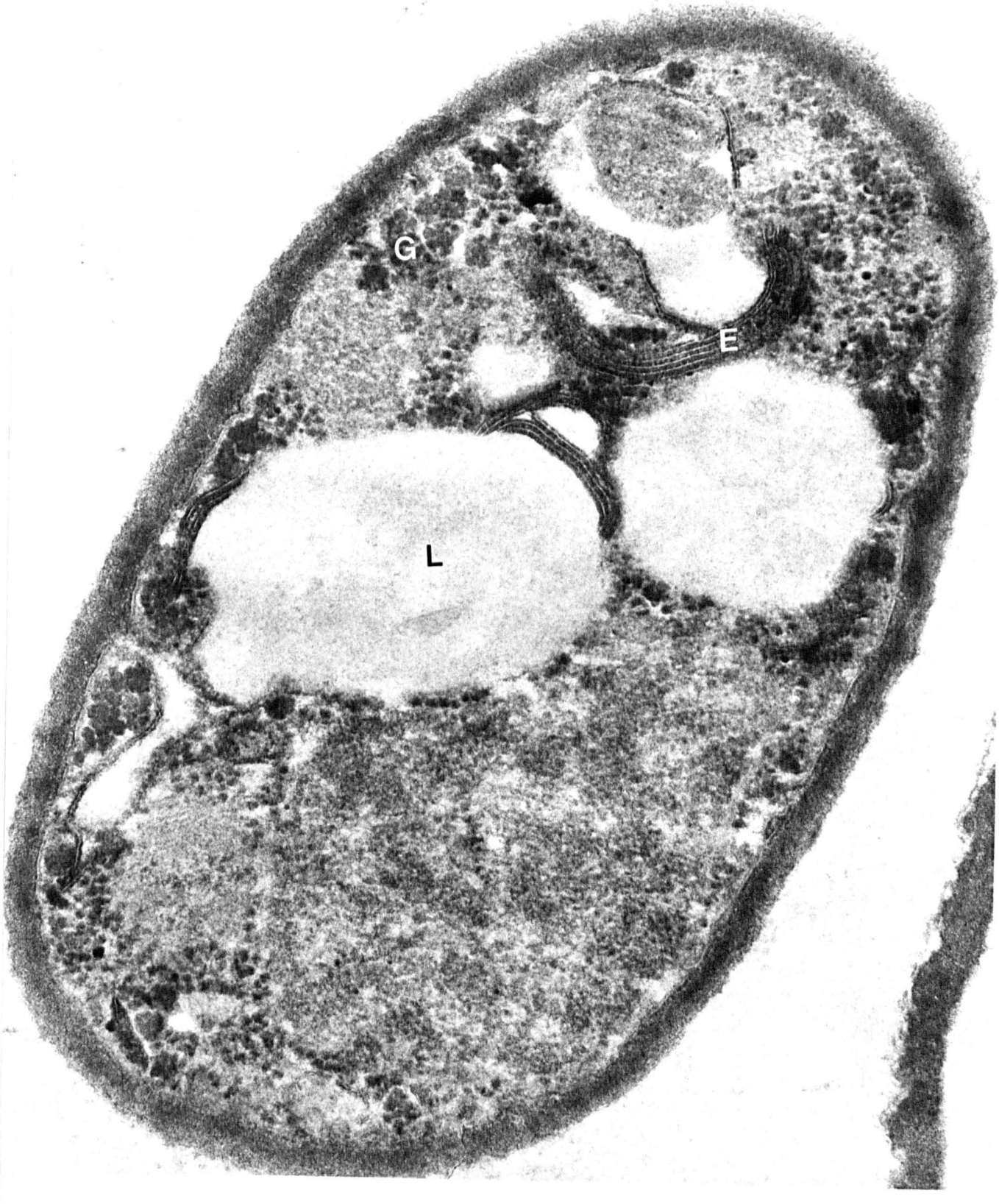
L, lipid globule;

G, glycogen granules;

E, endoplasmic reticulum.

1 μm

Plate 17.



Legend to Plate 18

Thin section of Rhodosporidium toruloides CBS 14 grown for 3 days on nitrogen-limited medium then for 24 hours on carbon starvation medium (Methods, Section 1.2) so that the accumulated lipid was utilized. The cells were fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2.).

Magnification: x 30,000.

Lipid and glycogen were absent from the cells and cell walls were much thinner than during lipid accumulation (Plate 17). The cells contained mitochondria and possibly peroxisomes but the fixation was not good enough to distinguish between the two. There was no endoplasmic reticulum visible in the sections in contrast to the sections of lipid accumulating cells (Plate 17).

M, mitochondria;

N, nucleus;

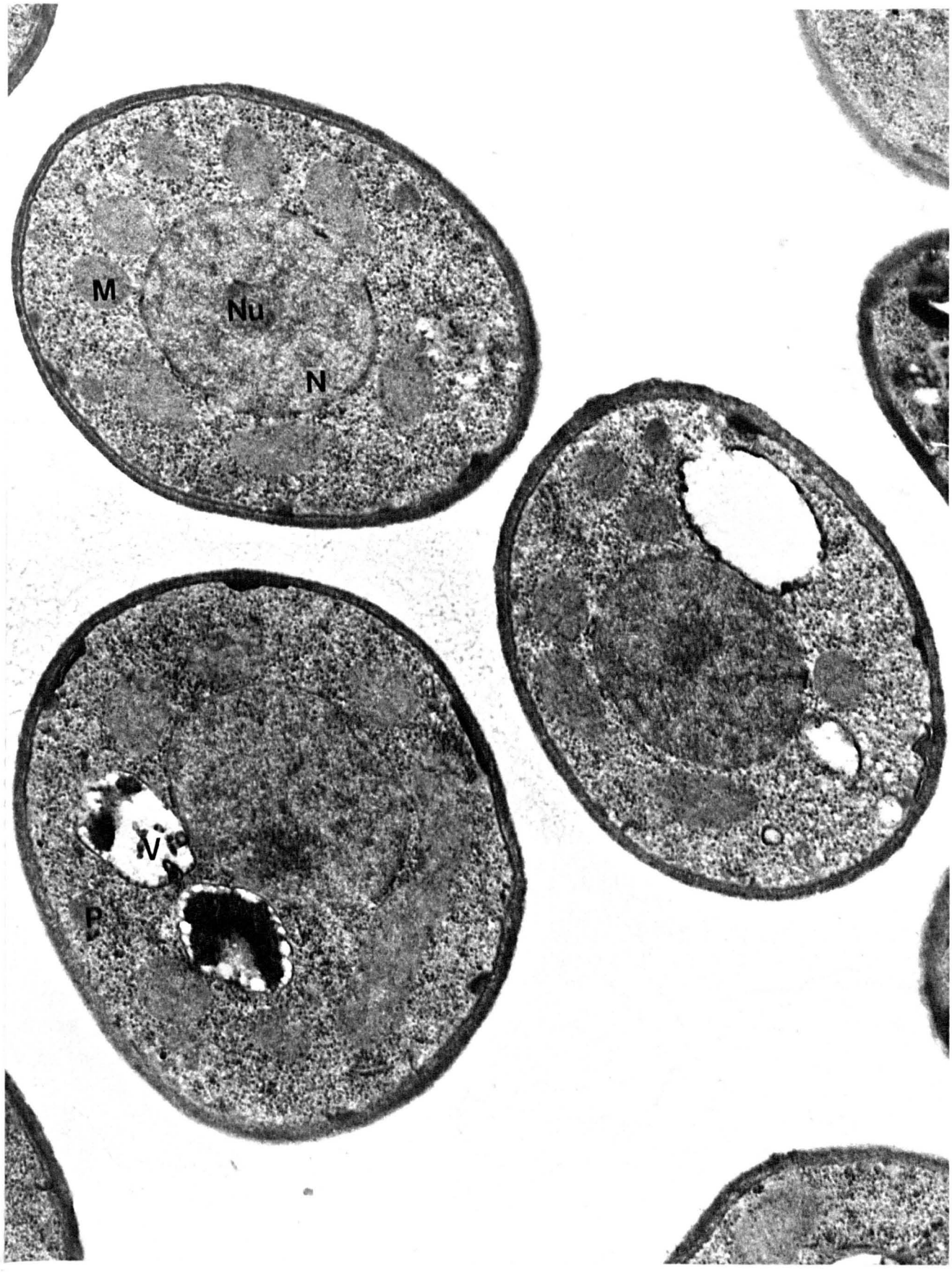
Nu, nucleolus;

V, vacuole;

P, peroxisome (?).

1 µm

Plate 18



Legend to Plate 19

Thin section of Rhodosporidium toruloides CBS 14 grown for 2 days on triolein medium (Methods, Section 1.2). The cells were fixed with glutaraldehyde-osmium according to Method A (Methods, Section 9.2).

Magnification: x 39,000.

The cell did not contain any lipid or glycogen. The nucleus and nucleolus were visible as were vacuoles. Bodies which were mitochondria or peroxisomes were present but it was not possible to distinguish between these organelles.

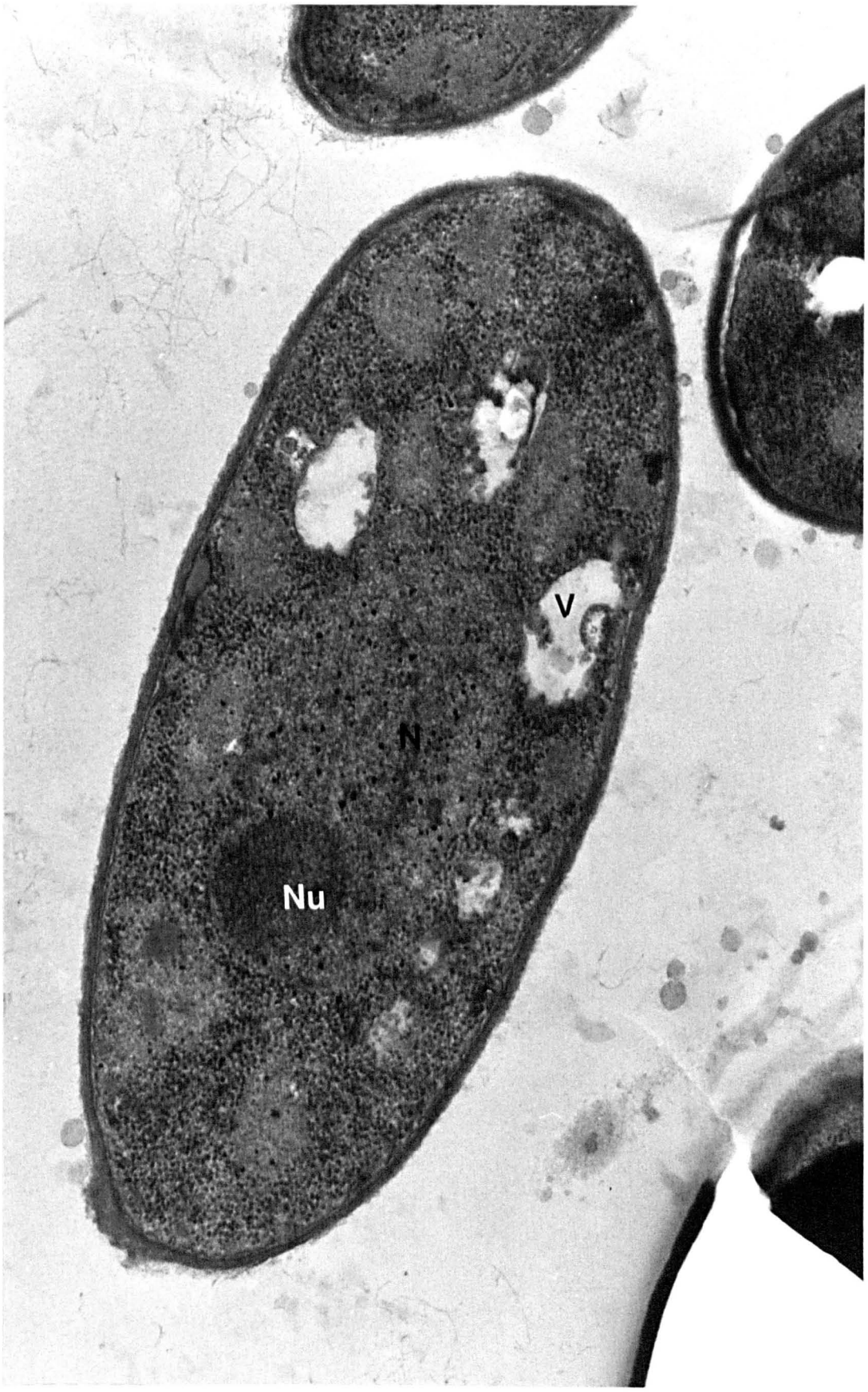
N, nucleus;

Nu, nucleolus;

V, vacuole

1 μm

Plate 19.



Legend to Plate 20

Thin section of Candida curvata D grown on glucose in complete medium (Methods, section 1.2) for 24 hours. The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 59,000.

Fixation of the membranes within the cell was very good due to the use of permanganate as fixative. The nucleus, mitochondria, vacuole and one small peroxisome were visible in the section. The cell did not contain lipid or glycogen granules.

