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The Biochemistry of Lipid Accumulation in Oleaginous Yeasts

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Lipids are, it transpires, a type of insoluble organic compound found in crisps.

The Guardian, 4th July 1980

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INTRODUCTION

1.1 Background perspective

At present we are at the dawn of an era of heightened biotechnological awareness when considerable interest is being expressed in the production of various commodities from microbial sources. One such area which might be considered ripe for exploitation is the production of microbially-derived oils and fats.

Conventional sources of oils and fats, excluding dairy and related products, are primarily plants which account for over 80%, the remainder being of animal and marine origin. Oils and fats are put to a multitude of uses, principally as human foodstuffs, for example in cooking oils, margarine, salad oils, etc. Technological uses include incorporation into products such as paints, soaps, detergents, waxes and polishes. Ratledge (1981) points out that Europe is obliged to import vast quantities of these raw materials, at great cost, from mainly Third World Countries. It follows, therefore, that as supplies are dependent on the climatic and political vagaries of the producers there is every likelihood that the trend of accelerating increase in such raw material costs will continue.

The technology has now been successfully developed for the large-scale continuous cultivation of micro-organisms for protein. Therefore, there seems little reason why a process for the production of microbial oils and fats could not compete on equal, or better, cost-effective terms compared with traditional sources of lipid. If this goal were achieved we would be assured of a stable home-produced supply of this vital commodity.

It is against this backdrop that the work of this thesis has been carried out. This has been an investigation into the underlying

biochemical mechanisms resulting in lipid accumulation in oleaginous yeasts. It is only by gaining a complete understanding of the metabolic processes leading to lipogenesis and lipid accumulation that the full fruits of any commercial undertaking may be harvested. In addition it is hoped that the results represent, on a more fundamental level, a modest advance in the elucidation of lipogenic pathways in biological systems.

1.2 Historical note

Lipid accumulation in micro-organisms has been recognized since the late nineteenth century when Nageli and Loew (1878) observed intracellular lipid bodies in yeast cells. During the First World War the blockade of Germany was the cause of a fat and oil shortage in that country and thereby provided the impetus for the initial investigation into the process. Consequently, Lindner (1922) considered, albeit unsuccessfully, the commercial production of microbially-derived lipid. This pioneering work was extended by other German workers (e.g. Damm, 1943 and Schmidt, 1947) in the years leading up to and during the Second World War, though once again no economically viable process resulted.

This initial period of enthusiasm declined once hostilities ceased and normal trading relations were resumed. The following three decades were characterized by a decline in interest in microbially-produced lipid. This is illustrated by the fact that until very recently the last authoritative review of the industrial potential of microbial lipid was published over 20 years ago (Woodbine, 1959).

Our laboratory has been one of the few to develop an interest in this field, as will be described later. However, a more general resurgence is now becoming increasingly evident as a consequence of the factors already outlined.

1.3 Oleaginous yeasts

An oleaginous micro-organism is possibly defined as one that has the potential to accumulate a substantial proportion of its biomass as lipid. The lipid may account for as much as 70% of the cell dry weight, in certain organisms, cultivated under optimal conditions. On purely practical grounds Ratledge (1981) includes in this category any micro-organism capable of amassing at least 25% of its biomass as lipid. This is an arbitrary delineation as there are many border-line cases but it is made in the knowledge that this is the probable lower limit acceptable in any future industrial process. It should be possible to decide if an organism is oleaginous on the basis of a biochemical test and as a result of the work described in this thesis I would consider that this may be realizable in the near future.

The majority of oleaginous micro-organisms are eukaryotic, representative types being found among the yeasts, fungi and algae. There are also, however, a few oleaginous prokaryotic algae and the phenomenon is seen in certain bacterial species. The investigations reported in this thesis were confined to oleaginous yeasts, a comparatively small group, the principal strains of which are shown in Table 1.

In common with plant and animal oils the major constituent of the lipid accumulated by oleaginous micro-organisms is triacylglycerol. This fraction typically accounts for 80% or more of the total cellular lipid. Although the fatty acid composition can be altered by varying the growth conditions (Thorpe and Ratledge, 1972), oleic acid (18:1) is usually the predominant fatty acid with palmitic (16:0) and linoleic acids (18:2) following in order of abundancy.

The remainder of the lipid is usually phospholipid, the three commonest types being phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol. In addition, sterols both free and esterified to

TABLE 1: Representative oleaginous yeasts (Ratledge, 1981)

Organism	Lipid content (% w/w)	Typical reference
Candida curvata D	58	Moon and Hammond, 1978
Candida lipolytica	36	Zvyagintseva and Pitryuk, 1976
Candida paralipolytica	32	Zvyagintseva and Pitryuk, 1976
Candida sp. 107	42	Thorpe and Ratledge, 1972
Cryptococcus terricolus	55-65	Pederson, 1961, 1962a, 1962b
Endomycopsis vernalis	65	Wither *et al.*, 1974
Hansenula saturnus	28	Fahmy *et al.*, 1962
Lipomyces lipofer CBS 944	37	McElroy and Stewart, 1967
Lipomyces starkeyi	37	Roy *et al.*, 1978
Lipomyces tetrasporus sp. 5011F	16-64	Pityruk *et al.*, 1975
Rhodosporidium toruloides 1FO 0559	56	Fuji Oil Co., 1977
Rhodotorula gracilis	57	Enebo *et al.*, 1946
Rhodotorula graminis 1FO 1422	36	Fuji Oil Co., 1978
Rhodotorula glutinis	59	Pidoplichko and Zalashko, 1977
Rhodotorula mucilaginosa	28	Borisova and Atamanyuk, 1980
Trichosporon cutaneum	45	Moon and Hammond, 1978
Trigonopsis variablis	40	Sentheshamuganathan and Nickerson, 1962

fatty acids may attain moderately high concentrations. It is interesting to note that deviations from this pattern have been observed in non-oleaginous micro-organisms, for example, Clausen *et al.* (1974) reported that oil droplets isolated from *Saccharomyces cerevisiae* contained 44% sterol esters.

Physically, lipid accumulates in oleaginous micro-organisms in the form of discrete droplets. Uzuka *et al.* (1974) isolated and analyzed the droplets from the yeast, *Lipomyces starkeyi* IAM 4753. Apart from triacylglycerol, they discovered that the droplets contained phospholipid and a protein component. The amino acid composition of this protein was similar to that reported for membrane proteins, and this finding led them to propose the existence of such structures surrounding the oil globules, a hypothesis they confirmed by scanning electron microscopy.

1.4 Conditions for and the course of lipid accumulation

As intimated earlier the quantity of lipid accumulated is dependent on cultural conditions. The prime requisite is for the culture medium to contain an excess of carbon over some other nutrient. Thus, when an oleaginous microorganism is cultivated in batch culture there is an initial period of cell proliferation, where lipid levels may be modest, followed by a period of 'fattening', initiated by the exhaustion of the limiting nutrient. In this second phase the excess carbon continues to be transported into the cell where it is converted into lipid. This behaviour is typical of oleaginous micro-organisms and was reported as long ago as 1946 by Enebo *et al.*, working with *Rhodotorula gracilis*.

Laboratory media for the growth of oleaginous micro-organisms usually contain glucose as the principal carbon source and nitrogen as the limiting nutrient. Lipid accumulation will also occur with other limiting nutrients, for example, phosphate (Nielson and Nilsson, 1953), sulphate (Maas-Forster, 1955), iron (Nielson and Rojowski, 1950) and

magnesium (Gill *et al.*, 1977).

A unique exception to this pattern was reported by Pederson (1961, 1962a, 1962b) working with a yeast, *Cryptococcus terricolus*, which he himself had isolated. He observed high lipid concentrations in this yeast even when it was grown on media containing low C:N ratios.

Initial work on the growth characteristics of oleaginous micro-organisms pre-dated the development of continuous culture techniques and, in fact, our laboratory has been one of the few to pursue this approach. In these studies (Gill *et al.*, 1977; Hall and Ratledge, 1977; Ratledge and Hall, 1979) it was demonstrated that oleaginous yeasts could attain lipid concentrations equal to those seen in batch cultures, providing a dilution rate of 0.03 to 0.15 h^{-1} was used and the medium had 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, from the medium, to lipid.

The most significant observation made in this series of reports was that the specific rate of lipid synthesis (g lipid per g lipid - free yeast per h) varied only slightly with dilution rate and more importantly, was similar when the yeast was grown under conditions of carbon- or nitrogen-limitation. Thus, the lipid content of the yeast did not correlate with the specific rate of lipid synthesis. Therefore, the conclusion was made that nutrient limitation restricted biosynthetic processes, in general, but did not necessarily increase the rate of lipid synthesis.

The series of reports discussed above illustrate the value of continuous culture techniques. It was appreciated at the outset of this investigation that the high degree of control attainable using a chemostat, provided a powerful tool for the elucidation of the biochemical changes

occurring during lipid accumulation.

2. The biochemistry of oleaginicity

The investigation of the biochemistry of oleaginicity in micro-organisms is a much neglected topic. In the majority of cases lipogenesis in yeasts has been studied in the 'classical' organisms *Saccharomyces cerevisiae* and *Candida utilis*, which are of course non-oleaginous, i.e. contain about 5% w/w lipid when grown on a medium with a high C:N ratio. It is convenient, therefore, to review the current knowledge of lipogenic processes in biological systems in general and compare this with the results of those studies applied specifically to oleaginous micro-organisms.

De novo long chain fatty acid biosynthesis is a cytosolic process (Van Baalen and Gurin, 1953; Popjak and Tietz, 1955) the basic building block being acetyl-CoA (Wakil, 1961). The sequence of events, therefore, may be considered in two parts. Firstly, a consideration of intermediate metabolism leading to the formation of cytosolic acetyl-CoA; and secondly, its subsequent utilization in fatty acid biosynthesis.