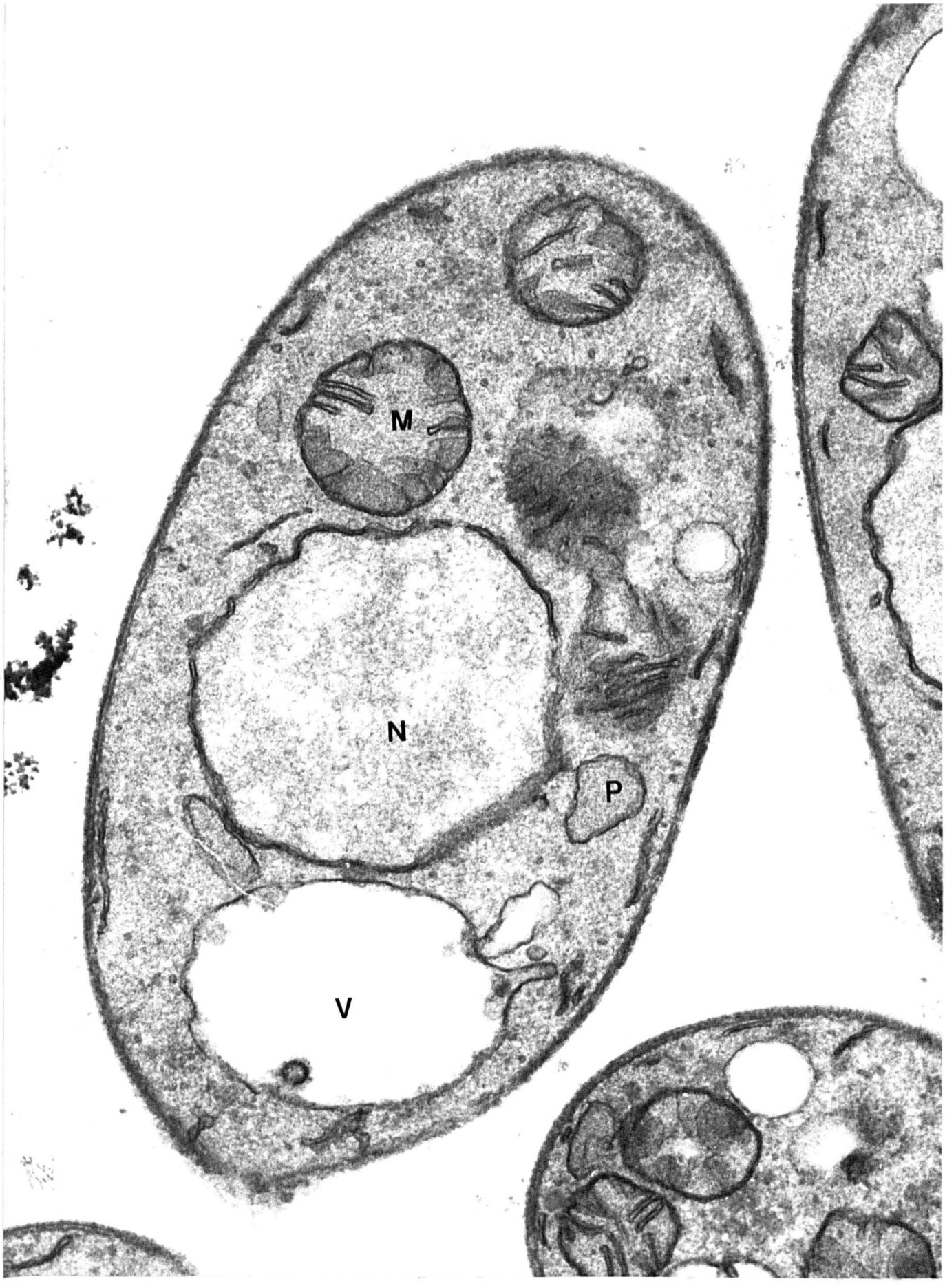
N, nucleus;

M, mitochondria;

V, vacuole;

P, peroxisome.

1 μm


M
N
P
V

Legend to Plate 21

Thin section of *Candida curvata* D grown on ethanol medium (Methods, Section 1.2) for 24 hours. The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 55,000.

Lipid globules were clearly visible in the section and were associated with the endoplasmic reticulum in many cases suggesting that this was a possible site for their synthesis. No glycogen was present in the section and it did not contain the nucleus. Mitochondria were present and peroxisomes were also visible. The peroxisome volume was slightly increased over that of glucose-grown cells (Plate 20).

M, mitochondrion;

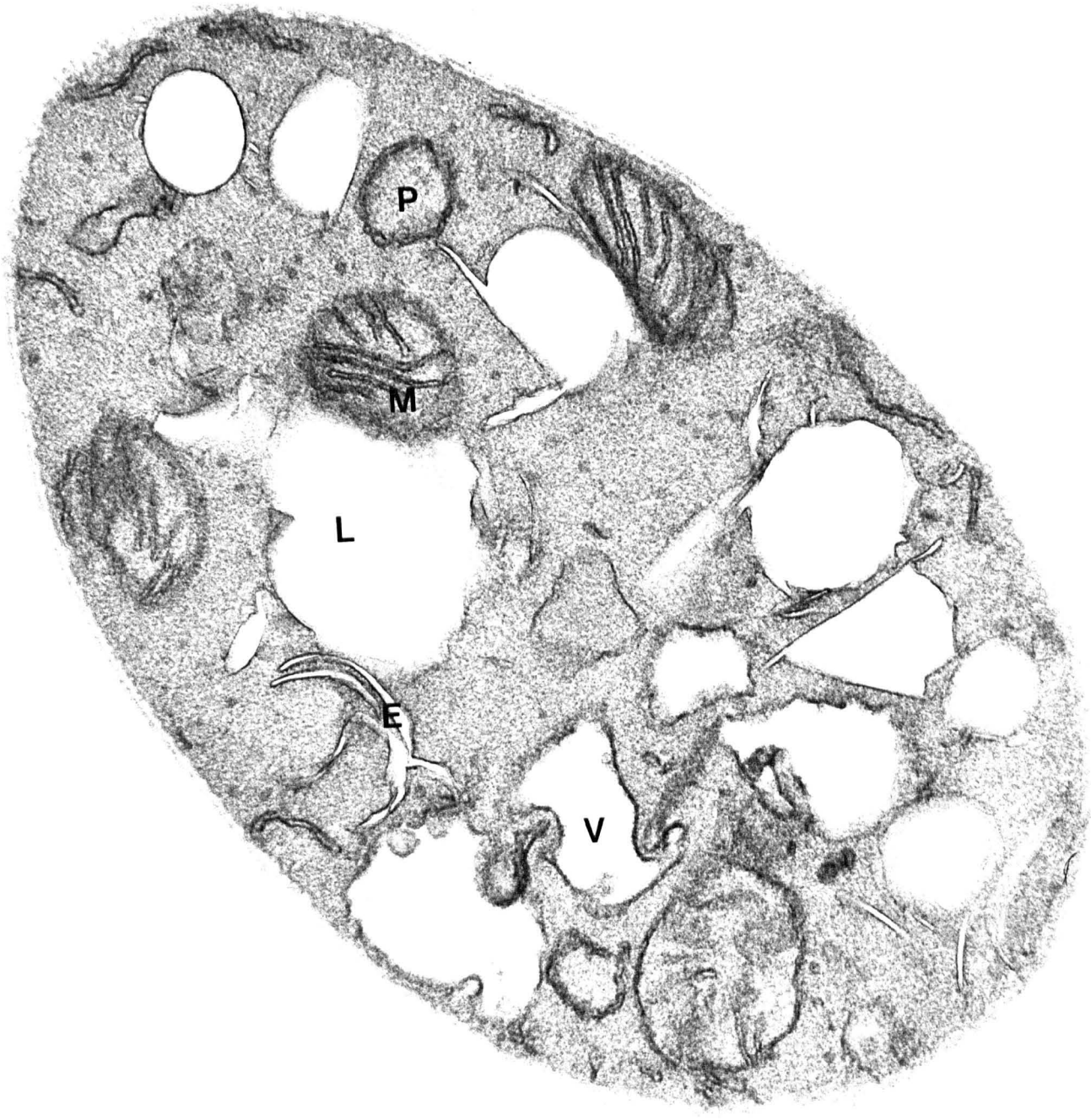
L, lipid globule;

V, vacuole;

E, endoplasmic reticulum;

P, peroxisome.

1 μm


P
M
L
E
V

Legend to Plate 22

Thin section of Candida curvata D grown for 2 days on nitrogen-limited medium (Methods, Section 1.2) such that lipid accumulated. The cells were fixed with $KMnO_4$ using Method B (Methods, Section 9.2).

Magnification: x 35,000.

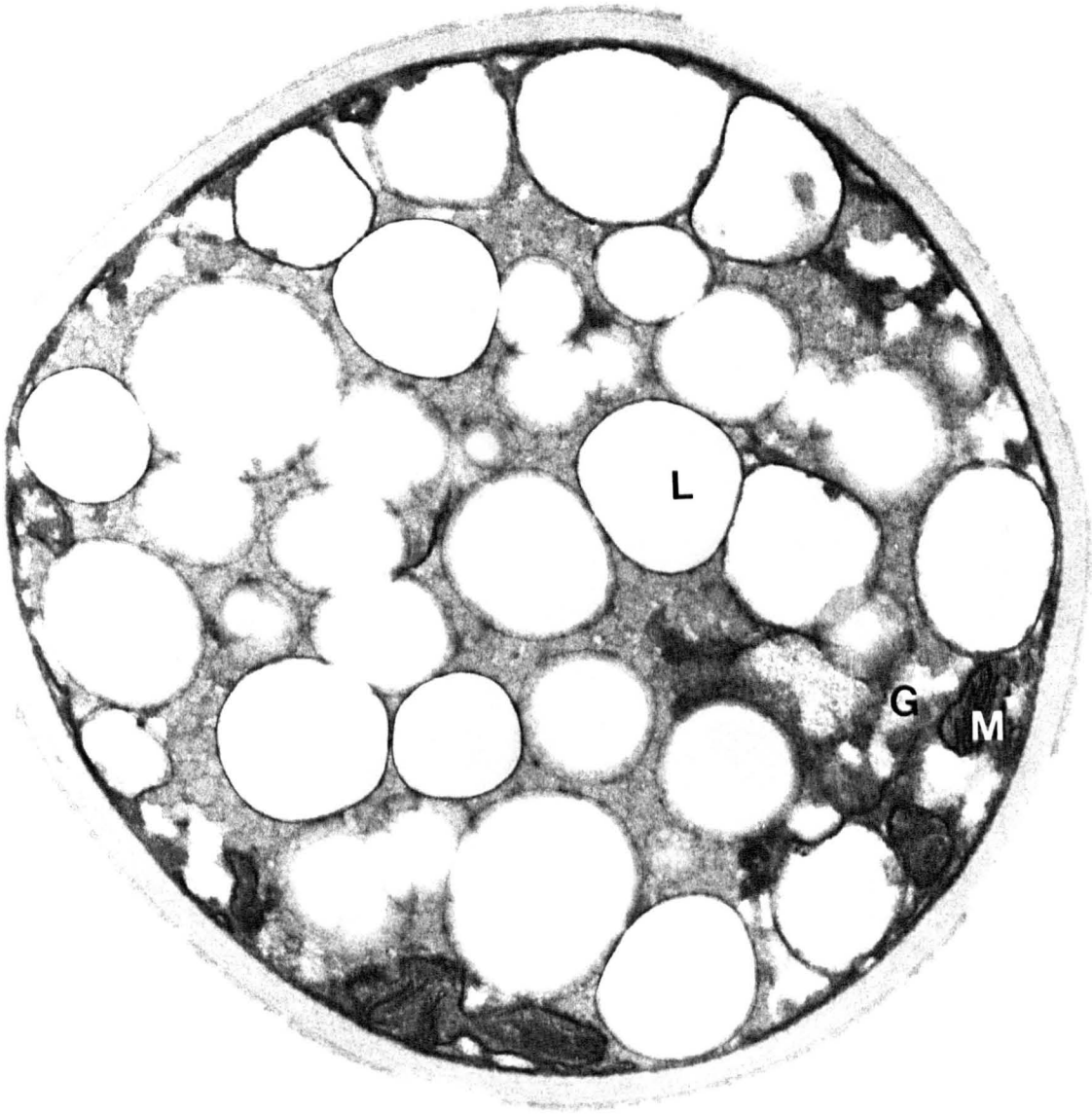
The cell contained a large number of lipid globules and also some glycogen granules which appeared as white grainy areas. A few mitochondria were also visible but no peroxisomes were detected.

L, lipid globule;

G, glycogen granules;

M, mitochondrion.

1 μm


L
G
M

Legend to Plate 23

Thin section of Candida curvata D grown for 2 days on nitrogen-limited medium, then for 18 hours on carbon starvation medium (Methods, Section 1.2) so that the accumulated lipid was utilized. The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 45,000.

The number of lipid globules within the cell had greatly decreased and the glycogen granules had disappeared. The nucleus was visible in the section as were many mitochondria and vacuoles. There was an increase in peroxisome number and volume fraction compared to glucose grown controls (Plates 20 and 22).

L, lipid globule;

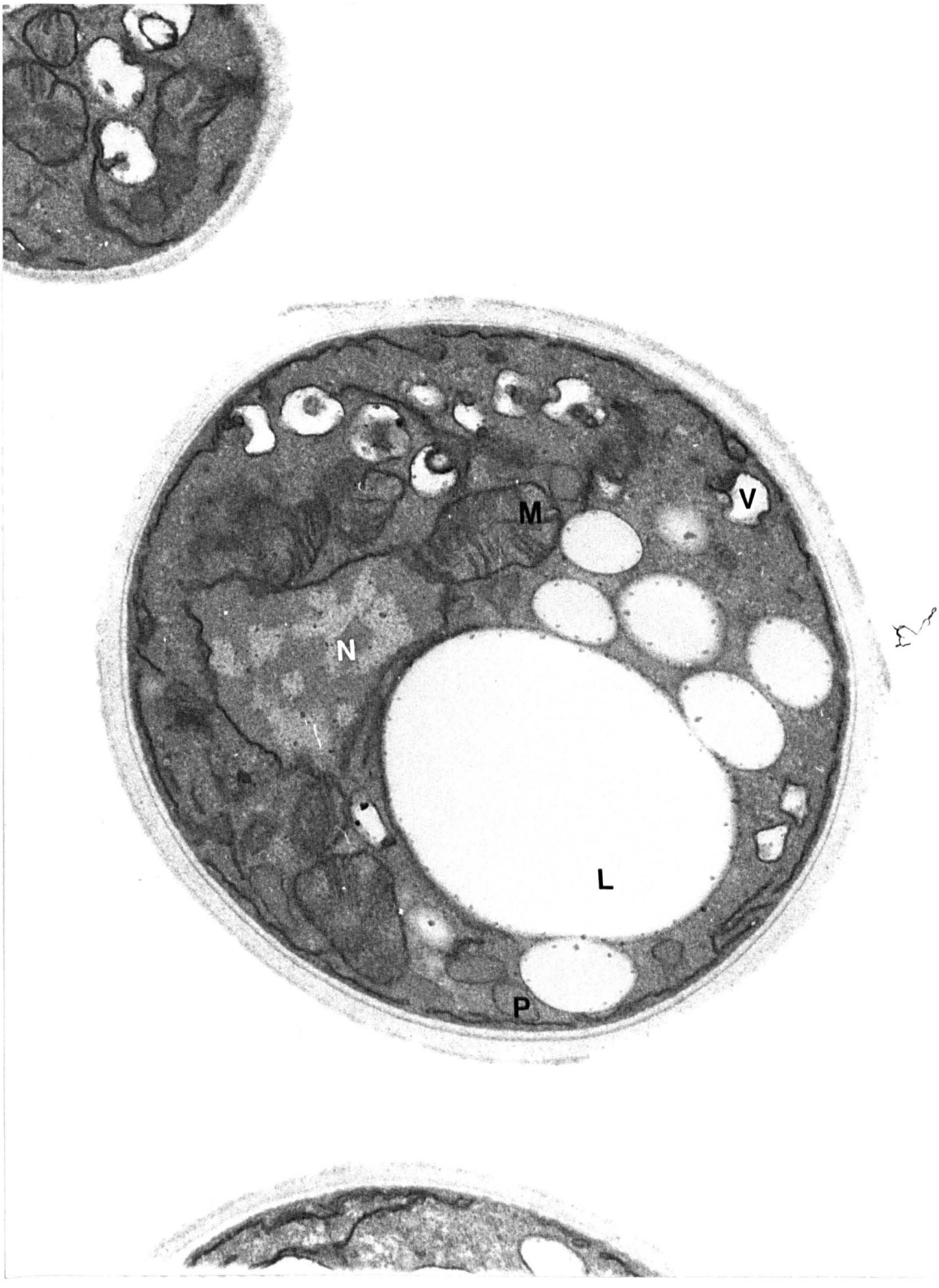
N, nucleus;

M, mitochondria;

V, vacuole;

P, peroxisome.

1 μm


M
V
N
L
P

Legend to Plate 24

Thin section of Candida curvata D grown for 24 hours on triolein medium (Methods, Section 1.2). The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 55,000.

The cell contained small lipid globules but no glycogen granules. There was an abundance of mitochondria and the nucleus and vacuoles were visible in the section. Many peroxisomes were present and there was a definite increase in the peroxisome number and volume fraction over the glucose-grown controls (Plate 20).

L, lipid globule;

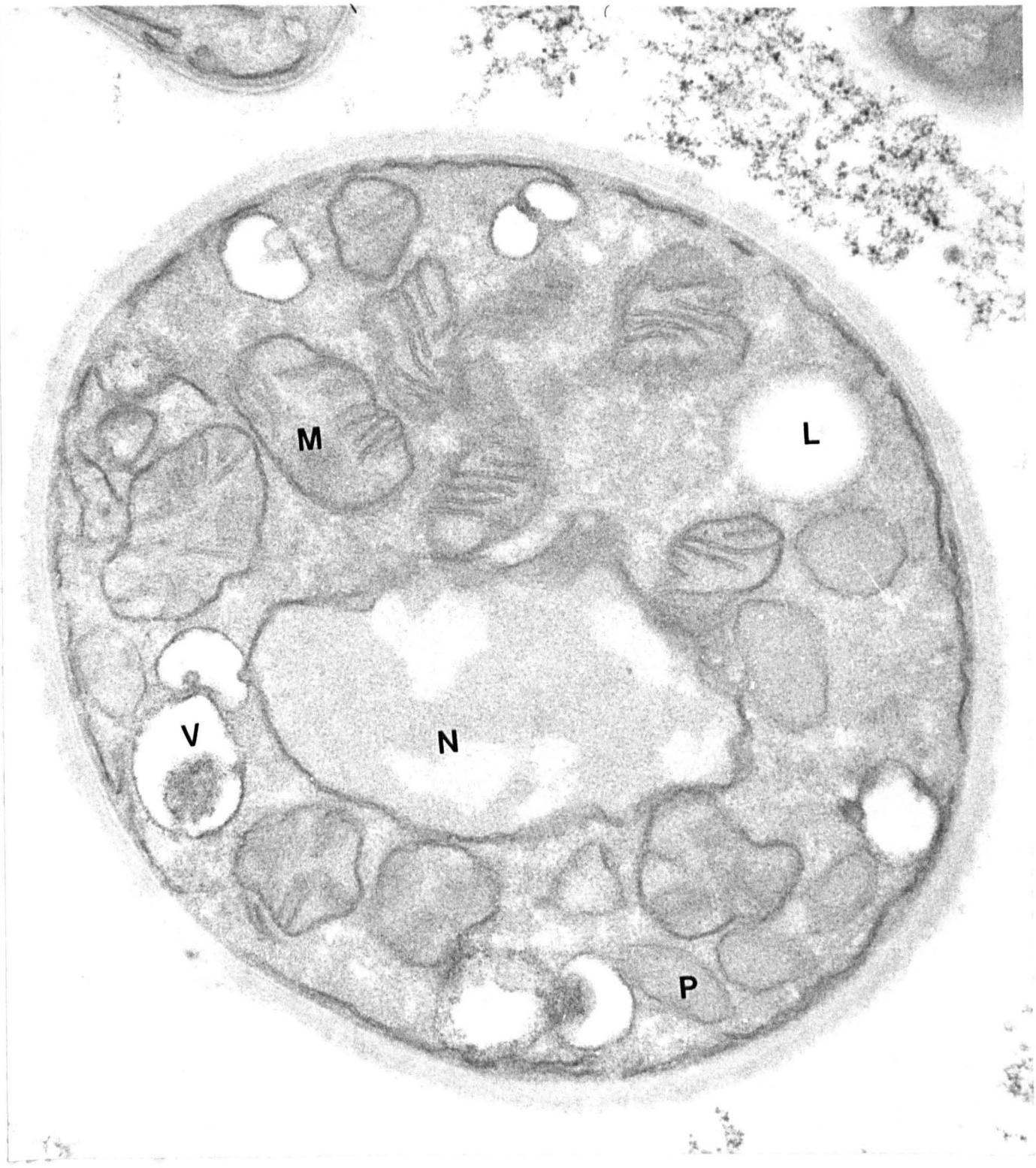
M, mitochondrion;

V, vacuole;

N, nucleus;

P, peroxisome

1 μm


M
L
V
N
P

Legend to Plate 25

Thin section of *Candida curvata* D grown for 24 hours on oleate medium (Methods, Section 1.2). Cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 45,000.

Cells contained a few small lipid globules but no glycogen granules. The nucleus, mitochondria and vacuoles were visible in the section. There was a definite increase in peroxisome number and volume fraction compared with glucose-grown controls (Plate 20). The cells, in general, had identical subcellular morphology to those grown on triolein medium (Plate 24).

L, lipid globule;

M, mitochondrion;

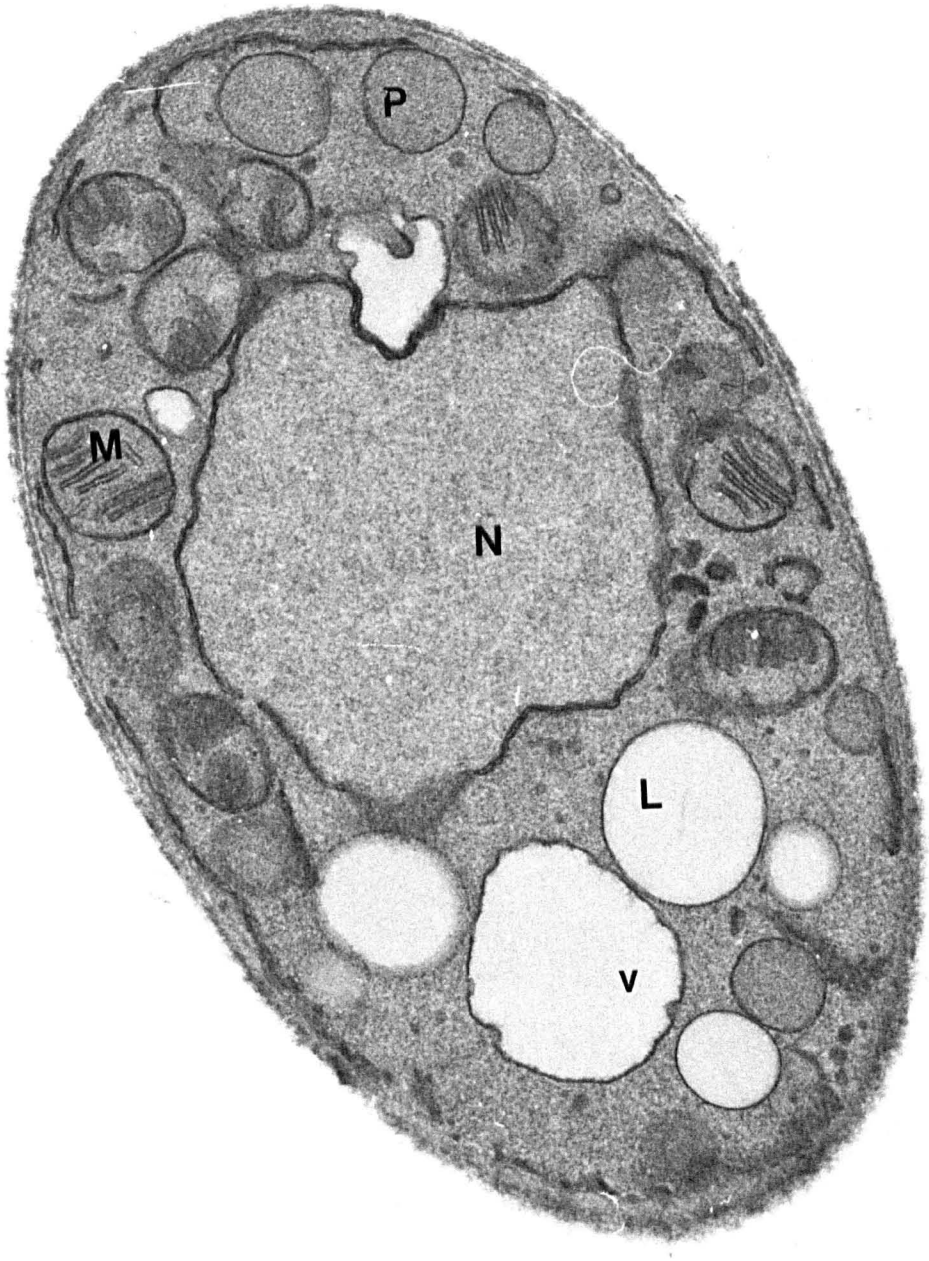
V, vacuole;

N, nucleus;

P, peroxisome.

1 μm

Plate 25.



Legend to Plate 26

Thin section of Lipomyces starkeyi CBS 1809 grown for 2 days on glucose in complete medium (Methods, Section 1.2). The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 30,000.

The cells contained small lipid globules but no glycogen granules. Many mitochondria and the nucleus were visible in the section. The cells usually contained one very small peroxisome.

L, lipid globule;

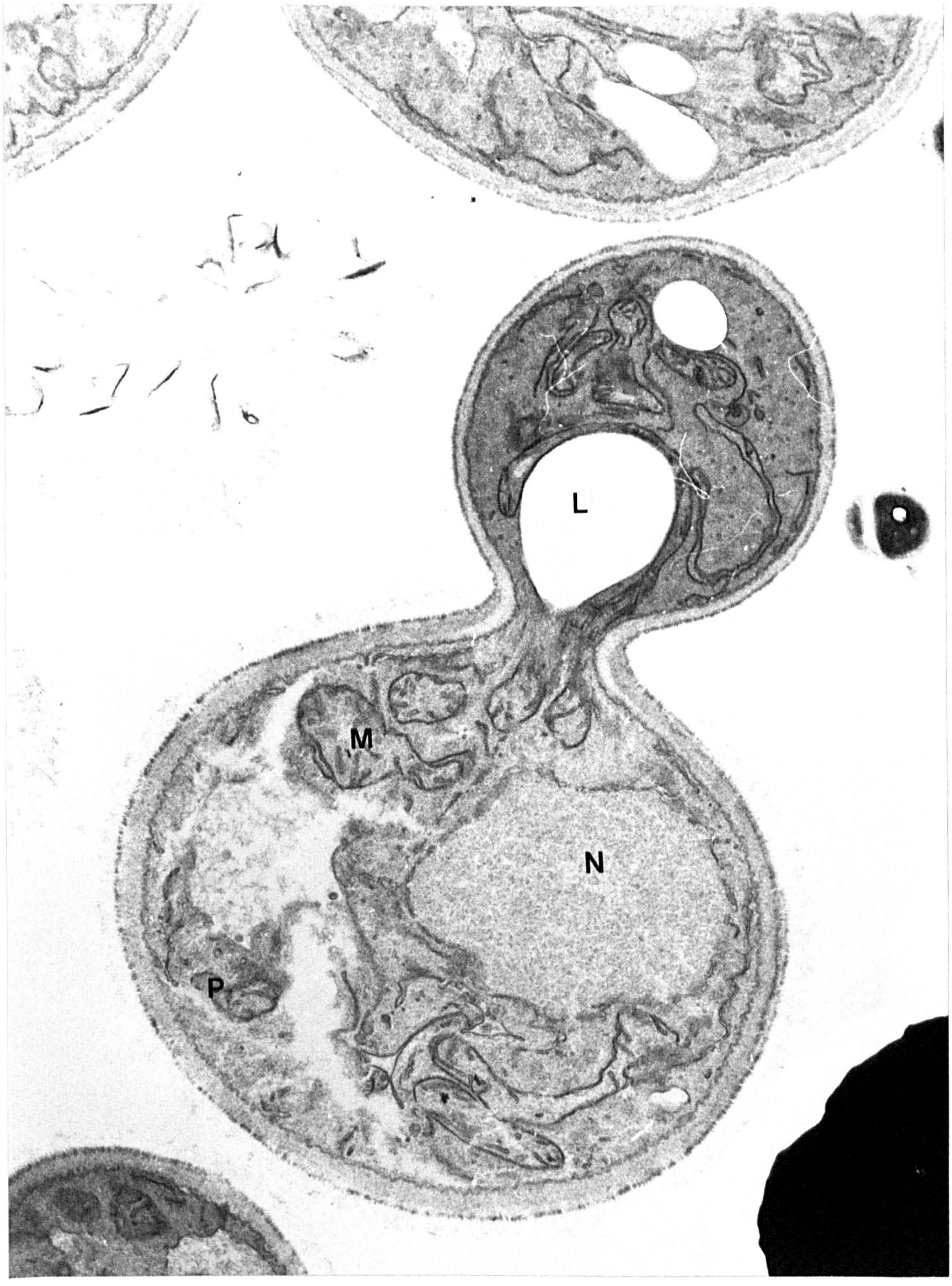
M, mitochondrion;

N, nucleus;

P, peroxisome.

1 μm

Plate 26.



Legend to Plate 27

Thin section of Lipomyces starkeyi CBS 1809 grown for 24 hours on ethanol medium (Methods, Section 1.2). The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 73,000.

The cells contained one lipid globule but no glycogen granules. The nucleus and mitochondria were visible in the section. There was a small increase in the peroxisome fraction over that of the glucose-grown control (Plate 26).