2.1.1 Pathways of glucose dissimilation

One criterion that has to be satisfied in order that lipid accumulation may occur is that the fatty acid synthesizing enzymes must be constantly primed with acetyl-CoA. It is important, therefore, to consider the contribution and possible regulation of the various pathways of glucose catabolism and the subsequent intermediate metabolism of oleaginous micro-organisms.

In previous studies with a number of oleaginous yeasts (Brady and Chambliss, 1967; Höfer, 1968; Whitworth and Ratledge, 1975 a,b) the failure to detect phosphofructokinase led to the conclusion that glucose was catabolized via the pentose phosphate pathway. Although this pathway could provide the reducing equivalents required for fatty acid biosynthesis it was appreciated that it could not account for the high molar growth and

lipid yields in these organisms, as only 1 mol acetyl-CoA arises from 1 mol glucose (Gill *et al.*, 1977; Höfer *et al.*, 1971; Brady and Chambliss, 1967). Consequently, it was proposed that the supply of C_2 units might be supplemented via the action of phosphoketolases (Höfer *et al.*, 1969, 1971; Höfer, Betz and Becker, 1970) viz:-

xylulose 5-phosphate → glyceraldehyde 3-phosphate + C_2 unit

or fructose 6-phosphate → erythrose 4-phosphate + C_2 unit

Whitworth and Ratledge (1977) detected these enzymes in the oleaginous yeasts, *Rhodotorula graminis*, *R. glutinis* and *Candida* 107 but not in the non-oleaginous yeast, *Saccharomyces carlsbergensis*. Thus, the hypothesis was advanced that the lack of phosphofructokinase resulted in an uncontrolled dissimilation of glucose, via the pentose phosphate cycle, obliging the organisms to utilize the excess carbon and NADPH by synthesizing lipid and that furthermore, the cytosolic pool of acetyl-CoA was augmented, to an unknown extent, via the action of phosphoketolases.

This hypothesis was later rejected when it was discovered that oleaginous micro-organisms did, in fact, possess a labile phosphofructokinase (Mazón *et al.*, 1974; Ratledge and Botham, 1977) thus, indicating that the glycolytic pathway is operative in these yeasts. Ratledge and Botham (1977) investigated the uptake of radioactive glucose into the oleaginous yeast, *Candida* 107, and concluded that 63% was metabolized via the pentose phosphate cycle, the remainder via the Embden-Myerhof pathway. In view of the high biomass yields and levels of lipid accumulation reported for *Candida* 107 (Gill *et al.*, 1977) this was a surprisingly low proportion of glucose being dissimilated via the Embden-Myerhof pathway and should be treated with some scepticism. The pathways of glucose dissimilation in *Candida* 107 are summarized in Fig. 1.

2.1.2 The origin of cytosolic acetyl-CoA

Whether glucose catabolism proceeds via the pentose phosphate or

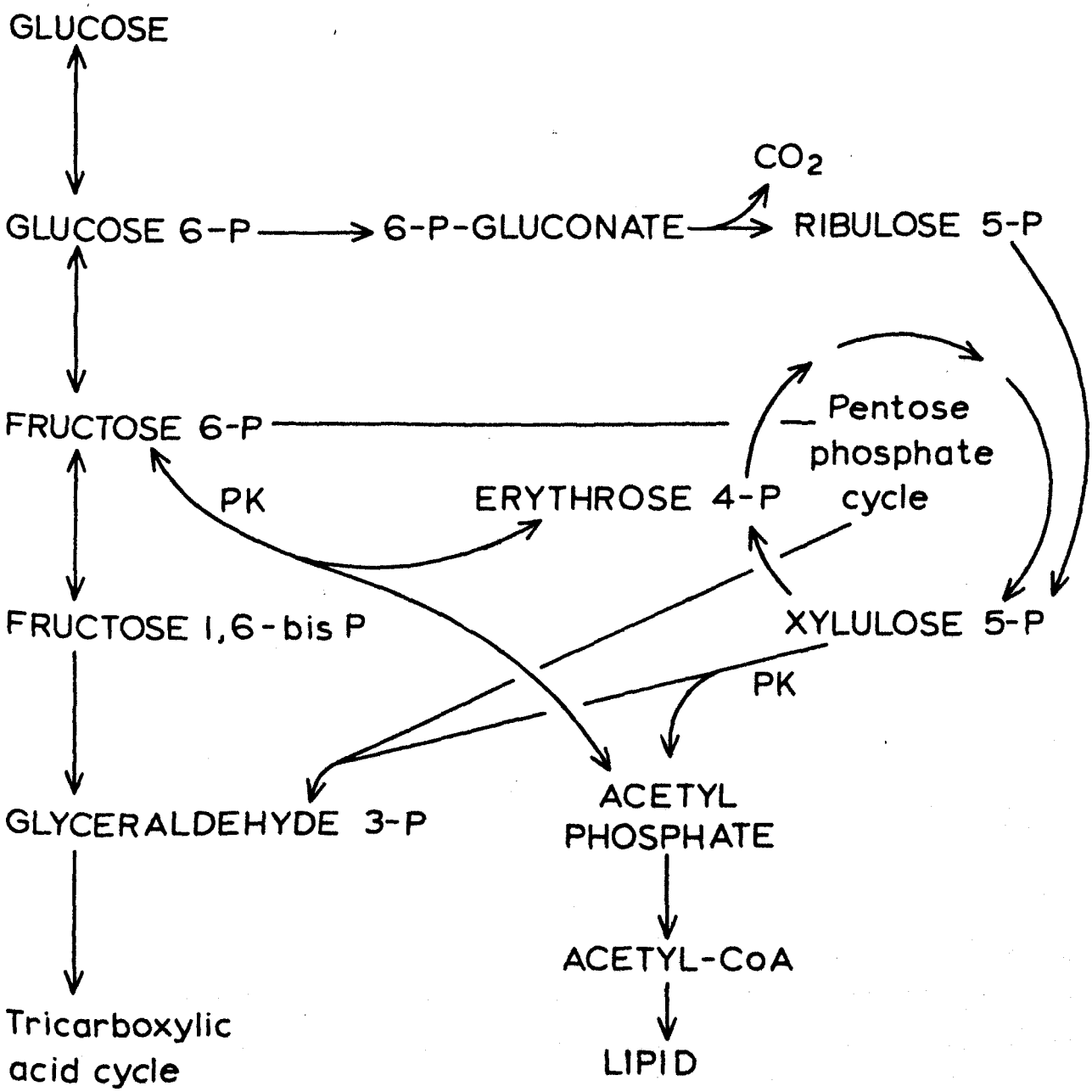


Fig.1 Pathways of glucose metabolism in *Candida* 107. (Ratledge and Botham, 1977)

PK, Phosphoketolase

Embden-Myerhof pathways the net result is the formation of pyruvate which is then oxidized to acetyl-CoA in the mitochondrion. Since the mitochondrial membrane is impermeable to acetyl-CoA (Spencer and Lowenstein, 1962) its mode of exit requires some explanation. The proposed mechanisms are summarized in Fig. 2.

Firstly, Kornacker and Lowenstein (1965a) postulated that the acetyl-CoA might be hydrolyzed to acetate, which can pass freely through the inner mitochondrial membrane, and then be regenerated by a cytosolic acetyl-CoA synthetase. This latter enzyme does occur in the cytosol but intramitochondrial activities of acetyl-CoA hydrolase are low in many tissues (Packter, 1973).

Bremer (1962) and Fritz (1963) suggested that the exit of acetyl-CoA might be carnitine-mediated. However, although the acetyl-CoA carnitine transferase is present in rat liver mitochondria it is absent from the cytosol. Furthermore, incorporation of acetyl-carnitine into lipid is much slower than incorporation of acetate and citrate, also activities of acetyl-CoA carnitine transferase are higher in the non-lipogenic tissues, rat heart and skeletal muscle than activities in rat liver (Beenakkers and Klingenberg, 1964). There is now general accord that the carnitine-mediated transport system is involved with the transfer of acyl groups into mitochondria, to the site of β-oxidation, and is not involved in the genesis of cytosolic acetyl-CoA.

The most convincing explanation is that acetyl-CoA passes across the mitochondrial membrane in the form of carbon atoms 1 and 2 of citrate. The citrate, formed from the condensation of acetyl-CoA with oxaloacetate, is accessible to the mitochondrial membrane and in the cytosol the acetyl-CoA may be regenerated by the action of ATP:citrate lyase (section 2.2.1). The evidence for this pathway is principally provided from studies on lipogenesis in mammalian systems.

KEY TO FIGURE 2

1. Citrate synthase
2. ATP:citrate lyase
3. Malate dehydrogenase
4. Malic enzyme
5. Acetyl-CoA synthetase
5. Acetyl-CoA hydrolase
7. Acetyl-carnitine transferase
8. Pyruvate dehydrogenase