N, nucleus;

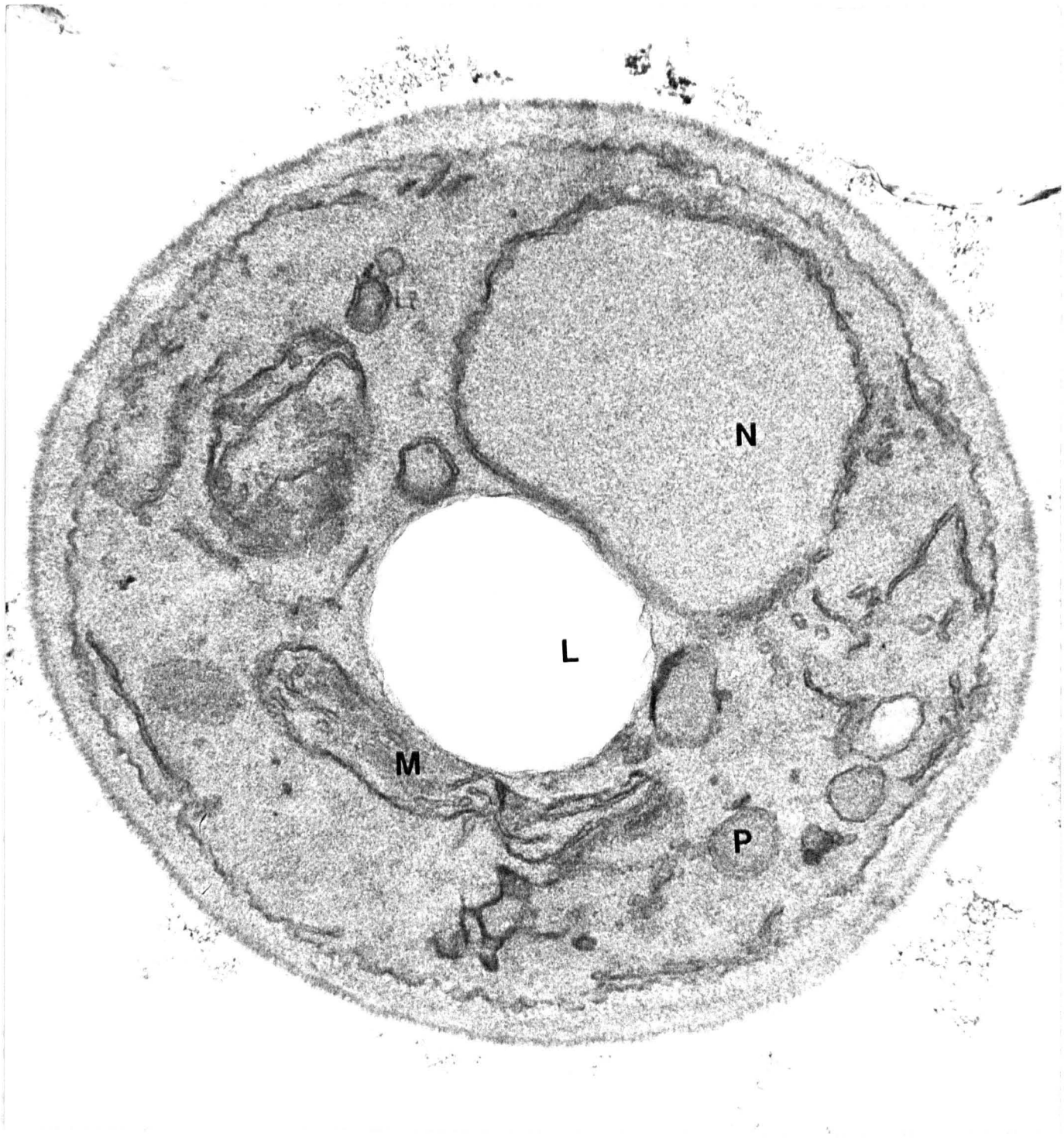
L, lipid globule;

M, mitochondrion;

P, peroxisome.

1 μm

Plate 27.



Legend to Plate 28

Thin section of Lipomyces starkeyi CBS 1809 grown on nitrogen-limited medium (Methods, Section 1.2) for 2 days. The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification: x 55,000.

The cells contained lipid globules as well as glycogen granules. Mitochondria and the nucleus were visible in the section. It was not possible to identify any microbodies.

L, lipid globule;

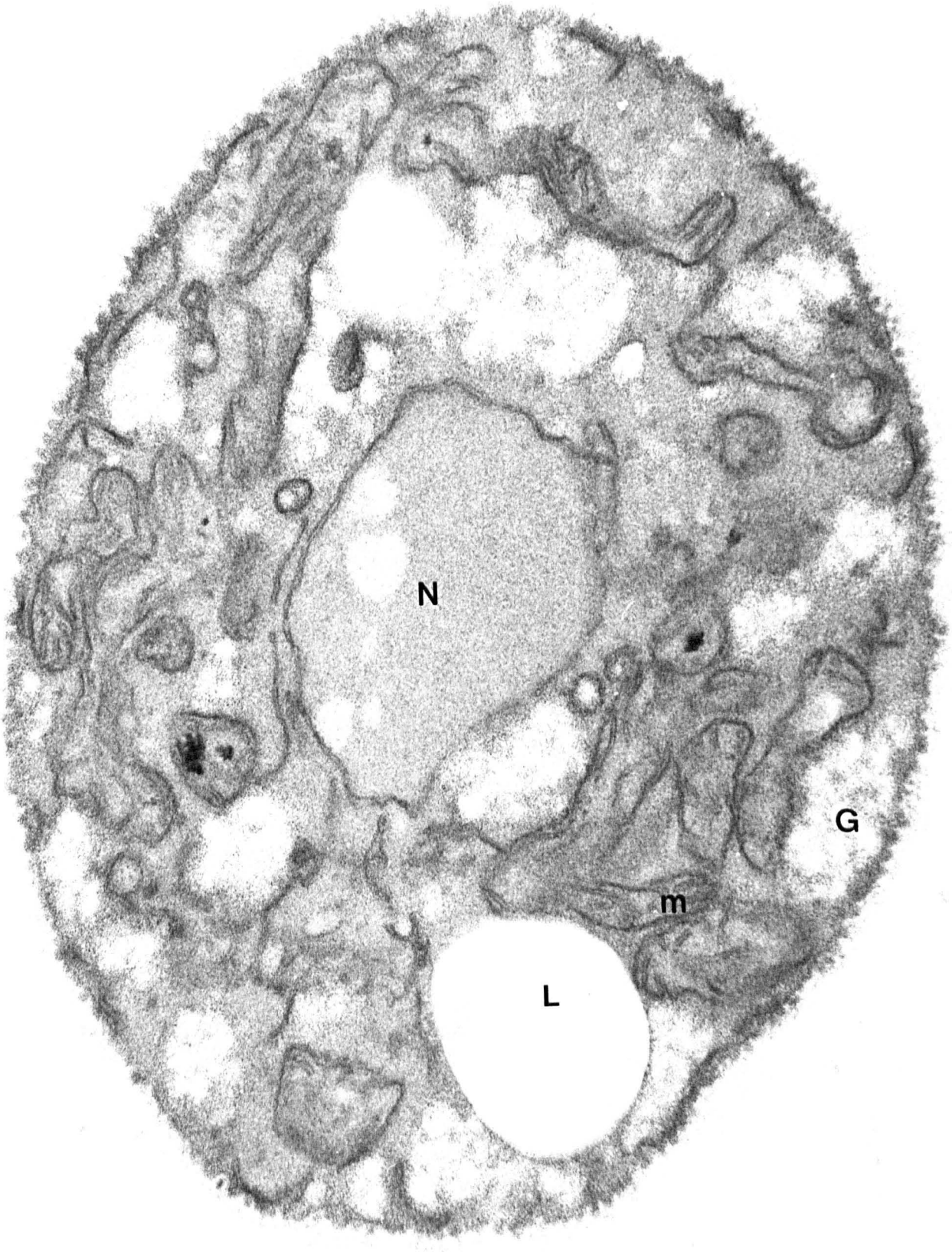
N, nucleus;

M, mitochondria;

G, glycogen.

1 µm

Plate 28.



Legend to Plate 29

Thin section of Lipomyces starkeyi CBS 1809 after growth for 2 days such that lipid accumulated (nitrogen-limited medium) followed by growth for 18 hours on carbon starvation medium (Methods, Section 1.2). The cells were fixed with $KMnO_4$ according to Method B (Methods, Section 9.2).

Magnification:: x 35,000.

The lipid globules were still visible within the cells but the glycogen granules were absent. The cells contained vacuoles in which cytoplasm appeared to be broken down (Marten Veenhuis, University of Groningen, Netherlands, personal communication). The nucleus and mitochondria were visible in the section.

N, nucleus;

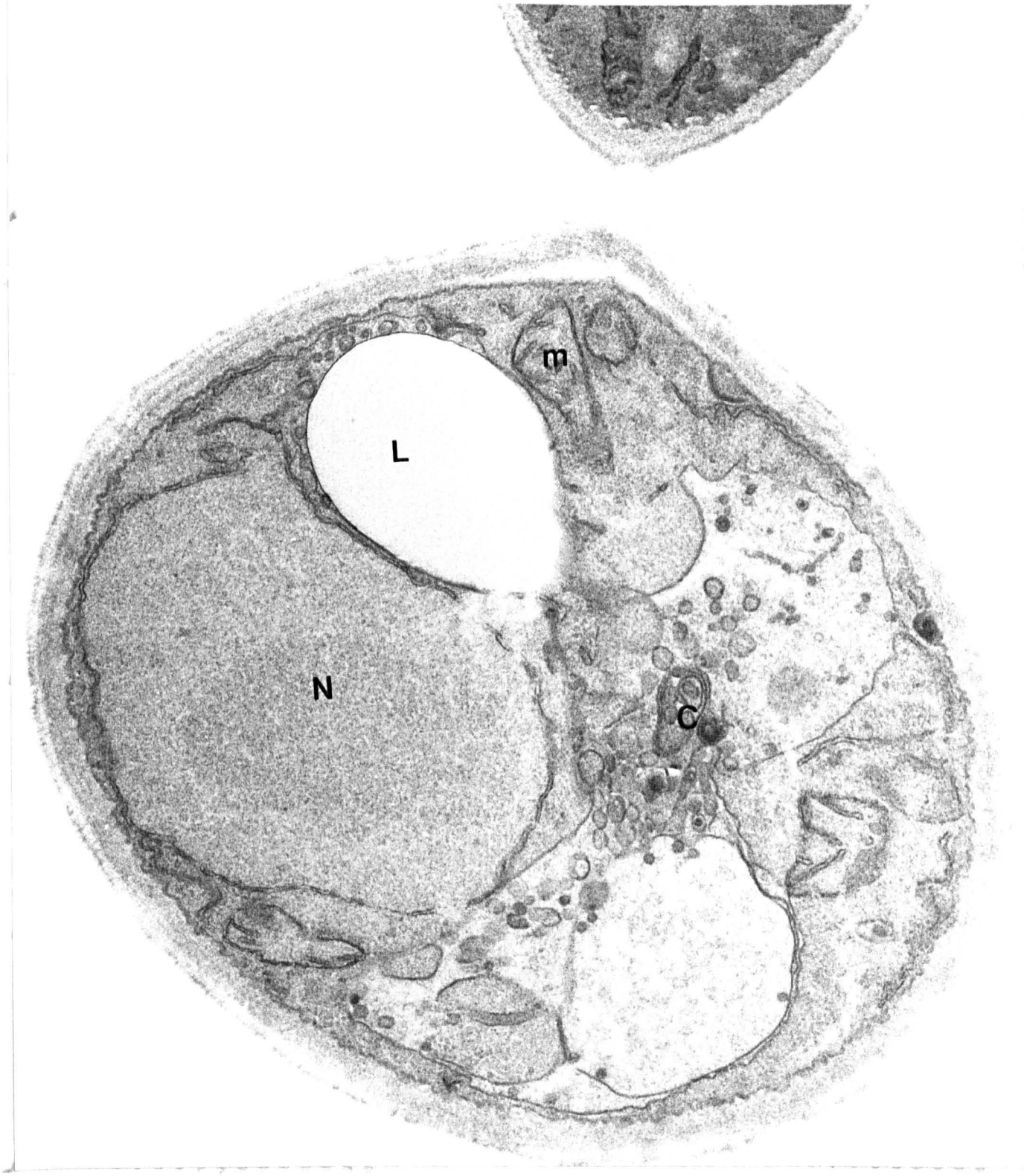
L, lipid globule;

M, mitochondrion;

C, cytoplasmic breakdown in vacuole.

1 μm ——————

Plate 29.



4.2.2. Freeze-etching

The yeast Candida curvata D was observed using the freeze-etching technique in an attempt to ascertain whether there was any difference in peroxisome ultrastructure during growth on triolein and growth on oleate as sole carbon sources.

The micrographs are shown in the subsequent pages and all details are provided in the legends to each photograph.

This work was kindly performed at the University of Groningen by Marten Veenhuis.

Legend to Plate 30

Freeze etched preparation of Candida curvata D grown on triolein medium (Methods, Section 1.2) for 24 hours.

Magnification: x 73,000.

The freeze-etched replicas showed normal smooth fracture faces through the microbodies. The nucleus, with nuclear pores, mitochondria and lipid globules were visible in the replica. The lipid showed micelle-like structure within the globules but there was no suggestion of a membrane around the globules.

N, nucleus;

Np, nuclear pore;

L, lipid globule;

V, vacuole;

P, peroxisome;

M, mitochondria.

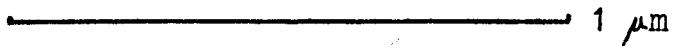


Plate 30.

Legend to Plate 31

Freeze-etched replica of Candida curvata D grown for 24 hours on oleate medium (Methods, Section 1.2).

Magnification: x, 73,000.

The replica showed identical subcellular morphology to the yeast grown on oleate medium (Plate 30). The fracture faces of the microbodies were normal and smooth.

Vacuoles, lipid globules, the nucleus and mitochondria were also visible in the replica.

N, nucleus;

Np, nucleus pore;

V, vacuole;

L, lipid globule;

M, mitochondria;

P, peroxisome.

Plate 31.