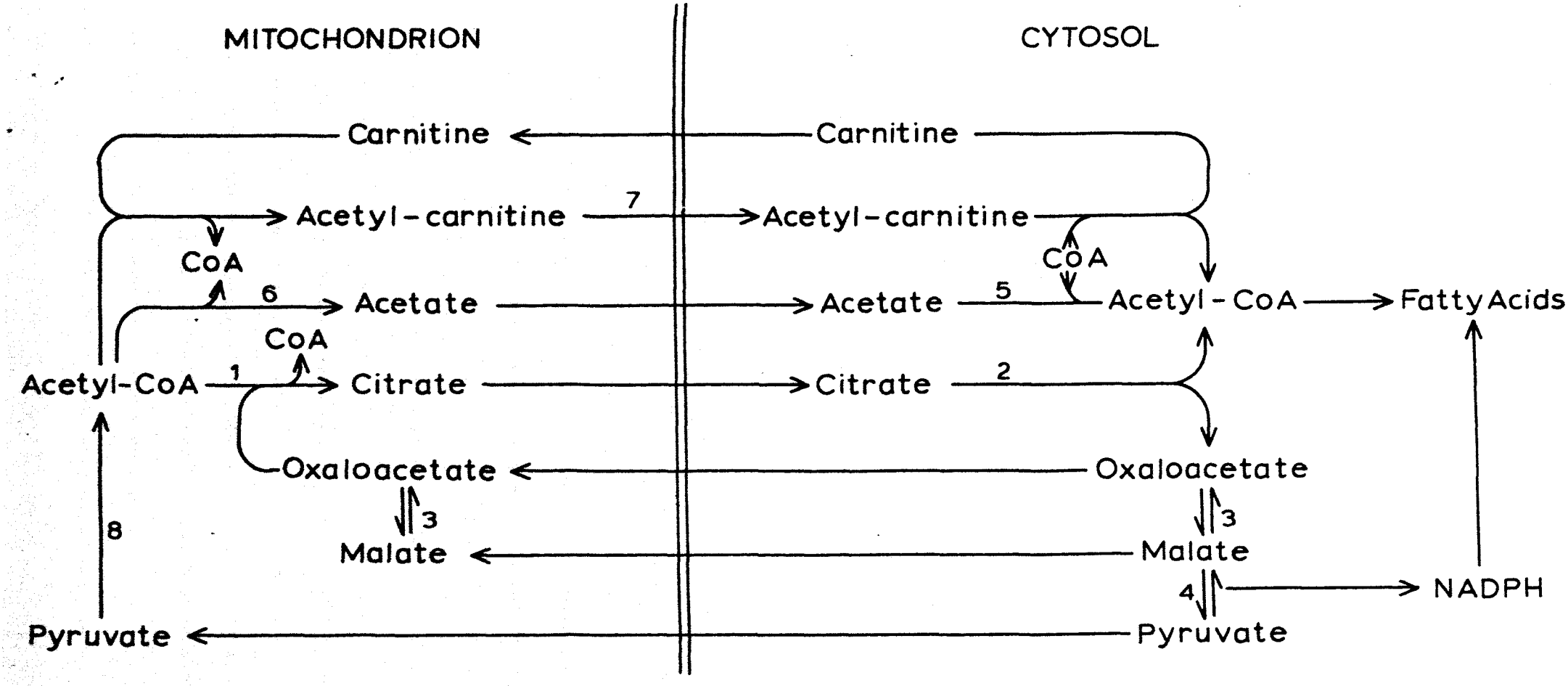


Fig.2 Possible mechanisms for the transfer of acetyl groups from mitochondria to the cytosol.

Isotopic uptake studies using rat liver extracts indicated that citrate was more rapidly incorporated into fatty acids than was acetate and that it was the acetyl portion of citrate that was involved. Furthermore, the effect was more pronounced in animals in which high lipogenic rates were induced by dietary regimens (Spencer and Lowenstein, 1962; Spencer *et al.*, 1964; Bhaduri and Srere, 1963)

There is considerable evidence, however, that this lipogenic pathway is not universal. Hanson and Ballard (1967) concluded that acetate was the prime precursor of fatty acid biosynthesis in ruminant liver and adipose tissue. Similarly, Goodridge (1973), working with neonatal chick hepatocytes reported that cytosolic acetyl-CoA synthetase activities were amply sufficient to accommodate the most rapid rates of observed fatty acid biosynthesis. Furthermore, the incorporation of [1-^{14}C] acetate into fatty acids could be demonstrated even though ATP:citrate lyase was inhibited by (-)-hydroxycitrate.

The mechanism by which cytosolic acetyl-CoA is generated in eukaryotic micro-organisms is difficult to surmise because of the paucity of published work. With very few exceptions, as will be described later, ATP:citrate lyase has not been detected (Srere, 1972). Therefore, the assumption must be made that the acetyl-CoA hydrolase/acetyl-CoA synthetase pathway is operative. However, in the few cases where the ATP:citrate lyase pathway has been reported in microbial systems, there also appears to be a greater than usual ability to synthesize lipids or related compounds (2.2.1).

There is a possibility that acetyl-CoA is generated in the cytosol of oleaginous micro-organisms by a novel mechanism, or by an unusual enzyme distribution such as the provision of a cytosolic pyruvate dehydrogenase. As this question has not yet been answered satisfactorily there remains scope for clarification.

2.1.3 Activities of TCA cycle enzymes during lipid accumulation

There are two essential prerequisites for the process of lipid accumulation to occur. Firstly, if the ATP:citrate lyase pathway is operative there must be no restriction in the flow of carbon from pyruvate to citrate in the mitochondrion. Secondly, the citrate must be channelled into the production of cytosolic acetyl-CoA and not oxidized via the TCA cycle. It follows, therefore, that citrate synthase must be active during lipid accumulation and there must be a mechanism restricting the oxidation of the citrate produced.

Citrate synthase is widely regarded as a probable control point of the TCA cycle and a variety of potential effectors have been nominated (for review see Weitzman and Danson, 1976). The enzyme from a variety of eukaryotic sources, for example, ox liver and ox heart (Jangaard *et al.*, 1968), rat liver (Shepherd and Garland, 1969), *Saccharomyces cerevisiae* (Hathaway and Atkinson, 1965), is strongly inhibited by ATP. The inhibition is competetive with respect to acetyl-CoA. As ATP can be regarded as an end-product of the TCA cycle this inhibition might be considered a plausible feed-back control mechanism.

Atkinson (1968) considered that the relative proportions of adenine nucleotides are of regulatory significance. He has developed the concept that the modulating effects of adenine nucleotides on the activities of many enzymes may be unified into a universal theory of the control of metabolism by changes in the adenylate 'energy charge'. This provides a measure of the energy status of the cell and is defined as follows:-

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

Enzymes whose activities are responsive to changes in energy charge are of two types. Firstly, those involved in pathways leading to the

formation of ATP (R-type) and secondly, those participating in sequences in which ATP is utilized (U-type). The theoretical response of the activities of those enzymes to changes in energy charge is shown in Fig. 3.

In vitro experiments with citrate synthases from rat liver (Shepherd and Garland, 1969) and *Saccharomyces cerevisiae* (Hathaway and Atkinson, 1965) have shown that activities do respond to mixtures of adenine nucleotides, simulating various energy charge values, as predicted for R-type enzymes. Therefore, the role of citrate synthase in the regulation of the TCA cycle was apparently confirmed. Whether or not this control mechanism also applies to oleaginous micro-organisms is far from clear. Citrate synthase must remain active during lipid accumulation for the reasons already discussed. Yet, Botham and Ratledge (1979) reported that the onset of nitrogen-limitation in cultures of *Candida* 107 was accompanied by a dramatic increase in the cellular energy charge, which would tend to restrict the activity of citrate synthase. Thus, there is an apparent paradox that any investigation into the mechanism of oleaginicity must resolve.

NAD^+-dependent isocitrate dehydrogenase is another TCA cycle enzyme reported to have regulatory properties. This was concluded when it was discovered that the enzyme from many eukaryotic sources was activated allosterically by AMP and or ADP (Hathaway and Atkinson, 1963; Goebell and Klingenberg, 1964; Bernofsky and Utter, 1966). Consequently, Atkinson (1977) classified NAD^+-dependent isocitrate dehydrogenase as another R-type enzyme.

The realization that energy charge, or more specifically AMP concentration, could alter the flux of the TCA cycle by modulating the activity of NAD^+-dependent isocitrate dehydrogenase led Botham and Ratledge (1979) to suggest that this formed the mechanism by which citrate was channelled into lipid production in oleaginous yeasts. In

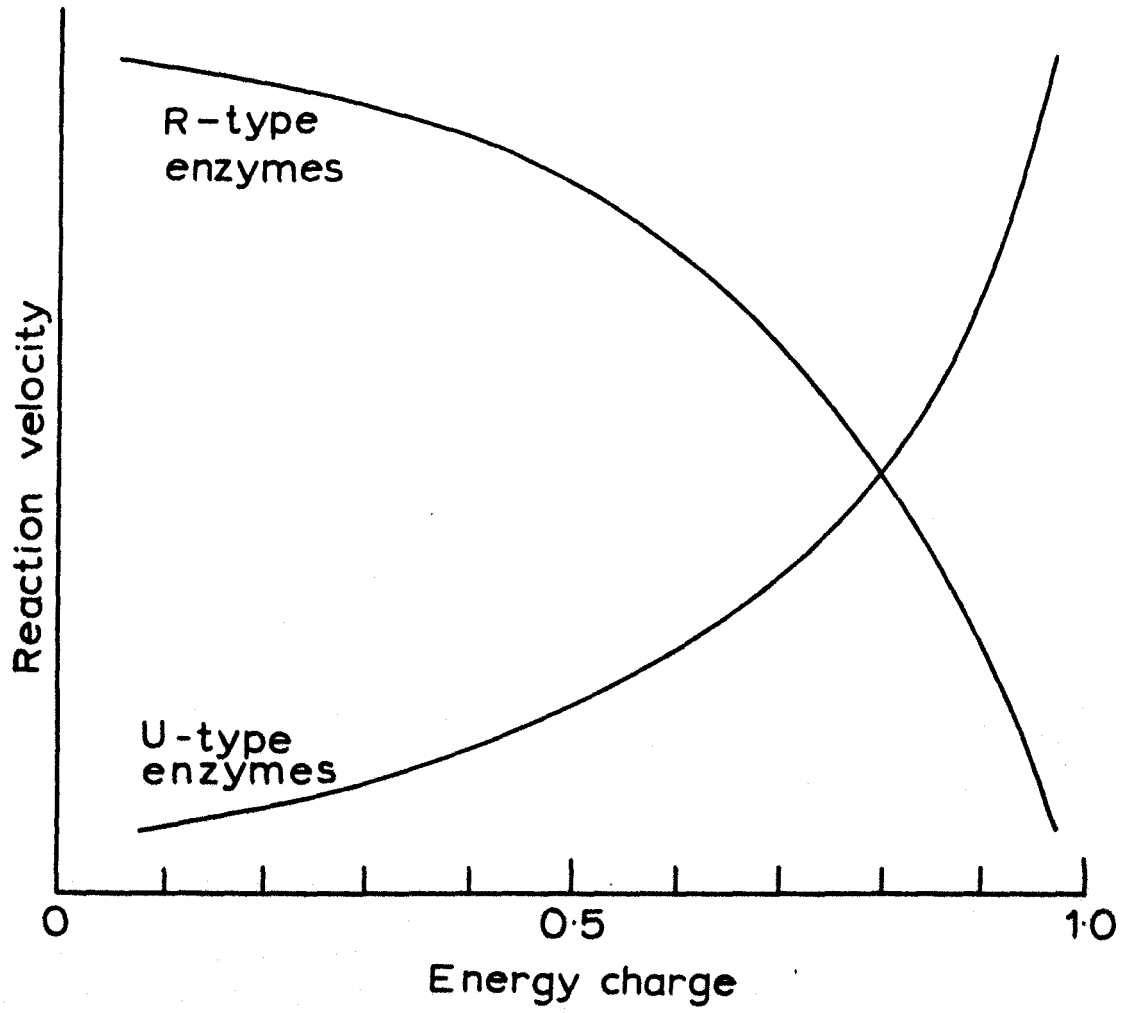


Fig. 3 Theoretical response of regulatory enzymes to changes in energy charge (Atkinson, 1968)

part this was based on other reports of a similar process occurring in citrate-accumulating micro-organisms. Several species of yeasts and moulds can accumulate large quantities of extracellular citric and isocitric acids (Marchal *et al.*, 1977). The acids accumulate in the stationary phase when growth is restricted by nitrogen-limitation; therefore, the phenomenon is analogous in some respects to lipid accumulation. Mitsushima *et al.* (1978) investigated citric acid production in the yeast *Saccharomycopsis lipolytica*. They reported that during the stationary phase there was an increase in the cellular ATP concentration accompanied by a decrease in intracellular levels of AMP and ADP. This depletion of AMP, in their opinion, resulted in the inactivation of the NAD^+-dependent isocitrate dehydrogenase and was, therefore, the prime reason for the diversion of carbon-flow into citric acid production.

Botham and Ratledge (1979) observed a similar requirement for AMP for the NAD^+-dependent isocitrate dehydrogenases of two oleaginous yeasts *Candida* 107 and *Rhodotorula glutinis* and an oleaginous mould, *Mucor circinelloides*. However, this enzyme from the non-oleaginous yeast, *Candida utilis*, was not activated by AMP or ADP nor did it require these adenine nucleotides for activity.

2.1.4 Transport of citrate from mitochondria to the cytosol in oleaginous micro-organisms

There is considerable evidence that the transport of citrate from mitochondria to the cytosol, in mammalian tissues, is mediated by a specific tricarboxylate carrier, situated in the inner mitochondrial membrane (Kingenberg, 1970). Early investigations had shown that mitochondria depleted of endogenous substrates did not oxidize citrate unless L-malate was also added. This finding could not be explained until it was demonstrated that L-malate facilitated the entry of citrate

into mitochondria (Ferguson and Williams, 1966). This was demonstrated by loading mitochondria with L-malate, which may enter via a dicarboxylic acid carrier (Palmieri *et al.*, 1971), and then measuring the exchange of L-malate with added citrate. The results indicated that citrate was transported into mitochondria in exchange with L-malate with a stoicheiometry of 1 to 1 and furthermore, the process was reversible (Palmieri and Quagliarello, 1968). The exchange of citrate^{3-} with L-malate^{2-} could in principle involve a net charge movement, however McGivan and Klingenberg (1971) demonstrated that 1 H^+ moves in the same direction as citrate so that the net exchange is electroneutral. Palmieri *et al.* (1977) have purified the tricarboxylate carrier and reported that it was protein in nature.

The suggestion is, therefore, that in lipogenic tissues and oleaginous micro-organisms exported citrate is cleaved to acetyl-CoA and oxaloacetate, in the cytosol, by ATP:citrate lyase. Malate dehydrogenase then catalyzes the reduction of the oxaloacetate and the resultant malate may exchange with more mitochondrial citrate. Thus, it is possible that citrate transport may be a regulated process. Indeed, Robinson and Chappell (1970) working with rat liver mitochondria concluded that even with saturating malate concentrations the transport system was the rate-limiting step in the oxidation of *cis*-aconitate and isocitrate.

There have been no reports of this process in oleaginous micro-organisms to date, consequently, it is an area worthy of further investigation.

2.2 Fatty acid biosynthesis in oleaginous micro-organisms

The remainder of the Introduction considers the actual process of fatty acid biosynthesis in oleaginous micro-organisms, the enzymes involved and their possible regulation.

2.2.1 ATP-citrate lyase

This cytosolic enzyme first discovered in chicken liver (Srere and Lipmann, 1953) catalyzes the ATP-dependent cleavage of citrate to oxaloacetate and acetyl-CoA. It is considered to occupy a key role in lipogenesis, in mammalian systems, by furnishing acetyl-CoA for fatty acid biosynthesis.

Evidence for the involvement of the enzyme in lipogenesis, in mammals, is provided by the fact that its activity in lipogenic tissues is dependent upon the nutritional and hormonal state of the animal. Thus, Kornacker and Lowenstein (1963, 1964, 1965a, 1965b) observed that starvation of rats for several days resulted in a reduction of liver ATP:citrate lyase activity, as did the production of alloxan diabetes. When fasted animals were fed on a high carbohydrate diet, hepatic ATP:citrate lyase activity was greatly increased. If the diet contained large amounts of fat there was no increase in enzyme activity although it was enhanced by injection of insulin into alloxan diabetic rats. Brown *et al.* (1966) reported that hypophysectomy caused a decrease in ATP:citrate lyase activity in rat liver, adrenal gland and adipose tissue which was restored after treatment with thyroxine. More recently, Yen and Mack (1980) using immunological techniques demonstrated that these variations in activity were a result of changes in the actual amounts of enzyme and not alterations in reaction velocity. Thus, it was concluded that rates of lipogenesis could be correlated with ATP:citrate lyase activity in these tissues.

However, ATP:citrate lyase does not appear to be involved in lipogenesis in all tisues. Goodridge *et al.* (1973) reported that there was no correlation between fatty acid biosynthesis and ATP:citrate lyase activity in neonatal chick hepatocytes. More recently, Hoffmann *et al.* (1980) claim that the citrate cleavage pathway does not contribute

significantly to lipogenesis in humans.

ATP:citrate lyase has been reported in certain plant tissues. For example, germinating castor bean endosperm (Fritsch and Beevers, 1979), ripening mango fruits (Mattoo and Modi, 1970) and developing soybean cotyledons (Nelson and Rinne, 1975). As all of these plant tissues contain high concentrations of lipid presumably the enzyme plays a similar role to that proposed for mammalian lipogenic systems.

Reports of ATP:citrate lyase from microbial sources are very scarce (see review by Srere, 1972). Indeed until very recently we were not aware that any had appeared. The enzyme has though been found in a yeast, *Rhodotorula gracilis* (Guerritore and Hanozet, 1970) and in the fungi, *Mortierella* spp. (Attwood, 1973) and *Penicillium spiculisporum* (Måhlén, 1973). The first two of these reports describe oleaginous micro-organisms whilst the latter concerns a mould that accumulates the anionic detergents, 2-decylcitric acid and 2-decylhomocitric lactone, thus requiring, according to the author, large amounts of lauroyl-CoA to be synthesized. Preliminary evidence was provided in each case that ATP:citrate lyase supplies acetyl-CoA for fatty acid biosynthesis and that there was a positive correlation between the activity of the enzyme and rates of fatty acid biosynthesis. However, these reports do not appear to have been developed further.

In our own laboratory Botham and Ratledge (1979) have detected ATP:citrate lyase in the oleaginous yeasts, *Candida* 107 and *Rhodotorula glutinis* also the oleaginous mould *Mucor circinelloides*. However there was some ambiguity in that the enzyme was also found in non-oleaginous strains of *M. circinelloides*. To my knowledge there have been no reports of any detailed studies of microbial ATP:citrate lyase, therefore, this was considered to be an area that should have a high priority in this investigation.

2.2.2 Malic enzyme

Malic enzyme catalyzes the oxidative decarboxylation of L-malate to pyruvate and CO_2. The enzyme is cytosolic and in eukaryotic organisms $NADP^+$ is the hydrogen acceptor (see Frenkel, 1975, for review). The purported role of malic enzyme, in lipogenesis, is that it provides reducing equivalents in the form of NADPH, which can be used for fatty acid biosynthesis (see Fig. 2). Evidence for this role, in mammalian systems, is similar to that reported for ATP:citrate lyase in that dietary or hormonal manipulation of rates of lipogenesis can be positively correlated with malic enzyme activities in many lipogenic tissues (Frenkel, 1975).

Malic enzyme has been detected in the oleaginous yeast, *Candida* 107 (Whitworth and Ratledge,1975a) but there was no correlation between enzyme activity and rates of lipid biosynthesis. In view of the fact that cytosolic malate may be involved in the exchange reaction with intramitochondrial citrate (see 2.1.4) and that reducing power may be supplied by the pentose phosphate cycle (see 2.1.1), the significance of malic enzyme in oleaginous micro-organisms remains unclear.

2.2.3 Acetyl-CoA carboxylase

Acetyl-CoA carboxylase is frequently considered to catalyze the first committed step in fatty acid biosynthesis (Volpe and Vagelos, 1976). It catalyzes the biotin-dependent carboxylation of acetyl-CoA to form malonyl-CoA, in a two-stage reaction summarized below:

$$ATP + HCO_3^- + \text{biotin}-\varepsilon \xrightarrow{Me^{2+}} CO_2^- - \text{biotin}-\varepsilon + ADP + P_i$$

$$CO_2^- - \text{biotin}-\varepsilon + CH_3CO - SCoA \rightarrow {}^-OOCCH_2CO - SCoA + \text{biotin}-\varepsilon$$

The enzyme is cytosolic and has been purified from many sources (Volpe and Vagelos, 1976). The majority of studies have been carried out with mammalian enzymes and in each case definable subunits have been

identified. Sumper and Riepertinger (1972) reported that the yeast enzyme exists as a tetramer of 6S subunits, each containing biotin, with a subunit molecular weight of 150,000.