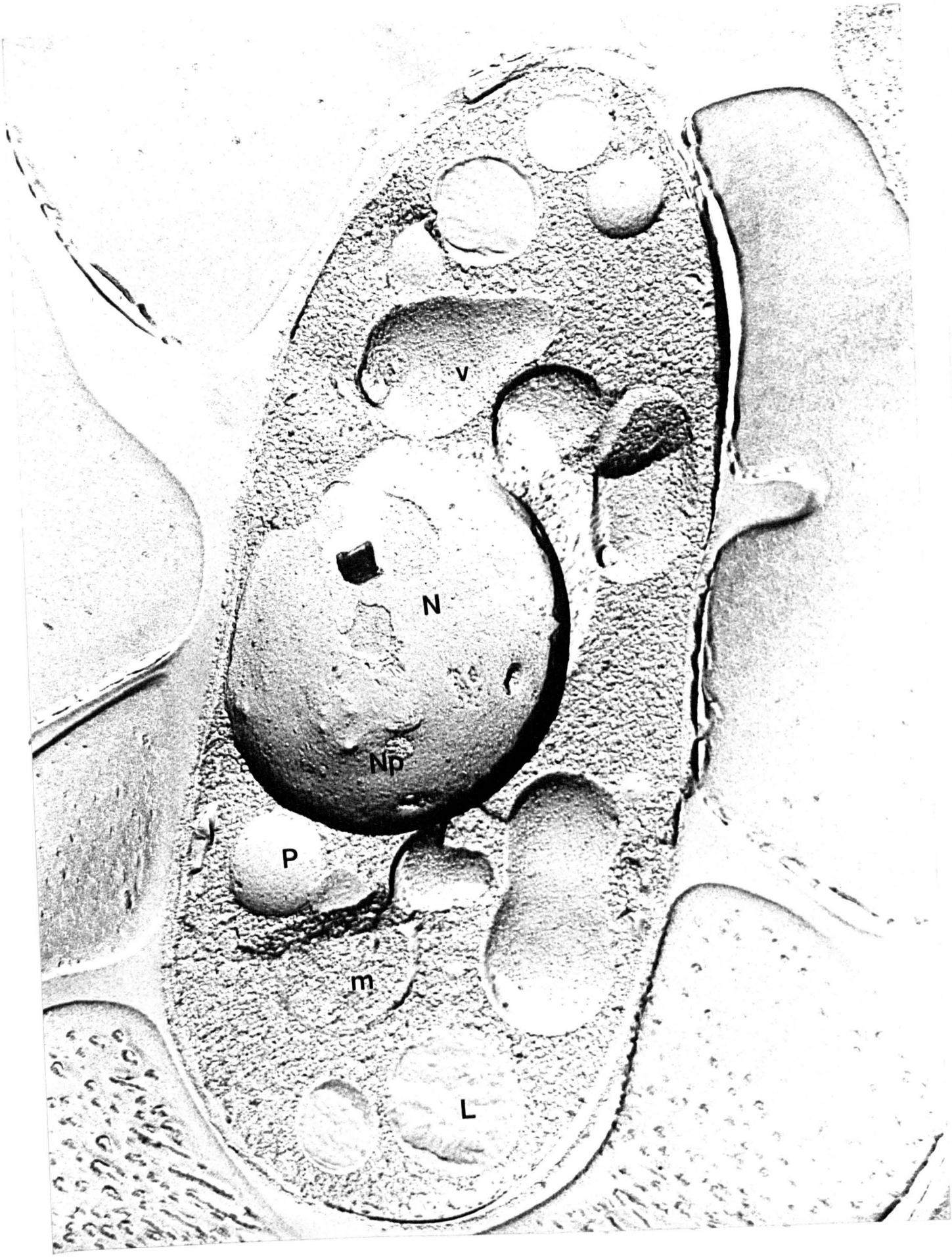


Table 34. Summary of ultrastructural observations in oleaginous yeasts

Organism	Growth source	Status	Main observations
C. curvata D	Glucose	C/N balanced	Low expression of peroxisomes all other organelles normal.
	Glucose	N-limited	Cells contained many small lipid globules, glycogen and usually one small peroxisome.
	Endogenous lipid	starved	Glycogen was absent and lipid content decreased. Slight increase in peroxisome number and volume over glucose-grown cells.
	Triolein	C/N balanced	Many peroxisomes present and also small lipid globules. Glycogen was absent.
	Oleate	C/N balanced	Identical morphology to triolein grown cells.
	Ethanol	C/N balanced	Small increase in peroxisome number over glucose grown controls. Evidence for lipid biosynthesis occurring on endoplasmic reticulum.
L. starkeyi CBS 1809	Glucose	C/N balanced	Cells contained one lipid globule but no glycogen or peroxisomes.
	Glucose	N-limited	Contained one large lipid globule and much glycogen. No peroxisomes were visible.
	Endogenous lipid	Starved	Lipid was still present, but glycogen had disappeared. Cell appeared to breakdown its cytoplasm in vacuoles.
	Ethanol	C/N balanced	Cells contained one large lipid globule and several peroxisomes.

Continued ...

Table 34 - continued

Organism	Growth source	Status	Main observations
T. cutaneum CBS 40	Glucose	N-limited	Contained many small lipid globules or one large one. Glycogen was present.
	Endogenous lipid	Starved	Decrease in lipid globule content, absence of glycogen. A few microbody profiles.
	Triolein	C/N balanced	Small lipid globule content, many microbody profiles.
R. toruloides CBS 14	Glucose	N-limited	Lipid accumulated in two or three large globules. Glycogen was present. High content of endoplasmic reticulum usually associated with the lipid globules.
	Endogenous lipid	Starved	Lipid and glycogen were absent. Endoplasmic reticulum was not as prominent. Increased content of vacuoles.
	Triolein	C/N balanced	Glycogen and lipid were absent. Some peroxisome profiles were present.

D I S C U S S I O N

1. The induction and properties of phosphoketolase in yeasts grown on xylose

The induction of pentulose 5-phosphate phosphoketolase in yeasts by growing them on xylose was first observed by Evans and Ratledge (1984), and this has been confirmed in this study.

All work performed by Evans and Ratledge (1984) used xylulose 5-phosphate (Xu5P) as assay substrate. The difference between Xu5P and ribulose 5-phosphate (Ru5P) only lies in the stereospecific orientation at C-3 which is lost when this carbon atom becomes C-1 of glyceraldehyde 3-phosphate (G3P). Therefore, there was no a priori reason why Ru5P should not also be considered as an alternative substrate and this possibility was investigated.

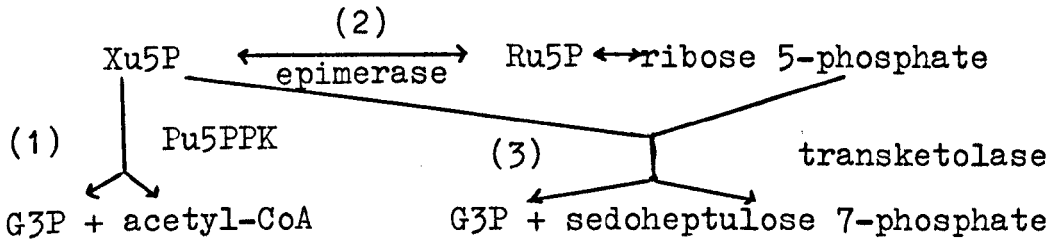
The apparent dual substrate specificity of nine yeasts of seven different genera was demonstrated when assays were carried out using either Xu5P or Ru5P as substrate.

No general trend of substrate preference was observed and five yeasts (Candida curvata, C. famata, Lipomyces starkeyi, Rhodotorula glutinis and Pachysolen tannophilus) were equally active with both substrates: three (C. boidinii, Pichia media and Yarrowia lipolytica) showed a marked preference for Ru5P. Only Rhodosporidium toruloides showed preference for Xu5P.

Attempts to purify the Pu5PPK from C. famata NCYC 33 to resolve the question of substrate specificity were made, but due to the high cost of substrates only partial purification could be achieved within the budget kindly allocated for this work by Cadbury Schweppes plc. Problems were also encountered

in assaying for contaminating enzymes and these are discussed below.

Xu5P, the putative substrate for Pu5PPK can react with three enzymes (1) Pu5PPK, (2) Ru5P-3-epimerase and (3) in the presence of ribose 5-phosphate, with transketolase.



Assay for the epimerase (Williamson and Wood, 1966) was dependent on having a preparation of phosphoketolase which was not reactive with Ru5P and thus was not possible in preparations which contained a phosphoketolase with dual specificity. Assay for transketolase was impossible in extracts containing a phosphoketolase as both enzymes produced glyceraldehyde 3-phosphate which formed the basis for the former assay. Therefore of the three enzymes capable of reacting with Xu5P it was only possible to assay for Pu5PPK. Absence of epimerase could only be inferred if purified Pu5PPK proved incapable of reacting with Ru5P. Competing activity of transketolase for Xu5P would disappear if either the epimerase or the pentose phosphate isomerase (converting Ru5P to ribose 5-phosphate) were eliminated. However for there to be an interfering transketolase, both Xu5P and R5P had to be simultaneously present, requiring the presence of two enzymes no matter which substrate was used in the Pu5PPK assay.

The partial purification of Pu5PPK resulted in a final preparation which was shown to have a specific activity for

Xu5P which was four-fold higher than that for Ru5P. The observed kinetic properties of Pu5PPK from C. famata NCYC 33 were determined using Xu5P as substrate for which a K_m of 2.4 mM and a V_{max} of 0.7 μmol min^{-1} (mg protein)$^{-1}$ were calculated.

The purification of the Pu5PPK did not therefore resolve between the two possibilities:

(a) that the enzyme had dual specificity for both Xu5P and Ru5P, with the suggestion that the former was the preferred substrate in C. famata NCYC 33;

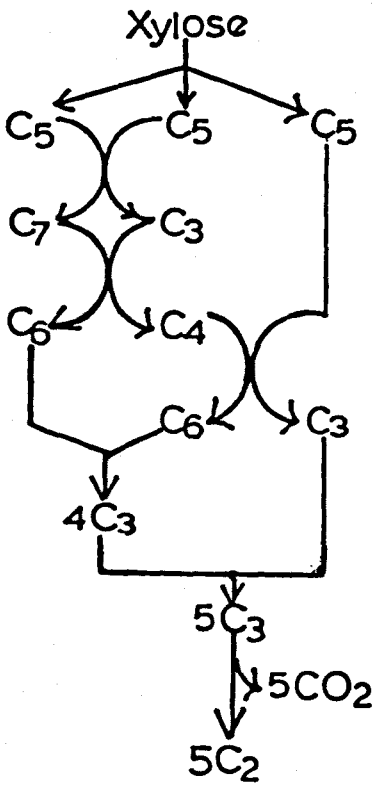
(b) that the enzyme had single substrate specificity for Xu5P and that activity towards Ru5P was due to the continued presence of Ru5P-3-epimerase converting Ru5P (when used as substrate) to Xu5P.

In view of the problems encountered in assaying competing enzymes which have already been discussed and the inability to resolve between the two alternatives, the enzyme must be regarded as a pentulose 5-phosphate phosphoketolase until further information to the contrary is found.

The enzyme had a large size (Mr~5 x 10^5 daltons) and was thus similar to the phosphoketolase from Lactobacillus plantarum (Heath et al., 1958) which has a molecular weight of 550,000. The bacterial enzyme however has no activity towards any substrate other than Xu5P and thus may prove to be different to the yeast enzyme. There was no evidence of product inhibition (acetyl-phosphate and glyceraldehyde 3-phosphate) or inhibition by citrate or acetyl-CoA with the yeast enzyme.

Scheme 5: Routes for pentose metabolism

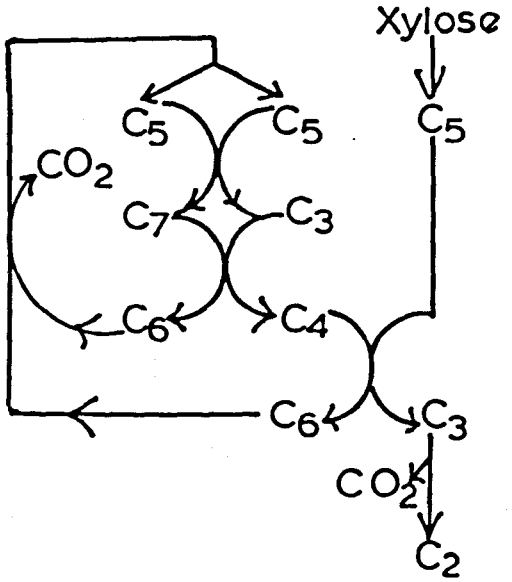
I



Net reaction

$C_5 \rightarrow 1{\cdot}67\,C_2 + 1{\cdot}67\,CO_2$

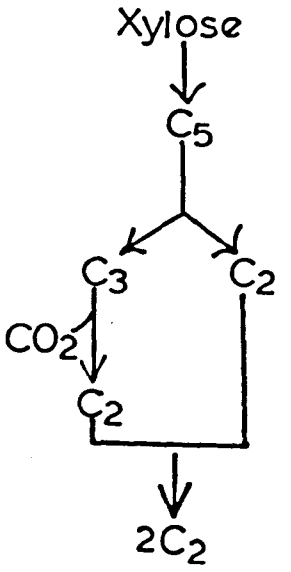
II



Net reaction

$C_5 \rightarrow C_2 + 3CO_2$

III



Net reaction

$C_5 \rightarrow 2C_2 + CO_2$

I Metabolism via the pentose phosphate pathway and glycolytic pathway for hexose and triose phosphate metabolism.

II Metabolism via pentose phosphate pathway with recycling of C_6 units (to provide NADPH).

III Metabolism via xylulose 5-phosphate phosphoketolase.

The initial metabolism of xylose in yeasts is usually its conversion to xylulose via intermediate formation of xylitol using xylose reductase and xylulose dehydrogenase (Chakravorty et al., 1962; Smiley and Bolen, 1982; Maleszka et al., 1983). The direct conversion of xylose to xylulose via xylose isomerase occurs mainly in bacteria (Gong et al., 1981) but it has been reported in the yeast Rhodotorula gracilis (Hofer et al., 1971b). In all cases xylulose is then phosphorylated to xylulose 5-phosphate by xylulose kinase (Gong et al., 1983).

It is currently accepted according to most textbooks and research papers that a yield of 5 mol of C_2 from 3 mol of xylose occurs by metabolism via the pentose phosphate pathway and by subsequent metabolism of triose and hexose via the Embden-Meyerhof-Parnas pathway (Scheme 5, Route I). Such a yield of C_2 units is however a theoretical maximum as it assumes no recycling of the C_6 units produced via the transketolase/transaldolase reactions is occurring.

The production of significant amounts of biomass, as well as accumulation of xylitol, from xylose as sole carbon source, does however require some recycling of these C_6 units from the pentose phosphate pathway to generate NADPH (Bruinenberg et al. 1983a). The first enzyme of xylose catabolism, xylose reductase, is strictly NADPH-dependent in all yeast except Pichia stipitis (Bruinenberg et al. 1983b; Smiley and Bolen, 1983) and so xylose-grown cells will require a higher concentration of NADPH to be produced than glucose-grown cells. Bruinenberg et al. (1983a) have calculated

that this may represent a doubling of the requirement in yeasts in general and that the pentose phosphate pathway, via glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, serves as the principal supplier of this reduced co-factor. Thus the true yield of C_2 units from xylose will be substantially less than the 1.67 mol indicated by Route I, though clearly not all the hexose units will be recycled as shown in Route II which provides for only 1 mol C_2 to be generated from each mol of xylose. If it is assumed that approximately 20 to 25% of hexose would need to be recycled, in keeping with the amount so processed when glucose is a carbon source (Wood et al. 1963), then the molar yield of C_2 from xylose would be approximately 1.5. This would then be insufficient to account for the high yields of ethanol (Chiang et al. 1981) and also biomass (Evans and Ratledge, 1983) which have been obtained from xylose.