Acetyl-CoA carboxylase is often regarded as catalyzing the rate-limiting step in the fatty acid biosynthetic pathway (Volpe and Vagelos, 1976) and there is a great deal of evidence for its allosteric regulation by a variety of effectors. A number of workers have reported that citrate and other TCA cycle intermediates cause polymerization and activation of the enzyme from various tissues, for example, rat adipose tissue (Martin and Vagelos, 1962), avian liver (Gregolin *et al.*, 1966) and bovine adipose tissue (Kleinschmidt *et al.*, 1969). The allosteric activation of acetyl-CoA carboxylase from yeast by citrate has been described but has also been disputed. Matsuhashi (1969) reported no activation of the enzyme from *Saccharomyces cerevisiae*. Conversely, Ramussen and Klein (1968) working with the same organism observed an activation similar to that reported for mammalian enzymes. Mishina *et al.* (1976) reported that there was no activation by citrate of acetyl-CoA carboxylase from *Candida lipolytica*..

There have been only two reports of the effect of citrate on acetyl-CoA carboxylase from oleaginous micro-organisms, both from our own laboratory. Gill and Ratledge (1973) and Botham (1978) both detected citrate activation of the enzyme from *Candida* 107 but this metabolite had no effect on acetyl-CoA carboxylase from the non-oleaginous yeast, *Candida utilis*.

Long chain fatty acyl-CoA esters are known to inhibit acetyl-CoA carboxylases from many sources and the inhibition has usually been observed to be competitive with respect to citrate (Numa *et al.*, 1965; Ogiwara *et al.*, 1978; Goodridge, 1972, 1973). Superficially this appears to be an attractive feed-back control mechanism but because of the

powerful detergent properties of these molecules many workers are sceptical of the significance of the phenomenon. The premise that the effect is of a general nature only and of no physiological significance is confirmed to a certain extent in that fatty acyl-CoA esters inhibit many enzymes. For example, yeast acetyl-CoA synthetase (Satyanarayana and Klein, 1973), yeast glucose 6-phosphate dehydrogenase (Kawaguchi and Bloch, 1974) and pig heart citrate synthase (Hsu and Powell, 1975). However, all of these authors considered that their evidence was sufficient to ascribe a specific regulatory role for fatty acyl-CoA ester inhibition, consequently, the phenomenon may not be lightly discounted.

In other studies it has been postulated that acetyl-CoA carboxylase might be controlled by reversible phosphorylation. Krakower and Kim (1980) reported that the rat epididymal enzyme was activated by a phosphorylase phosphatase, a process accompanied by a loss of ^{32}P from the labelled enzyme. Similarly, Hardie and Guy (1980) observed a reversible phosphorylation and inactivation of the enzyme from lactating rat mammary gland by a cyclic AMP-dependent protein kinase. Thus, the control of acetyl-CoA carboxylase, in mammals, may be regulated by hormone-sensitive enzymes.

The degree of control of acetyl-CoA carboxylases from oleaginous micro-organisms and its importance as a determinant of oleaginicity remain obscure. However, Botham and Ratledge (1979) concluded that there were no major differences between the activities or regulation of the enzymes from *Candida* 107 and *Candida utilis*, with the possible exception of citrate activation, that correlated with the profound differences in their ability to accumulate lipid.

2.2.4 Fatty acid synthetase

Fatty acid synthetase is a multienzyme complex that catalyzes the synthesis of saturated fatty acids from malonyl-CoA, as indicated

$$CH_3CO - S\,CoA + 7HOOCCH\,CO - S - CoA + 14NADPH + 14H^+ \rightarrow$$

$$CH_3CH_2(CH_2CH_2)_6CH_2COOH + 7CO_2 + 14NADP^+ + 8CoA - SH + 6H_2O$$

Originally Lynen (1961) postulated that the enzyme from *Saccharomyces cerevisiae* was an aggregate of seven uni-functional subunits. It now appears, however, that the enzyme consists of multiple copies of only two multi-functional polypeptides (Schweizer *et al.*, 1973). The fatty acid synthetases of animals catalyze a reaction in which the products are free fatty acids. The enzyme from plant sources (including yeasts) differs, however, in that there is a terminal transacylation reaction to CoA so that fatty acyl-CoA esters are produced (Bloch and Vance, 1977).

It is doubtful that the enzyme has any role in the short term regulation of fatty acid biosynthesis. Although fatty acid synthetases from many tissues are inhibited by long chain fatty acyl-CoA esters, in general, the acetyl-CoA carboxylase from the same source shows a greater sensitivity (Volpe and Vagelos, 1976). It is possible that the enzyme is implicated in the long-term regulation of fatty acid biosynthesis by adaptive changes in its activity (Bloch and Vance, 1977).

There have been no studies, to date, on the fatty acid synthetases from oleaginous micro-organisms except the report of Gill and Ratledge (1973). They compared the activities of fatty acid synthetase and acetyl-CoA carboxylase from *Candida* 107 grown on glucose and n-alkanes. Growth on n-alkanes completely repressed acetyl-CoA carboxylase and partially repressed fatty acid synthetase and they concluded that the principal site of regulation of fatty acid biosynthesis, in this yeast, was acetyl-CoA carboxylase. This work was done, however, before the realization that the ATP:citrate lyase pathway of lipogenesis might be operative in oleaginous micro-organisms. Consequently, these authors were not in a position to comment with any certainty on the regulation of

lipogenic enzymes in those organisms.

3. Summary and aims of investigation

At the outset of this investigation a hypothesis had been advanced by Botham and Ratledge (1979) to explain the phenomenon of lipid accumulation in oleaginous micro-organisms. Briefly, this stated that when growth ceases as a result of nutrient limitation, glucose utpake continues at an undiminished rate. This causes an increase in the intracellular adenylate energy charge with a concomitant decrease in the intramitochondrial AMP concentration. Consequently, the carbon flow, which must be unrestricted, is diverted into the formation of cytosolic acetyl-CoA by mechanisms discussed previously. This acetyl-CoA together with reducing equivalents produced by glucose dissimilatory pathways, is then utilized to synthesize lipid. It follows, therefore, that lipogenic enzymes must remain active under these conditions and that once formed the lipid must accumulate and not be oxidized.

As this hypothesis was based on the results of studies with a restricted number of oleaginous micro-organisms my aims were to test its validity and universality and furthermore, to compare the process of lipogenesis in oleaginous yeasts with other lipogenic systems. Finally, it was hoped that an insight might be gained into what limits the absolute quantity of lipid accumulated as the amount of lipid which an oleaginous micro-organism can accumulate appears to have an upper limit which varies from species to species and even from strain to strain.

The investigations were pursued along two lines. Firstly, an investigation of the properties of isolated key enzymes with special reference to any possible regulatory properties. Secondly, an in-depth study of the sequence of events leading to lipid accumulation in oleaginous yeasts using continuous culture techniques.

MATERIALS AND METHODS

1. Organisms, media and cultural conditions

1.1 Organisms

Lyophilized 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, U.K.). Cultures were revived in accordance with the vendors' instructions.

1.2.1 Media for batch culture experiments

Medium for batch culture experiments contained the following constituents ($g l^{-1}$) : 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; $ZnSO_4.7H_2O$, 0.0001, with, for carbon-limiting medium, ammonium tartrate at 3 $g l^{-1}$ and glucose at 10 $g l^{-1}$ [Medium A]; and for nitrogen-limiting medium ammonium tartrate at 1.5 $g l^{-1}$ and glucose at 30 $g l^{-1}$ [Medium B] or ammonium tartrate at 0.5 $g l^{-1}$ and glucose at 100 $g l^{-1}$ [Medium C]. The pH was adjusted to pH 5.5 prior to the addition of glucose and micro-nutrients.

1.2.2 Media for continuous culture experiments

For the majority of continuous culture experiments the media used were of the same composition as those used for batch culture experiments (1.2.1) but ammonium chloride was substituted for ammonium tartrate. In certain transition (shift up from low carbon to high carbon) experiments a defined medium was used of the following composition ($g l^{-1}$) : KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4.7H_2O$, 1.5; $CaCl_2.2H_2O$, 0.1; $FeCl_3.6H_2O$, 0.008; $ZnSO_4.7H_2O$, 0.001; thiamine, 0.005; calcium pantothenate, 0.005; 4-aminobenzoic acid, 0.005; nicotinic acid, 0.005; riboflavin, 0.005; pyridoxin HCl, 0.005; biotin, 5 µg and NH_4Cl, 1.5. For carbon-limiting

medium glucose was added at 10 $g l^{-1}$ and for nitrogen-limiting medium glucose was at 100 $g l^{-1}$ [Medium D]. Medium was adjusted to pH 5.5 with HCl prior to sterilization.

1.3 Sterilization of media

Batch culture medium was sterilized *in situ* by autoclaving at 121 °C for 15 min. 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 sterile 20 l aspirators. Filters were sterilized in stainless steel holders for 30 min at 121 °C.

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). In one experiment (Results 1.1) yeast was grown in batch culture, using a 10 l fermenter fabricated in our own laboratory. The vessel contained 7.5 l medium and the culture was maintained at 30 °C by water circulation through baffles immersed in the medium and pH 5.5 by the automatic addition of NaOH. Air was delivered to the vessel at 7.5 $l min^{-1}$ and the culture was stirred at 600 rpm. Foaming was controlled by the timed addition of polyglycol P2000 antifoam (Bevaloid Ltd., Beverley, U.K.) to a final concentration of approximately 0.1% (v/v).