Such deductions as these led to the original discovery of phosphoketolase by Evans and Ratledge (1984) in a wide variety of yeasts. The activity of phosphoketolase is shown in Route III. This Route obviously plays a key role in pentose metabolism, along with channelling through the pentose phsophate pathway to provide C_6, C_5 and C_4 intermediates and the recycling process (Route II) to provide NADPH for biosynthetic purposes. How a xylose-utilizing organism regulates its metabolism of pentulose phosphates between the various Routes, remains for the time being, an intriguing and open question.

Phosphoketolase activity in relation to lipid levels

The discovery of phosphoketolase (PK) activity in oleaginous yeasts grown on xylose which could provide C-2 units

directly in the cytosol led to the postulation of two theories:

(a) That these yeasts may no longer have a requirement for the enzyme ATP:citrate lyase, which cleaves citrate to acetyl-CoA and oxaloacetate and provides the building blocks for fatty acid biosynthesis in oleaginous yeasts;

(b) That yeasts grown on xylose may be capable of accumulating greater percentages of lipid than those grown on glucose, due to the provision of C-2 units by PK as well as by ATP:citrate lyase.

The latter proposal was postulated due to the findings of Evans and Ratledge (1984) who demonstrated that lipid levels in C. curvata D were increased from 33.2% to 48.6% (w/w) when grown on xylose instead of glucose as sole carbon source.

Lipid levels in oleaginous yeasts grown on xylose in this study were found to be the same as those observed when grown on glucose in all yeasts except L. starkeyi CBS 1809. This yeast showed a slight increase in levels when grown on xylose. Therefore, proposal (b) was thought unlikely to have a significant effect.

ATP:citrate lyase activities in yeasts grown on xylose were the same as those grown on glucose and hence it appeared unlikely that proposal (a) had any significance.

Therefore it was concluded that the additional acetyl-CoA formed by the phosphoketolase reaction did not exert any control over ATP:citrate lyase activity. Furthermore, as the additional acetyl-CoA formed from xylose did not lead to increased fatty acid biosynthesis (and hence more lipid),

the factors influencing the amount of lipid accumulated by the cells must lie outside the control of the size of the acetyl-CoA pool.

Phosphoketolases therefore play a key role in growth on xylose by acting with the pentose phosphate pathway in the breakdown of xylose to produce metabolic intermediates for the production of biomass and extracellular products such as xylitol and ethanol.

2. Degradation of storage lipid by oleaginous yeasts

The potential for developing a system for the commercial production of microbial fats and oils by fermentation was discussed in the Introduction to this thesis. One important aspect of such a process would be the effect that downstream processing would have on the microbial oil product. The action of lipases within the cell could, for example, lead to the unwanted degradation of this product.

The turnover or utilization of the accumulated lipid was therefore studied in starved yeasts in order to assess whether or not lipid degradation for cell proliferation could occur. The time scale for such a process was also studied.

Experiments were carried out in batch culture, in two-stage continuous culture and in single stage continuous culture using a transition from N-limited (high fat) medium to carbon-free/nitrogen high medium. In each type of experiment the effect of starving a yeast which had previously accumulated lipid was examined.

Batch culture studies were used to screen nine yeasts of four different genera for their potential to degrade

their storage lipid for cell proliferation. All yeasts examined except those of the species Lipomyces were capable of such utilization of storage lipid for the production of new biomass (Results, Chapter 2, Tables 5 and 6).

The reason for the inability of the Lipomyces to degrade their storage lipid was not apparent. One possibility which was examined was that this species of yeast may lack a lipase. Examination for the presence of lipases in the four species of yeast was undertaken, but unfortunately the detection of these enzymes proved to be very difficult, even in cells which were known to be degrading lipid. Therefore the reason for the inability of the Lipomyces to degrade their storage lipid remains uncertain. However, the fact that this may be of value in the commercial production of microbial oil was noted. Owing to the lack of product degradation in the Lipomyces species which could potentially occur during downstream processing in the other three species, they may be more suitable organisms for such a process.

Values for the yield of increase in biomass per decrease in lipid (g/g) were calculated for all yeasts which were capable of lipid degradation in batch culture and these ranged from 0.9 to 1.21 g biomass per g lipid. At the time of performing these experiments the fact that these yeasts simultaneously accumulate another storage product, namely glycogen was not known. This fact only came to light towards the end of the period of study when the transmission electron microscopy studies were performed. These revealed a granular

material within the cells which accumulated together with the lipid (Results, Chapter 4, Plates 9, 12, 14, 17, 22, 28). Subsequent analysis revealed that this was glycogen (Results, Chapter 2, Table 7).

The glycogen accounted for quite high percentages of biomass in some yeasts and as much as 26% (w/w) was recorded for the yeast Trichosporon cutaneum CBS 40. The accumulated glycogen was shown to be degraded rapidly after the onset of carbon starvation in the yeasts C. curvata D and T. cutaneum CBS 40 and only approximately 5% remained after two hours starvation. Disappearance of glycogen occurred slowly in the yeast L. starkeyi CBS 1809 and the percentage had only fallen from 23% (w/w) to 13% (w/w) after 24 hours.

Therefore it is apparent that the yields of increase in biomass per decrease in lipid (g/g) recorded in this thesis are overestimates as some of the increase in biomass would have been due to glycogen degradation.

Glycogen has long been known as a storage polysaccharide in yeast cells (Ling et al., 1925; Northcote, 1953; Trevelyn and Harrison, 1952). The accumulation of glycogen simultaneously with lipid has previously been reported by Pitryuk et al (1974). They observed that species of Lipomyces, Cryptococcus and Rhodotorula could accumulate up to 6.5% (w/w) of glycogen under nitrogen-limited conditions. However most of the values they reported were significantly lower than those detected in the yeasts examined in this study.

In order to assess the true amount of cell proliferation due to lipid degradation it would be necessary to repeat all lipid turnover studies and monitor glycogen levels within the cells as well as biomass and lipid levels.

The discovery of accumulated glycogen within these yeast is an interesting finding. It opens doors for the consideration of how these yeasts might be manipulated, such that all glucose is converted into lipid and hence might lead to the development of a yeast which has the potential to accumulate much greater percentages of lipid.

Two-stage chemostat studies with the yeast C. curvata D revealed similar results to those obtained in batch culture, (Results, Chapter 2, Table 8). The greatest yields of increase in biomass per decrease in lipid (g/g) were observed at a dilution rate of 0.053 h^{-1}. Similar considerations regarding simultaneous accumulation of glycogen with lipid to those in batch culture apply. Hence the actual yields for increase in biomass per decrease in lipid (g/g) were probably overestimates. The experiment would require repeating with the simultaneous monitoring of glycogen levels in order to give accurate values.

Single stage chemostat transition studies were attempted in order to establish a time scale for the process of lipid degradation and to try and identify a trigger event. These revealed that lipid was broken down soon after the depletion of glucose in the external medium and the supply of a nitrogen source. The lipid levels were observed to fall from 30% to 23% within 1.5 hours of the onset of carbon starvation (Results, Chapter 2, Figure 11).

The rapidity with which the yeast is capable of ceasing lipid accumulation and commencing lipid degradation is interesting and would suggest rigorous control of the pathways.

In mammals, many of the controls of rapidly regulated pathways lie at the level of phosphorylation, dephosphorylation covalent modification. An example is the control of glycogen metabolism where the activity of a cascade reaction links cyclic AMP with the crucial enzymes of glycogen metabolism.

It is possible that such a cascade reaction may be responsible for the high degree of control of lipid metabolism in the oleaginous yeast.

The results of the radioactive transition experiment indicated that, although part of the increase in biomass observed in two stage batch and continuous culture may have been due to glycogen turnover, at least part of the new biomass arose from breakdown of lipid. This was demonstrated by the appearance of ^{14}C label from the lipid in the biomass fraction within 1.5 hours of transition from lipid accumulation to carbon starvation (Results, Chapter 2, Figure 13).

Therefore, although the yields of increase in biomass per decrease in lipid (g/g) may be overestimates there can be no doubt that lipid degradation for synthesis of cell components did occur. The process of lipid degradation was also rapid and highly controlled.

3. Enzymic changes accompanying lipid degradation

The ability of oleaginous yeasts to rapidly degrade their storage lipid under carbon starvation conditions

indicated a highly controlled lipid metabolism.

It was therefore expected that key enzymes activities involved in lipid biosynthesis and degradation would be tightly controlled within the cell. Study of seven of these enzymes was carried out during growth such that lipid accumulated, lipid was degraded and during growth on an external lipid source (triolein) in batch culture. Activities were also examined in two-stage continuous culture.

3.1. ATP:Citrate Lyase

This enzyme which plays a key role in lipid biosynthesis by virtue of the fact that it furnishes acetyl-CoA units in the cytoplasm for fatty acid biosynthesis and hence lipid biosynthesis was examined in four yeasts of three species (Results, Chapter 2, Tables 10 and 11).

Activities were high during lipid accumulation but were greatly decreased during growth on either internal storage lipid or the external lipid source. The enzyme is known to be inhibited by fatty acyl-CoA esters (FACES) Boulton (1982) which are one of the breakdown products of triacylglycerol degradation. Originally it was thought that this lowering of activity was probably due to such an inhibition. However, addition of extract which exhibited low activity to that which exhibited high activity did not alter the observed activity of the latter. Hence it was thought that the extract which showed low activity did not contain FACES. Therefore it was thought that the effect was possibly due to a repression of enzyme synthesis.

3.2. Peroxisomal Enzymes

The enzymes catalase, isocitrate lyase and $NADP^+$-dependent isocitrate dehydrogenase were studied under the three growth conditions because these enzymes are reported to be located in the peroxisome (Fukui and Tanaka, 1979).

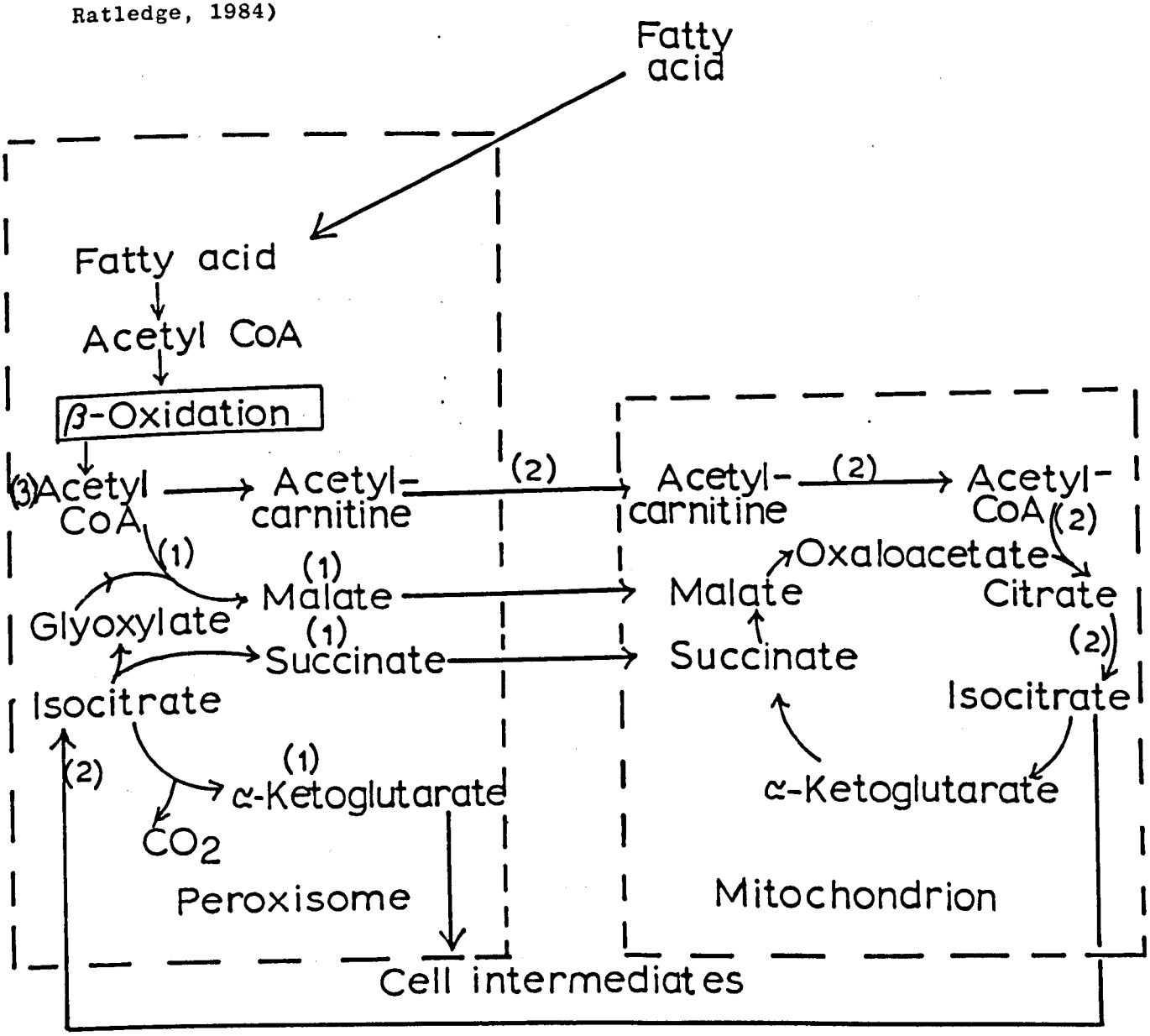
Peroxisomes are subcellular organelles characterised biochemically by the presence therein of one or more hydrogen peroxide-producing oxidases and catalase. Morphologically, these organelles are characterised by a single limiting membrane, a fine granular matrix, and often a crystalline or tubular core. The development of peroxisomes in yeasts is closely correlated to the increase in catalase activity and the assimilation of alkanes or higher fatty acids (Teranishi et al, 1974). This indicates the participation of these organelles and catalase in alkane and fatty acid metabolism. Degradation of fatty acids to acetyl-CoA via the β-oxidation pathways is reported by Fukui and Tanaka (1981) to exclusively occur in the peroxisome. The physiology of hydrocarbon utilising yeasts was reviewed by Boulton and Ratledge (1984)and Scheme 6 represents the proposed role for peroxisomes and mitochondria in the degradation of fatty acids in a yeast.

Specific activities of catalase and isocitrate lyase and $NADP^+$-dependent isocitrate dehydrogenase were found to be enhanced in both C. curvata D and T. cutaneum CBS 40 during lipid degradation and growth on triolein. Similar effects were seen in the yeasts R. toruloides CBS 14 and R. toruloides ATCC 26217 but there was no enhancement of

Scheme 6

Proposed role for peroxisomes and mitochondria in the degradation of fatty acids

(adapted from Fukui and Tanaka, 1979a,b, 1981; by Boulton and Ratledge, 1984)



A possible stoichiometric flow of carbon is given: for every three molecules of acetyl-CoA produced by β-oxidation in the peroxisome, one molecule of α-ketoglutarate and one molecule of CO_2 can be produced. Other products, e.g. oxaloacetate to give aspartate etc. could equally be proposed.

activity of catalase in these yeasts (Results, Chapter 2, Tables 10 and 11).

The enhancement of catalase activity was slight in all yeasts grown on their own storage lipid, and was markedly higher during growth on the external lipid source.

These results indicated that peroxisomes were induced in these yeasts during growth on both endogenous and exogenous lipid sources. These findings were also confirmed using electron microscopy studies (Results, Chapter 4, Plates 10 to 31). These revealed that in L. starkeyi CBS 1809 and C. curvata D, peroxisomes did proliferate during carbon starvation conditions. They were also shown to proliferate in both yeasts grown on ethanol and in C. curvata D during growth on oleate and triolein (L. starkeyi would not grow on either of these substrates).

3.3. Malic Enzyme

This enzyme, which is thought to have an integral role in lipid biosynthesis in the production of the reducing power in the form of NADPH for fatty acid biosynthesis was examined.

Specific activity of this enzyme was greatly reduced in yeasts grown on triolein, and was undetectable in two of the yeasts examined. The specific activity during utilization of endogenous lipid reserves was similar to that observed during lipid accumulation both in continuous and in batch culture (Results, Chapter 2, Tables 10 and 11).

The proposal of this role for malic enzyme in oleaginous yeasts was first put forward by Botham and Ratledge (1979).

They confirmed earlier findings by Whitworth and Ratledge (1975a) who detected the enzyme in the yeast Candida 107. However, Botham and Ratledge (1979) failed to detect malic enzyme in two oleaginous yeast strains of Lipomyces (CBS 1807 and CBS 1809). Furthermore, the observed activities from Candida 107 did not correlate with the prevailing lipid content.

Malic enzyme therefore appears not to be essential to the process of lipid accumulation. In yeasts where it is present, its role appears to be in contribution to the supply of reducing power (NADPH) for fatty acid biosynthesis. In yeasts where it is absent, this role is probably performed by the pentose phosphate pathway.

3.4. Carnitine acetyl-transferase (CAT)

Transfer of acetyl-CoA units into the mitochondrion for subsequent degradation by the action of the TCA cycle is achieved by transfer of the acyl group to carnitine. Acyl-carnitines unlike acyl-CoAs are able to cross the permeability barrier of the inner mitochondrial membrane. Once inside the mitochondrion, the acyl group is returned to CoA.

The work of Kohlaw and Tan-Wilson (1977) proposed that this pathway was also of significance in transfer of acetyl-CoA out of the mitochondrion for the synthesis of fatty acids. Recent work by Ratledge and Gilbert (1985) has shown that in the oleaginous yeast this is unlikely to be the major method of supply of acetyl-CoA units to the cytoplasm and that there was no correlation between the specific

activity of CAT and the amount of lipid in an oleaginous yeast. However, the enzyme was present even in yeasts with the highest ATP:citrate lyase activity.

Carnitine acetyl transferases of both peroxisomes and mitochondria have been isolated (Ueda et al, 1982) and are similar enzymes with respect to molecular size, general properties and restricted specificity for acetyl- and propionyl-CoAs.

Specific activities of CAT were greatly enhanced during growth on triolein in all yeasts examined in this study (Results, Chapter 4, Tables 10 and 11). This was in accordance with the findings of Ratledge and Gilbert (1985).

Specific activities were not significantly enhanced, however, during growth on endogenous lipid reserves. The reason for this was not apparent but a possibility is that during this process only slight induction of peroxisomes occurs and enhancement of the activity is too small to be detectable.

The key enzymic changes occurring during lipid utilization in oleaginous yeasts grown in batch and continuous culture are summarised in Figure 17.

4. Triacylglycerol synthesis in oleaginous yeasts

Early information on the biochemistry of the neutral lipids of yeast came from light and electron microscopic studies of lipid granules (Matile, et al, 1969). These structures were observed to accumulate in stationary phase cells, during sporulation and under some conditions of unusual unbalanced growth. Later work led to the isolation of these lipid granules and the discovery that their principal component was triacylglycerol.

Figure 17

Summary of enzymic changes during lipid utilization in oleaginous yeasts grown in batch and continuous culture

(1) ATP:citrate lyase

$$\text{Citrate} \xrightarrow{(-)} \text{Oxaloacetate} + \text{Acetyl-CoA}$$

(2) Carnitine acetyl-transferase

$$\text{Carnitine} + \text{Acetyl-CoA} \xrightarrow{(+)} \text{Acetyl-carnitine} + \text{CoA}$$

3. Malic enzyme

$$\text{L-Malate} + NADP^{+} \xrightarrow{(-)} \text{Pyruvate} + NADPH + H^{+}$$

4. Isocitrate lyase

$$\text{Isocitrate} \xrightarrow{(+)} \text{succinate} + \text{glyoxylate}$$

5. Catalase

$$2H_2O_2 \xrightarrow{(+)} 2H_2O + O_2$$

6. $NADP^{+}$-Dependent isocitrate dehydrogenase

$$\text{Isocitrate} + NADP^{+} \xrightarrow{(+)} \alpha\text{-ketoglutarate} + NADPH + H^{+} + CO_2$$

Key

(-) Activity decreases

(+) Activity enhanced

The endoplasmic reticulum is considered to be the site for triacylglycerol synthesis in mammalian cells. However, membrane-bound lipid particles of beef heart and lipid micelles of rat adipose tissue have also been reported to contain enzymes necessary for triacylglycerol synthesis (Christiansen, 1975; Matsuoka et al, 1974). Such enzymes have also been detected in association with triacylglycerol containing particles in the fat fraction of plant cells such as castor bean seeds and Crambe seeds (Harwood et al, 1971; Gurr et al, 1974).

In yeasts, little study has been made of the triacylglycerol biosynthesizing system. All work which has been performed used non-oleaginous yeasts and Christiansen (1978) has reported that the lipid particles are the site of synthesis in S. cerevisiae. Belov and Davidova (1982) reported similar findings for the yeast C. tropicalis.

Initial study of triacylglycerol biosynthesis in C. curvata D and L. starkeyi CBS 1809 was largely unsuccessful. It was not possible to detect activity capable of accounting for the known specific rate of synthesis in these yeasts. Although assays were attempted using cell-free extracts and subcellular-fractions, activities equal to those obtained by Christiansen (1978) in S. cerevisiae were never detected (Results, Chapter 3, Tables 12, 13, 14, 16, 17 and 18). Other unsuccessful attempts to detect the enzymes of triacylglycerol biosynthesis in cell-free extracts involved using a variety of different acyl acceptors, use of a more hydrophobic environment for assays and addition of

protease inhibitors (Results, Chapter 3, Tables 19 and 20). Use of extracts prepared from cells grown on different media did not lead to success in detecting the enzymes. The only addition to assays which showed any stimulation of activity was the use of cyclodextrins as emulsifiers for oleate. α- And β-cyclodextrins are composed of cyclohexamylose and cycloheptamylose respectively. They are capable of binding lipid derivatives below the critical micellar concentration and it is thought that the lipid is included within the hydrophobic space of the helical chain (Yabusaki and Ballou, 1978). Addition of these compounds to the assay mixture led to vast stimulation of incorporation of label into acyl-CoA and slight incorporation of label into phosphaditidic acid (Results, Chapter 3, Table 21). Therefore it appeared that use of these cyclodextrins led to the provision of a more hydrophobic environment which stimulated action of the acyl-CoA synthetase enzymes, but not the acyltransferase enzymes of the α-glycerol 3-phosphate pathway.

Final attempts to detect enzymes of triacylglycerol biosynthesis involved the use of whole sphaeroplasts as protein source in the assay mixture. This proved to be a system which was successful and incorporation of label into all intermediates of the α-glycerol 3-phosphate pathway was observed (Results, Chapter 3, Table 22). The amount of label incorporated into triacylglycerol (117 nmol oleate incorporated/h/mg protein) was not however sufficient to account for the observed specific rate of lipid synthesis calculated at 490 nmol [1-^{14}C]oleate incorporated/h/mg protein from the

work of Boulton (1982), and documented in Results, Section 3.1.1. However, rates of incorporation were markedly higher than those previously observed with these yeasts and it was initially hoped to increase values by standardising the assay system. The endogenous acylation level was consistently high implying that the levels of acyl acceptor were also high in the cell.

A linear rate of incorporation of label into diacylglycerol and triacylglycerol was observed over 30 minutes in C. curvata D and L. starkeyi CBS 1809. Incorporation of label into acyl-CoA and other intermediates of the pathway was not linear over this time scale but did not limit flow of label through the pathway (Results, Chapter 3, Figures 14 and 15). This finding was consistent with phosphatidic acid phosphatase being the rate-limiting step in the biosynthetic pathway as demonstrated in liver (Smith, Weiss and Kennedy, 1957).

Assays performed using whole sphaeroplasts as protein source and those using lysed sphaeroplasts yielded similar results. Therefore it was concluded that the reason for the inability to detect activity in previously used extracts was due to the slow speed centrifugation (2000 xg for 5 minutes) which was intended to remove whole cells and debris, sedimenting a subcellular component necessary for biosynthetic activity. Confirmation that this loss of activity was not due to a co-operative effect between constituents of pellet and extract being lost on fractionation was achieved by demonstrating that there was no rate enhancement

on mixing these fractions.

It was finally demonstrated that gentle homogenisation (10 strokes of the homogeniser) could be used to prepare extracts from the sphaeroplasts which had triacylglycerol biosynthesizing activity. However care had to be taken in the use of this technique as a greater number of strokes (20) was shown to lead to partial loss of activity (Results, Chapter 3, Table 24). A possible explanation for this is that the lesser number of strokes simply led to gentle separation of the subcellular components and that the greater number led to actual homogenisation of the cell and some of the subcellular components resulting in the loss of activity.

The initial investigation of triacylglycerol biosynthesis in C. curvata D and L. starkeyi CBS 1809 followed the methods of Christiansen (1978) whose investigation was carried out using the non-oleaginous yeast S. cerevisiae. The work of Christiansen (1978) used subcellular fractions of S. cerevisiae and hence could not be directly compared with activities determined in crude extracts of C. curvata D and L. starkeyi CBS 1809. Assays were therefore performed using whole sphaeroplasts prepared from three strains of S. cerevisiae in order to generate data which could be directly compared with the data for C. curvata D and L. starkeyi CBS 1809. A marked difference between strains was noted (Results, Chapter 3, Table 25). Triacylglycerol biosynthesis was negligible in extracts from strain NCYC 1006 although a small activity of acyl-CoA synthetase was detected. Activity with strain NCYC 24860 was high and equalled that

observed in the oleaginous yeast C. curvata D. Activity in strain NCYC 87 was lower than that in strain NCYC 24860 and resembled that of L. starkeyi CBS 1809.

Christiansen (1978) claimed that the amount of protein associated with the crude lipid particle fraction of S. cerevisiae constituted only 0.3% of the total protein of the cytoplasmic extract. His observed rate of triacylglycerol biosynthesis in the lipid fraction of this yeast was 380 nmol [1-^{14}C]oleate incorporated/h/mg protein. Therefore if 1 mg of lipid droplets contains 0.3% of the total cytoplasmic protein, 100% would equal 333.3 mg and the rate of lipid synthesis in a whole cytoplasmic extract would be of the order of 1.14 nmol [1-^{14}C]oleate incorporated/h/mg protein. This value is much lower than that observed in the strains of S. cerevisiae examined in this study. Therefore, the possibility arises that Christiansen (1978) may only have observed high rates of triacylglycerol biosynthesizing enzymes because the values were expressed in terms of specific activity. Christiansen (1978) gives no indication of the number of units of activity associated with each fraction, only the specific activity. Therefore although the lipid fraction has the highest specific activity due to the low amount of protein units associated with it, it may have contained less actual activity than one of the other fractions. Activity may also have been lost as in the early work documented in this thesis due to the harsh homogenisation of the cells which was employed to prepare extracts.

Subcellular fractionation of the yeast *C. curvata* D resulted in six fractions. Mitochondrial and microsomal fractions were characterised by specific activities of marker enzymes. The highest activities of the enzyme acyl-CoA synthetase were detected in the pellet and lipid fractions respectively. Assay for triacylglycerol biosynthesis, by measuring incorporation of [1-^{14}C]oleate or [U-^{14}C]-glycerol 3-phosphate, yielded specific activities similar to those obtained using whole sphaeroplasts. Activity was mainly observed in the pellet fraction with a small amount of incorporation in both the intermediate and mitochondrial fractions (Results, Chapter 3, Tables 30, 31 and 32). The pellet fraction also contained most actual units of activity (Results, Chapter 3, Figure 16) confirming that this was the fraction in which triacylglycerol biosynthesizing activity was mainly located. The lipid fraction showed negligible incorporation of label into intermediates of the α-glycerol 3-phosphate pathway and hence was concluded not to be the site of triacylglycerol biosynthesis in the yeast *C. curvata* D.

Recombination of fractions in various proportions was attempted, but activity of the order seen in whole sphaeroplasts was only observed when all fractions were present. Maximal activity seen in the pellet was thought to be due to the fact that this fraction would also contain whole sphaeroplasts and hence all subcellular components.

The reason for the loss of activity on fractionation is a mystery and indicates a high degree of association between organelles.

Therefore, in conclusion, it appears that synthesis of triacylglycerols in C. curvata D and L. starkeyi CBS 1809 occurs via the α-glycerol 3-phosphate pathway. Enzyme activity being mainly in the pellet fraction with a small amount of activity in mitochondrial and intermediate fractions.