1.4.2 Continuous culture conditions

Continuous culture experiments were performed in an 8 l chemostat (Modular Microferm, New Brunswick Scientific Co. Inc., New Jersey, U.S.A.) with an operating volume of 5.2 l, as described by Gill (1973). Yeasts were grown at 30 °C and the culture maintained at pH 5.5 by the automatic addition of NaOH. Air was delivered to the vessel at 5 $l min^{-1}$ and the culture stirred at 600 rpm. Foaming was controlled by the timed addition

of antifoam, as described (1.4.1).

1.4.2.1 Sampling procedures

In the majority of cases 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. The subsequent handling of samples is described in the relevant analytical section.

In certain experiments, however, a rapid sampling device was fitted to the chemostat, as described by Knowles (1977). This consisted of a long hypodermic needle, passing into the culture vessel by way of the sampling port, to which a three-way tap was attached. Rapid sampling was achieved by attaching a 20 ml syringe, containing 4 ml quenching agent, to the needle and withdrawing the plunger to the desired volume with the tap sealed. This created a partial vacuum in the syringe, consequently, when the tap was placed in the appropriate position culture was forced into the syringe with concomitant rapid quenching of enzyme activities. Returning the tap to its previous position allowed removal of the syringe without danger of contaminating the remainder of the culture. Knowles (1977) has reported that using this procedure quenching times are faster than 1 sec.

1.5 Monitoring of growth

For routine measurements growth was monitored by measuring the A_{570} of suitably diluted samples of cell suspensions. The biomass was then calculated from a calibration curve of A_{570} against cell dry weight. When more accurate measurements were required, yeast biomass was determined directly. Washed yeast samples were dried in tared vials, either in a vacuum oven at 60 °C for 48 h or by treatment in a microwave oven for 5 min, prior to weighing.

1.6 Analyses

In this section analytical methods are described that were employed

during batch and continuous culture experiments.

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). The kit contains glucose oxidase, peroxidase and the chromogen, 2,2'-azino-di-[3-ethyl-benzythiazoline sulphonate] (ABTS). The principle of the method is indicated below:

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

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

Suitably diluted medium samples in 0.2 ml were mixed with 5 ml GOD-Perid reagent and after incubating at room temperature for 30 min the resultant colour read against a reagent blank at 610 nm. The unknown glucose concentration was calculated from the A_{610} due to a standard glucose solution (containing 91 $\mu g\ ml^{-1}$) taken through the above procedure.

1.6.2 Determination of NH_4^+

The concentration of NH_4^+ in medium samples was determined using 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 min the A_{625} was read against a reagent blank. A calibration curve, constructed using NH_4Cl, was linear at concentrations up to 20 $\mu g\ ml^{-1}$ when taken through the same procedure.

1.6.3 Determination of total lipid content of yeast

Total lipid content of yeast 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 H_2O. 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 a vacuum oven at 50 °C and the lipid weighed.

1.6.4 Determination of total protein content of yeast

Total cellular protein content was determined by the biuret method (Robinson and Hogden (1940). Yeast was washed in distilled H_2O to remove medium contaminants then lyophilized. Pre-weighed samples were then suspended in 2 ml distilled H_2O (1-5 mg ml^{-1}) in 5 ml centrifuge tubes. One ml 3 M NaOH was added to each tube and the contents treated in a boiling water bath for 5 min. After cooling, 1 ml $CuSO_4.5H_2O$ (2.5% w/v) was added to each tube and the contents mixed thoroughly. Yeast debris was then removed by centrifuging for 5 min at 5000 *g* and the A_{555} of the supernatant read against a reagent blank. A calibration curve was constructed using bovine serum albumin (0-5 mg ml^{-1}).

1.6.5 Determination of total intracellular amino acid pool

The amino acid pool was extracted from *Candida* 107, cultivated in continuous culture, by the procedure of Dawson (1965). 20 ml of culture were removed from the chemostat and immediately filtered under a slight negative pressure using a Millipore filtration assembly (47 mm type HA filter, 0.45 µm porosity). The pellet was washed with 5 ml ice cold distilled H_2O and the combined filtrates retained for subsequent analysis. Amino acids were extracted by treatment with 20 ml boiling distilled H_2O for 5 min. After filtration, as described, the pellet was washed

with 10 ml boiling distilled H_2O. The combined filtrate, containing extracted amino acids, was made up to 35 ml and stored at -30 °C for subsequent analysis.

Total amino acids were determined by the procedure of Yemm and Cocking (1955). A 1 ml sample, containing 0.1 to 0.5 µmol amino acid was mixed with 1 ml KCN (0.2 m$\underline{M}$ in methoxyethanol), 0.2 ml ninhydrin (5% w/v in methoxyethanol) and 0.5 ml 0.2 $\underline{M}$ citrate buffer, pH 5.0, and the mixtures incubated in a boiling water bath for 20 min. Samples were then allowed to cool to room temperature, 10 ml ethanol (60% v/v) added to each, and the resultant A_{570} read against a reagent blank. A calibration curve was constructed using glycine and this was linear up to 0.5 µmol min^{-1}. NH_4^+ interfered in this method (A_{570} 1 ≡ 4.2 µmol NH_4^+) consequently, the concentration of this ion was determined, as described previously, (1.6.2) and a correction factor calculated.

1.6.6 Determination of intracellular adenine nucleotide concentration and calculation of *in vivo* energy charge

Yeast was removed from the chemostat using a rapid sampling device, as previously described (1.4.2.1). The chosen quenching agent was 1.4 $\underline{M}$ H_2SO_4. It was not possible to process samples immediately, consequently, they were stored at -30 °C until such time as it was convenient to perform assays. Adenine nucleotides slowly decompose under such conditions, therefore, internal standards were added prior to freezing.

The following sampling procedure was used. An 18 ml sample of culture was removed from the chemostat and quenched with 4 ml 1.4 $\underline{M}$ H_2SO_4. To 3 ml of quenched sample was added 27 ml 5 m$\underline{M}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.3. To 10 ml diluted sample was added 10 ml of the above buffer and the mixture neutralized with 1 $\underline{M}$ NaOH (unknown sample). A second 10 ml aliquot of diluted sample was mixed with 10 ml 5 m$\underline{M}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.3, containing AMP, ADP and ATP each at a concentration of 1 µ$\underline{M}$ and

neutralized as described (unknown sample + internal standards). Neutralized samples were held at -30 °C after removal of debris by centrifugation. In addition, samples of spent medium were retained for the determination of extra-cellular adenine nucleotides.

Adenine nucleotides were determined using the modified luciferin-luciferase procedure of Chapman *et al.* (1971). In this method AMP and ADP are converted enzymically to ATP. To 1 ml adenine nucleotide-containing extract was added 0.2 ml of the following incubation mixtures: for ATP only (per 1 ml), 100 µmoles Na_2HPO_4/KH_2PO_4 buffer, pH 7.3; 15 µmoles $MgCl_2$; 0.5 µmole phospho(enol) pyruvate; 108 µmoles $(NH_4)_2SO_4$, pH 7.3: for ADP + ATP (per 1 ml), 100 µmoles Na_2HPO_4/KH_2PO_4, pH 7.3; 15 µmoles $MgCl_2$; 0.5 µmoles phospho(enol) pyruvate; 108 µmoles $(NH_4)_2SO_4$, pH 7.3; 11 units pyruvate kinase (Sigma type II): for AMP + ADP + ATP (per 1 ml), 100 µmoles Na_2HPO_4/KH_2PO_4 buffer, pH 7.3; 15 µmoles $MgCl_2$; 0.5 µmole phospho(enol) pyruvate; 108 µmoles $(NH_4)_2SO_4$, pH 7.3; 11 units pyruvate kinase (Sigma type II); 38.5 units adenylate kinase (Sigma type III). Reaction mixtures were incubated at room temperature for 30 min.

ATP was determined by the luminescence produced on the addition of luciferin-luciferase in the form of extract of firefly lanterns (Photinus pyralis). The assay mixture of 1 ml distilled H_2O, 1 ml $MgSO_4.7H_2O$ (1.5 $mgml^{-1}$) and 1 ml sample incubation mixture was placed in a plastic vial. At zero time, 0.1 ml lantern extract was added and simultaneously a pen recorder, attached to an instrument which incorporated a photo-multiplier tube (EMI type 60975) [Damoglou and Dawes, 1968], was switched on. The vial was immediately placed in the ATP machine and the deflection of the pen recorder caused by the bioluminescence was measured after 15 s.

Firefly lantern extract (FLE-250, Sigma London Chemical Co.) was

reconstituted by suspending in distilled H_2O (18 $mgml^{-1}$) then clarified by centrifugation at 5000 *g* for 5 min.

Each sample was assayed in triplicate and the deflections due to AMP and ATP were calculated by difference. In addition, blank determinations were carried out on samples taken through the above procedure, without added adenine nucleotides, in order to correct for adenine nucleotides in the assay reagents.

1.6.7 Determination of intracellular citrate concentration

Yeast (18 ml) was removed from the chemostat using a rapid sampling device, as previously described (1.4.2.1) and quenched with 4 ml 1.4 M H_2SO_4. After neutralizing the sample with 1 M NaOH, precipitated protein and cell debris was removed by centrifugation and the supernatant retained for analysis. At the time of sampling, aliquots of spent medium were also retained in order to determine extracellular citrate concentrations.

Citrate was determined by the method of Williamson and Corkey (1969) in which oxaloacetate produced by citrate lyase was coupled to malate dehydrogenase with the concomitant oxidation of NADH. Reaction mixtures contained in 3 ml:1 ml 0.05 M triethanolamine-HCl buffer, pH 7.4, containing 10 mM $MgSO_4$ and 5 mM EDTA; 0.05 ml NADH (10 mg ml^{-1}); 1 unit citrate lyase (from *Enterobacter aerogenes*, Sigma London Chemical Co.) and 10 units malate dehydrogenase (porcine heart, mitochondrial, Sigma London Chemical Co.) Reactions were initiated with citrate lyase and allowed to go to completion. A calibration curve was constructed using tripotassium citrate (0-100 µmoles).