Reports other than that of Christiansen (1978) have demonstrated the enzymic activity of lipid synthesis to be located mainly in particulate fractions (Kuhn and Lynen, 1965; Davidson and Stanacev, 1972). However, these studies were mainly confined to examination of phospholipid synthesis. The pellet fractions, which showed highest activities in this study may have contained cytoplasmic membrane fragments along with nuclei and whole sphaeroplasts. Such cytoplasmic membranes have previously been cited as the location of lipases in yeasts (Nurminen and Soumalainen, 1970). Also, Marinetti (1970) has reported that centrifugation of a homogenate from rat liver at low speed markedly decreases incorporation of glycerol into neutral lipids implying that the enzymes involved in neutral-lipid synthesis are situated in the low speed pellet.

The actual component of the pellet fraction which carries out the triacylglycerol biosynthesis therefore remains unknown. Electron microscopy studies did however shed some light on the possible location of triacylglycerol biosynthesis. They revealed a close association between endoplasmic reticulum and lipid globules in all yeasts examined and this was particularly evident in C. curvata D when grown on ethanol and fixed with permanganate. This may

indicate that lipid is in fact synthesized on the endoplasmic reticulum. It was possible that during fractionation the endoplasmic reticulum (which should have sedimented in the microsomal fraction) was damaged and hence lost its lipid biosynthesizing activity. An explanation for the fact that activity remains in the pellet is that this would contain some whole sphaeroplasts in which the endoplasmic reticulum was still intact and hence actively synthesizing lipid.

4.1. Fatty acid specificity in triacylglycerol biosynthesis

The fatty acids of triacylglycerols in oleaginous yeasts are unexceptional in their nature and relative proportions (Ratledge, 1982). Conditions of growth may bring about slight changes in fatty acid composition.

Oleic acid (18:1) is usually the predominant fatty acid in all oleaginous yeasts; palmitic acid (16:0) tends to be the second most abundant acid with linoleic (18:2) often being third (Ratledge, 1982).

Relative rates of incorporation of [1-^{14}C]fatty acids into intermediates of the α-glycerol 3-phosphate pathway and the activities of acyl-synthetases for four different fatty acids were determined using whole sphaeroplasts from C. curvata D and L. starkeyi CBS 1809.

Similar activities of fatty acyl-CoA synthetases were observed for linoleic, oleic and palmitic acids in both yeasts. However activity of the acyl-CoA synthetases for stearic acid (18:0) was very low in C. curvata D and totally absent in L. starkeyi CBS 1809 (Results, Chapter 3, Tables 26

and 27). A possible explanation for this was that due to the insoluble nature of stearic acid it was not readily available to the fatty acyl-CoA synthetase. However a similar argument could have applied to palmitic acid, which showed good activity in both yeasts.

Rates of incorporation of label into intermediates of the α-glycerol 3-phosphate pathway in both C. curvata D and L. starkeyi CBS 1809 were similar when using palmitic acid, oleic acid or linoleic acid. Little incorporation of label was seen using stearic acid, this was not surprising due to the low specific activities of acyl-CoA synthetases observed for this fatty acid in both yeasts.

Experiments using [U-^{14}C]glycerol 3-phosphate and fatty acyl-CoA esters (FACES) of oleic acid and stearic were performed to elucidate whether or not the yeasts could incorporate stearyl-CoA into α-glycerol 3-phosphate pathway intermediates. Experiments using oleoyl-CoA were also performed. C. curvata D showed similar rates of incorporation of corresponding fatty acids and FACES into triacylglycerol intermediates. Very little incorporation of label was seen for stearyl-CoA or stearic acid when compared with incorporation of oleoyl-CoA or oleic acid. L. starkeyi CBS 1809 showed greatly increased incorporation of label into diacylglycerol when using either steaoyl-CoA or oleoyl-CoA compared to their respective fatty acids. However no increase of incorporation of label into any other intermediate of the α-glycerol 3-phosphate pathway was seen (Results, Chapter 3, Table 28). This observation was not understood.

The activation of fatty acids to acyl-CoA esters by one of several chain length-dependent fatty acyl-CoA synthetases is required for nearly all metabolic fates of fatty acids in eukaryotic tissues. Triacylglycerols contain predominantly long chain fatty acids and hence the long chain fatty acyl-CoA synthetase is mainly involved in their biosynthesis.

The most detailed knowledge of the long chain fatty acyl-CoA synthetase of yeasts comes from work on the oleaginous yeast Yarrowia (Candida) lipolytica, (Kamiryo et al, 1977; Mishina et al, 1978a; Mishina et al, 1978b; Hosaka et al, 1979). These studies have indicated the presence of two long-chain fatty acyl-CoA synthetases, termed acyl-CoA synthetases I and II. Synthetase I was present in cells grown on glucose and oleate (Mishina et al, 1978a), and was absent in mutant strains (Kamiryo et al, 1977). Synthetase I was distributed between mitochondrial and microsomal fractions and was reported to be responsible for the production of acyl-CoA to be used in the synthesis of cellular lipids. The enzyme was purified from Y. lipolytica (Hosaka et al, 1979) and was shown to have a subunit molecular weight of 84,000 and exhibit specificity for fatty acids containing 14-18 carbon atoms, regardless of the degree of unsaturation. It was also noted that the activity of synthetase I towards stearic acid was half that observed for palmitic, oleic or linoleic acids in Y. lipolytica (Hosaka et al, 1979).

Synthetase II was immunologically distinct from synthetase I and was induced by fatty acids and dependent

on phosphatidylcholine (Mishina et al, 1978b). Synthetase II was reported to be located in the microbodies (Mishina et al, 1978b) and its main function was in the production of acyl-CoA for degradation via β-oxidation.

In the present study it was noted that the specific activity of fatty acyl-CoA synthetase for oleic acid was increased 40-fold when C. curvata D was grown on its own intracellular storage lipid compared to growth on glucose (Results, Chapter 3, Table 29). The activity of this enzyme in L. starkeyi CBS 1809 was increased 10-fold under the conditions of carbon starvation (although no breakdown of lipid occurred). These increases in enzyme activity during starvation conditions may have been due to induction of synthetase II in peroxisomes of C. curvata D and L. starkeyi CBS 1809 as described for C. lipolytica (Mishina et al, 1978b).

Incorporation of label into intermediates of the α-glycerol 3-phosphate pathway was very low in both L. starkeyi CBS 1809 and C. curvata D during starvation conditions compared to that observed during lipid accumulation (Results, Chapter 3, Table 29). This was consistent with the lipid biosynthetic pathway being repressed or inhibited.

5. Lipid globules in oleaginous yeasts

Lipid globules were first isolated from the yeast S. cerevisiae by disintegration of protoplasts under mild conditions (Clausen et al, 1974) and from L. starkeyi by osmotic lysis of protoplasts (Uzuka et al, 1974). More recently isolation from the non-oleaginous yeast Candida tropicalis was achieved (Belov and Davidova, 1982). All

globules isolated were reported to be surrounded by a membrane, with an uneven laminar structure on the outside. Globules from L. starkeyi were reported to be rich in lipid, represented mainly by triacylglycerol (85%) (Uzuka, et al 1975). They were thought to be surrounded by a phospholipid membrane composed mainly of phosphatidylethanolamine. Lipid globules from S. cerevisiae contained triacylglycerol (47%), a large amount of sterol esters (44%) and small amounts of phospholipid (1-4%) and protein (Clausen et al, 1974). The protein was thought to form part of the membrane surrounding the globule.

Lipid globules isolated from L. starkeyi CBS 1809, Candida curvata D and Trichosporon cutaneum CBS 40 in this study had a similar appearance to those previously isolated from L. starkeyi (Uzuka et al, 1975) when viewed under the scanning electron microscope (Results, Chapter 4, Plates 1 to 8). The outer surface of the globules appeared rough and laminated in structure. Diameters of globules ranged from 1.3 to 5 μm, and were similar to those previously reported by Uzuka et al (1975) for L. starkeyi (1 to 4 μm). 'Scars' were sometimes observed in the outer surface of globules, in particular those isolated from T. cutaneum CBS 40 (Results, Chapter 4, Plates 5 and 6). Initially it was thought that these were tears in the outer phospholipid membrane surrounding the globule, but later it was decided that they were an artifact resulting from the fixation method. Lipid on the surface of the globule was probably fixed adequately, however that in the centre of the globule was probably still

liquid, and it is likely that it is this which appears to emerge from the outer fixed surface of the globules.

Determination of the chemical composition of oil globules isolated from C. curvata D revealed that the major constituent was triacylglycerol (78%). The globules also contained sterol (11.1%), diacylglycerol (5.25%), free fatty acid (3.1%) and sterol ester (2.1%), (Results, Chapter 4, Table 33). The high percentages of diacylglycerol and free fatty acid will probably have resulted from breakdown of triacylglycerol during the isolation procedure. The most significant finding was that the globules contain a negligible amount of phospholipid (0.1%). This would make the possibility that they are surrounded by a bilayer or unit membrane extremely unlikely. Freeze-etch studies of C. curvata D grown on oleate and triolein also failed to show the presence of a membrane around the globules.

The method of labelling the lipid globules with [1-^{14}C]-acetate may have limitations in that the labelled fatty acyl-CoA may preferentially enter one particular class of lipid (eg sterols). However, the fact that no evidence for a membrane around the globules was provided by the freeze-etch studies supports the theory that the globules are not surrounded by a membrane.

Transmission electron microscopy studies revealed that in L. starkeyi CBS 1809 lipid tends to accumulate as one large globule, whereas in C. curvata D and T. cutaneum CBS 40, the existence of multiple small globules is more common.

Therefore it may be possible that in L. starkeyi a unit membrane or bilayer surrounds the globule keeping it intact, whereas in C. curvata D and T. cutaneum CBS 40, there is no membrane and hence lipid arises as small distinct globules.

SUMMARY

The most significant findings of this thesis may be summarised as follows:

1. Phosphoketolase studies

Pentulose 5-phosphate phosphoketolase was shown to be induced in nine yeasts grown on xylose. The enzyme was active with both ribulose 5-phosphate (Ru5P) and xylulose 5-phosphate (Xu5P) as assay substrate. Partial purification of the enzyme from Candida famata NCYC 33 led to a decrease in activity for Ru5P. Distinction between the possibility that pentulose 5-phosphate phosphoketolase enzyme may have dual substrate specificity and the possibility that Ru5P 3-epimerase activity may be closely associated with the phosphoketolase enzyme was not possible.

Pentulose 5-phosphate phosphoketolase had a K_m of 2.4 mM for Xu5P, a pH optimum of 7.2-7.4 and a Mr of 5×10^5 daltons. It was not sensitive to inhibition by citrate or acetyl-CoA at physiological concentrations.

Lipid levels and ATP:citrate lyase activities were shown to be similar in oleaginous yeasts grown on xylose and grown on glucose. It was concluded that the presence of a pentulose 5-phosphate phosphoketolase did not affect the process of lipid accumulation in these yeasts.

2. Lipid turnover studies

Yeasts of the genera Candida, Trichosporon and Rhodosporidium were shown to be capable of turning over storage lipid for cell proliferation under conditions of carbon starvation. Yeasts of the genus Lipomyces were unable to breakdown their storage lipid, the reason for this was not discovered.

Oleaginous yeasts were also shown to accumulate glycogen simultaneously with lipid. This was also degraded during carbon starvation conditions even in the Lipomyces species.

Lipid turnover was shown to be a rapid process in the yeast C. curvata D which occurred within 1.5 hours of switch from conditions which facilitated lipid accumulation to those of carbon starvation. Carbon from lipid degradation was shown to be converted to new lipid-free biomass by a radioactive transition experiment.

The enzyme ATP:citrate lyase was shown to be repressed during conditions of carbon starvation in three species of yeast. It was also repressed during growth on an external lipid source.

Peroxisomes were shown to proliferate in oleaginous yeasts under carbon starvation conditions and during growth on exogenous lipid sources or ethanol. This was demonstrated by the increase in the peroxisomal enzymes catalase, isocitrate lyase and $NADP^+$-dependent isocitrate dehydrogenase under these conditions. This was also illustrated by transmission electron microscopy.

Activity of malic enzyme was shown to be reduced during growth on external lipid sources but not during endogenous lipid utilisation.

Carnitine acetyl-transferase activity was shown to be enhanced during growth on exogenous or endogenous lipid sources and was thus thought to be the likely medium for the transport of acetyl-CoA units across the mitochondrial membrane.

3. Triacylglycerol biosynthesis

Triacylglycerol biosynthesizing activity was demonstrated in whole sphaeroplast preparations of Candida curvata D and Lipomyces starkeyi CBS 1809 by incorporation of [1-^{14}C]oleate into lipids.

Biosynthesis was shown to occur by the α-glycerol 3-phosphate pathway and incorporation of label into lysophosphatidic acid, phosphatidic acid, diacylglycerol and triacylglycerol was observed.

Activities of triacylglycerol biosynthesizing enzymes were demonstrated in some strains of Saccharomyces cerevisiae. Significant strain variation was observed, activities in some cases being shown to be similar to those observed in C. curvata D and L. starkeyi CBS 1809.

Sub-cellular fractionation of C. curvata D did not reveal the location of triacylglycerol biosynthesis. Activity was mainly recovered in the 'pellet' fraction which was likely to contain whole sphaeroplasts.

Fatty acyl-CoA synthetases were shown to be equally active with oleic, palmitic, and linoleic acids, but showed a marked decrease in activity towards stearic acid in both C. curvata D and L. starkeyi CBS 1809.

Oleic, palmitic and linoleic acids were equally well incorporated into intermediates of the α-glycerol 3-phosphate pathway in L. starkeyi and C. curvata D. Steaoyl-CoA was not readily incorporated into α-glycerol 3-phosphate intermediates in C. curvata D. However it was readily incorporated

into diacylglycerol in L. starkeyi CBS 1809.

The specific activity of fatty acyl-CoA synthetase for oleic acid was increased 40-fold in C. curvata D during growth on endogenous lipid. This activity was increased 10-fold in L. starkeyi CBS 1809 during carbon starvation conditions.

Triacylglycerol biosynthesis was markedly decreased during carbon starvation in both C. curvata D and L. starkeyi CBS 1809.

4. Lipid globules in oleaginous yeasts

Lipid globules were isolated from L. starkeyi CBS 1809, T. cutaneum CBS 40 and C. curvata D. All globules were shown to have an uneven laminar surface when viewed under the scanning electron microscopy and a diameter of 1.3 to 5 μm.

The major constituents of lipid globules from C. curvata D were shown to be triacylglycerol (78%), sterol (11%), diacylglycerol (5.3%), free fatty acid (3%) and sterol ester (2%).

Existence of a unit membrane or bilayer around the lipid globules in C. curvata D was shown to be unlikely due to the absence of significant amounts of phospholipid in the globules. Electron microscopic studies also failed to give any evidence for the presence of such a membrane.

PUBLICATIONS

PUBLICATIONS

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