2. Methods used in attempts to isolate mitochondria from oleaginous yeasts

In this section methods are described which were used to isolate mitochondria from oleaginous yeasts.

2.1 Assessment of preparations

2.1.1 Use of marker enzymes

Since citrate synthase is known to be exclusively of mitochondrial origin its presence in cytosolic fractions was taken to indicate mitochondrial damage. The assay procedure is described in the relevant section (3.1.4).

2.1.2 Demonstration of respiratory control

The demonstration of a high respiratory control ratio is considered to be the best criterion for assessing the quality of mitochondrial preparations (Chappell and Hansford, 1972). Rates of O_2 consumption were measured using a 3 ml Perspex reaction vessel, maintained at 30 °C by means of a water jacket, and fitted with a Clark-type oxygen electrode (Model 777, Beckman Instruments, Glenrothes, Fife, U.K.) attached to a potentiometric recorder (Servoscribe, Smith Industries Ltd., Wembley, U.K.).

Reaction mixtures contained 2.9 ml 50 m$\underline{\underline{M}}$ HEPES buffer, pH 7.4, containing 0.5% (w/v) bovine serum albumin and 0.1 ml 0.1 $\underline{\underline{M}}$ HEPES/succinate, pH 7.4. Mitochondrial suspension (0.3 ml) was added and the recorder switched on to establish a base line after which 2.5 µl 0.1 $\underline{\underline{M}}$ ADP was added and the rate of O_2 consumption monitored. When the rate of O_2 consumption returned to the basal level a further aliquot of ADP was added. The respiratory control ratio was the ratio of the rates of O_2 consumption observed after the addition of the second aliquot of ADP and the basal rate.

2.2 Attempts to produce sphaeroplasts from oleaginous yeasts

In early attempts the procedure of Ohnishi *et al.* (1966) was employed. Yeast was harvested in the early exponential phase of growth by centrifugation at 1000 *g* for 10 min and 4 °C. The pellet was washed 4 times in distilled H_2O by repeated resuspension and centrifugation, as

described. This was followed by 2 washes, in a similar manner, in 10 m$\underline{M}$ EDTA. Yeast was then resuspended in the same medium (0.5 g wet wt/ml). Snail gut enzyme (see 2.2.3) was added and the mixture incubated at 30 °C with gentle agitation in a shaking water bath. Samples were removed at 10 min intervals and the yeast either examined microscopically for morphological changes, or diluted 500-fold in distilled H_2O then examined microscopically for cell lysis.

2.2.1 Pre-treatment with sodium thioglycollate

In certain experiments yeast was treated with sodium thioglycollate prior to snail gut enzyme exposure, as described by Lebeault *et al.* (1969). Yeast was harvested and washed in 50 m$\underline{M}$ Tris/HCl, pH 8.0 by centrifugation and resuspension, as described (2.2). The pellet was suspended (0.5 g wet wt/ml) in 50 m$\underline{M}$ Tris/HCl, pH 9.0, containing 0.5 $\underline{M}$ sodium thioglycollate and the mixture incubated for 30 min at 25 °C. Yeast was recovered by centrifugation at 1000 g for 10 min and 25 °C then resuspended (0.5 g wet wt/ml) in 50 m$\underline{M}$ citrate/phosphate buffer, pH 4.1, containing 1 $\underline{M}$ $MgSO_4.7H_2O$. Snail gut enzyme (see 2.2.3) was added and the mixture incubated at 30 °C with gentle agitation in a shaking water bath. Samples were removed and examined for evidence of sphaeroplast formation, as described (2.2).

2.2.2 Treatment with mercaptoethylamine

In certain experiments the procedure of Duell *et al.* (1964) was employed. Yeast was harvested and washed in 50 m$\underline{M}$ Tris/HCl buffer, pH 8.0, as described (2.2). The pellet was resuspended in 0.1 M citrate/phosphate buffer, pH 5.8, containing 0.63 $\underline{M}$ sorbitol, 0.4 m$\underline{M}$ EDTA and mercaptoethylamine (6 mg ml^{-1}). Snail gut enzyme was added (see 2.2.3) and the mixture incubated at 30 °C with gentle agitation in a shaking water bath. Samples were removed and examined for sphaeroplast formation, as described (2.2).

2.2.3 Snail gut enzyme

Three commercial preparations of snail gut enzyme were used: β-glucuronidase (Sigma London Chemical Co. Ltd.); β-glucuronidase (Boehringer Corporation (London) Ltd.); and helicase (Industrie Biologique Francaise S.A., Gennevilliers, France). The preparations supplied were used at a concentration of 0.3 ml per g wet wt yeast.

In addition, snail gut enzyme was prepared from live *Helix pomatia* by the procedure of Poole and Lloyd (1976). Digestive tracts were removed from 6 live snails (Gerrard and Co. Ltd., Dorking, Surrey, U.K.), which had been first de-shelled, and then ground in a pre-cooled mortar and pestle after suspending in ice-cold distilled H_2O (1 ml per digestive tract). The homogenate was centrifuged at 30,000 *g* for 30 min and 4 °C and the supernatant dialyzed against 100 vol distilled H_2O for 6 h and 4 °C. Solid $MgSO_4.7H_2O$ was added to the dialysate to a final concentration of 0.9 M and after adjusting to pH 6.8 the material was stored at -20 °C until required for use.

2.3 Mechanical methods of yeast disruption used in attempts to isolate mitochondria

Various mechanical cell disruption methods used to effect the initial cell breakage step in the isolation of mitochondria from oleaginous yeasts are described here. All the methods shared in common, the harvesting of yeast from exponentially growing cultures, washing and then resuspension in isotonic medium prior to treatment by the disruption method under test.

2.3.1 Use of the Chaikoff press

This apparatus was fabricated in our own workshops according to the specifications of Emanuel and Chaikoff (1957). It consists of two cylinders, one upper, one lower, subdivided by a stainless steel partition. The upper cylinder contains a closely fitting piston terminating in a rod, such that as the piston is depressed the rod passes through a

precisely machined orifice in the partition. Rods are interchangeable giving a range of gap sizes between 6 and 130 μm. A cell suspension is placed in the upper reservoir and when the piston is depressed cells are forced into the lower collecting reservoir, via the gap between the rod and orifice. With a knowledge of cell size a gap size may be selected that is small enough to rupture the wall but large enough to allow free passage of organelles.

Washed yeast was suspended (0.5 g wet wt per ml) in 50 mM Tris/HCl, pH 7.5, containing 1.3 M sorbitol, and 30 ml subjected to two treatments in the chilled Chaikoff press, with a selected gap size of 6 μm. After treatment, samples of the suspension were examined microscopically for evidence of breakage.

2.3.2 *Use of a cell disruption bomb*

Attempts were made to disrupt suspensions of oleaginous yeasts using a cell disruption bomb (Kontes, Vineland, N. Jersey, U.S.A.), as described by Hunter and Commerford (1964). The apparatus consists of a stainless steel vessel into which a cell suspension is placed and then the whole compressed to several atmospheres with gaseous nitrogen. After a period of equilibration, the apparatus is rapidly decompressed, thereby generating intracellular N_2 bubbles which rupture cell membranes as they come out of solution.

Washed yeast suspension (100 ml, 0.5 g wet wt per ml in 50 mM Tris/HCl buffer, pH 7.5, containing 1.3 M sorbitol) was subjected to 30 min treatment at 2000 psi and 4 °C. After decompression, whole cells and debris were removed by centrifugation at 2000 *g* for 10 min and 4 °C. Prior to decanting the supernatant, solidified lipid was removed from the surface and sides of centrifuge tubes by wiping with tissue. Mitochondria were then sedimented by centrifuging at 19,000 *g* for 20 min and 4 °C. The pellet was resuspended, by gentle agitation, in 50 mM Tris/HCl buffer,

pH 7.5, containing 1.3 M sorbitol, and mitochondria recovered by centrifugation, as above. Washed mitochondria were then resuspended in 2 ml 50 mM HEPES buffer, pH 7.5, containing 0.25 M sucrose and retained for analysis.

2.3.3 Use of an agitator ball mill

Labbe and Chambon (1977) described a method using a Dyno Mill KDL (Glen Creston Machinery Ltd., London, U.K.) for the large scale preparation of mitochondria from *Saccharomyces cerevisiae* exhibiting acceptor control. This method was applied to oleaginous yeasts.

The apparatus consists of a stainless steel drum of 600 ml capacity fitted with a cooling jacket through which chilled ethylene glycol is circulated. The cylinder contains glass beads typically occupying 80 to 85% of the volume. Rotating agitator discs within the cylinder transfer their kinetic energy to the beads creating impact and shear forces which disrupt the yeast cells. It is a continuous flow device; exit of the beads being prevented by an adjustable separator gap. The degree of disruption can be controlled by selection of bead size, rotation rate, and by varying the flow rate and hence, the residence time.

The chamber was loaded with 520 ml 0.5 mm glass beads after fitting 4.64 cm rotating agitator discs and 0.05 mm dynamic slit separator. After cooling the apparatus to -5 °C the yeast suspension (500 g wet wt in 1 l 50 mM HEPES buffer, pH 7.5, containing 1.3 M sorbitol) was pumped in at a rate of 70 ml min^{-1}. When 200 ml was in the vessel, the mill was switched on at a rotation speed of 3000 rpm. Effluent was collected in a chilled beaker. When all of the suspension was in the breakage chamber the apparatus was flushed with 500 ml of the above buffer and the washings combined with the original effluent. The mitochondrial fraction was recovered from the effluent as previously

described (2.3.2).

2.3.4 Use of the Braun disintegrator

The Braun disintegrator (Shandon Scientific Co. Ltd., London, U.K.) achieves disruption by the creation of shear and impact forces when cells are violently agitated with glass beads. The heat which is produced is dissipated by pulses of high pressure CO_2.

Washed yeast was suspended in 50 mM HEPES buffer, pH 7.5, containing 1.3 M sorbitol (0.5 g wet wt per ml) and chilled in ice. Ballotini beads (60 mesh) were added to the suspension (1 part suspension to 2 parts beads, v/v) and 50 ml of the mixture placed in a chilled disruption bottle which was then subjected to 5 min treatment in the disintegrator. Whole cells and beads were sedimented by centrifuging at 2000 g for 10 min and 4 °C. Mitochondria were recovered from the supernatant, as described (2.3.2).

2.3.5 Use of the MSE homogenizer

This instrument (MSE Co. Ltd., Crawley, Sussex, U.K.) consists of a motor-driven bladed impeller. Disruption is achieved by the creation of shear and impact forces created when mixtures of cells and glass beads are violently agitated.

Washed yeast was suspended in 50 mM HEPES buffer, pH 7.5, containing 1.3 M sorbitol (0.5 g wet wt per ml). Acid washed 60 mesh glass beads were added (1 part suspension to 1 part glass beads, v/v) and 30 ml of the mixture subjected to 5 min treatment at maximum rotation. Heat was dissipated by immersing the vessel in a salt/NaCl mixture. Mitochondria were separated from the resultant homogenate, as described (2.3.2).

2.3.6 Use of the ultrasonic disintegrator

Ultrasonic extracts were prepared by suspending washed yeast (0.5 g wet wt per ml) in 50 mM HEPES buffer, pH 7.5, containing 1.3 M sorbitol, and after cooling in an ice/NaCl mixture subjecting the yeast to a series

of 30 s exposures to the full output of a Dawe Soniprobe ultrasonic disintegrator (Dawe Instruments Ltd., London, U.K.). The total treatment time was 3 min and in between exposures the suspension was cooled to 4 °C in an ice/NaCl mixture. Mitochondria were recovered from the sonicate by differential centrifugation, as described (2.3.2).

2.3.7 Use of the French pressure cell

Washed yeast was suspended (1 g wet wt per 1 ml) in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, containing 1.3 M sorbitol and subjected to 1 passage through a pre-cooled French pressure cell operated at 35 MPa. Mitochondria were recovered from the homogenate by differential centrifugation, as described (2.3.2).

2.4 Preparation of cytosolic and mitochondrial extracts from *Lipomyces starkeyi* CBS 1809

Yeast was harvested from a 48 h vortex-aerated batch culture, cultivated on nitrogen-limiting medium by centrifuging at 2000 *g* for 10 min and 4 °C. After washing, the cell paste was suspended (0.5 g wet wt per ml) in 50 mM Tris/HCl buffer, pH 7.5, containing 1.3 M sorbitol, 1 mM $MgCl_2$ and 10 mM dithiothreitol. Yeast was disrupted by 1 passage through a chilled French pressure cell operated at 35 MPa. Whole cells were removed by centrifuging at 2000 *g* for 10 min and 4 °C. Solidified lipid was removed from the sides of the centrifuge tube by wiping with tissue and the supernatant carefully decanted. Mitochondria were sedimented by centrifuging at 19,000 *g* for 20 min and 4 °C. The supernatant (cytosolic fraction) was retained for analysis and the crude mitochondrial fraction resuspended in the above medium and then re-sedimented, as described.

The washed mitochondrial pellet was suspended in 2 volumes of the above medium. Glass beads (100 mesh) were added to the mitochondrial suspension (1 part suspension to 2 parts beads) and the mixture treated for 1 min in a motor-driven homogenizer at 4 °C. The beads were

removed by centrifuging at 2000 *g* for 5 min and 4 °C and the supernatant (mitochondrial extract) retained for analysis.

3. Enzyme assays

3.1 Enzyme assays used in experiments to demonstrate the intracellular location of pyruvate dehydrogenase and ATP:citrate lyase

3.1.1 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 buffer, 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.), 10 units and extract. Reactions were initiated by the addition of CoA.

3.1.2 Pyruvate dehydrogenase complex

[E.C.1.2.4.1; pyruvate: lipoamide oxidoreductase (decarboxylating and acceptor-acetylating); E.C.1.6.4.3; dihydrolipoamide reductase (NAD^+) (NADH:lipoamide oxidoreductase); E.C.2.3.1.12; dihydrolipoamide acetyltransferase (acetyl-CoA:dihydrolipoamide-s-acetyltransferase)] was assayed by a procedure based on that of Reed and Mukherjee (1969) following the increase in A_{340} due to the reduction of NAD^+.

The reaction mixture contained in 1 ml: KH_2PO_4/Na_2HPO_4 buffer, pH 8.0, 50 mM; $MgCl_2$, 5 mM; NAD^+, 0.5 mM; dithiothreitol, 1 mM; thiamine pyrophosphate, 0.2 mM; CoA (made up in distilled H_2O containing 30 mM dithiothreitol), 0.1 mM; sodium pyruvate, 1 mM and extract. The enzyme was assayed at 30 °C and reactions were initiated by the simultaneous addition of CoA and sodium pyruvate.

3.1.3 NAD^+-dependent isocitrate dehydrogenase

[E.C.1.1.1.41; threo-D_s-isocitrate : NAD^+ oxidoreductase] was assayed at 30 °C according to Kornberg (1955) by measuring the increase in A_{340} due to the reduction of NAD^+.

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

3.1.4 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 (Sigma London Chemical Co. Ltd.), 100 µM and extract.

Assays were performed at 25 °C using a blank with all components but 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 A_{412}.

3.1.5 Glucose 6-phosphate dehydrogenase

[E.C.1.1.1.49; D-glucose 6-phosphate:$NADP^+$ oxidoreductase] was assayed at 30 °C by the procedure of Noltmann *et al.* (1961) measuring the rate of reduction of $NADP^+$ at 340 nm.

The reaction mixture contained in 1 ml: glycylglycine buffer, pH 8.0, 70 mM; $MgSO_4$, 10 mM; $NADP^+$, 0.5 mM; glucose 6-phosphate, 1 mM and extract. Reactions were initiated by the addition of glucose 6-phosphate.

3.2 Phosphoketolases

3.2.1 Fructose 6-phosphate phosphoketolase

[E.C.4.1.2.22; D-fructose 6-phosphate D-erythrose 4-phosphate lyase (phosphate-acetylating)] was assayed as described by Botham (1978) based on an original method of Goldberg *et al.* (1966). The acetyl phosphate product is allowed to react with neutral hydroxylamine to give aceto-hydroxamate, which in turn reacts with ferric chloride to give a complex with a λ_{max} at 540 nm. Botham (1978) made two modifications to the original method. Firstly, hydroxylamine was present during the incubation to prevent further metabolism of acetyl phosphate to acetate. Secondly, sodium arsenite was included to inhibit pyruvate dehydrogenase which would produce interfering acetyl-CoA.

The reaction mixture contained in 1 ml: histidine buffer, pH 7.0, 40 m$\underline{M}$; $MgCl_2$, 0.8 m$\underline{M}$; dithiothreitol, 1.4 m$\underline{M}$; thiamine pyrophosphate 0.3 m$\underline{M}$; sodium arsenite, 4 m$\underline{M}$; fructose 6-phosphate, 30 m$\underline{M}$; 500 μl 2 $\underline{M}$ hydroxylamine hydrochloride, pH 5.4 and extract.

Reactions were initiated by the addition of fructose 6-phosphate and the mixture incubated at 30 °C. After 15 min 1.5 ml 20% (w/v) $FeCl_3$ in 0.1 $\underline{M}$ HCl added and after centrifuging, the A_{540} of the supernatant compared with a calibration curve produced from acetyl phosphate (0-1 μmol) taken through the above procedure.

3.2.2 Xylulose 5-phosphate phosphoketolase

[E.C.4.1.2.9; D-xylulose 5-phosphate D-glyceraldehyde 3-phosphate lyase (phosphate-acetylating)] was assayed by the two-stage method of Goldberg *et al.* (1966). In the first stage, aldolase and triose-phosphate isomerase are 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 is 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 0.15 ml: KH_2PO_4/ Na_2HPO_4 buffer, pH 6.0, 33 mM; $MgCl_2$, 3.3 mM; thiamine pyrophosphate, 1.3 mM; xylulose 5-phosphate, 1.3 mM; aldolase, 0.14 unit; triose phosphate isomerase, 0.62 unit; α-glycerophosphate dehydrogenase, 0.08 unit and extract. The mixture was incubated at 30 °C for 5 min then the reaction stopped by immersion in a boiling water bath for 1 min. Precipitated protein was removed by centrifuging at 10,000 g for 5 min.

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, 0.05 mM, aldolase, 0.14 unit; triose-phosphate isomerase, 0.62 unit; α-glycerophosphate dehydrogenase, 0.08 unit.

3.3 Enzymes assayed as possible contaminants of purified citrate synthase preparations

3.3.1 Malate dehydrogenase

[E.C.1.1.1.37; L-malate:NAD^+ oxidoreductase] was assayed by the method of Englard and Siegel (1969) following the reduction of NAD^+ at 340 nm.

The reaction mixture contained in 1 ml: glycine/NaOH buffer, pH 10.0, 70 mM; NAD^+, 1 mM; sodium L-malate, 10 mM and extract. Assays were performed at 30 °C and initiated by the addition of L-malate.

3.3.2 Adenylate kinase

[E.C.2.7.4.3; ATP:AMP phosphotransferase] was assayed as described by Sottocasa *et al.* (1967) in which the ATP produced in the reaction is used to synthesize glucose 6-phosphate, from glucose, via the action of hexokinase. The glucose 6-phosphate is then converted to 6-phospho-gluconate, by the addition of glucose 6-phosphate dehydrogenase with the concomitant reduction of $NADP^+$ which is monitored at 340 nm.

The reaction mixture contained in 1 ml: Tris/HCl buffer, pH 7.5, 50 mM; ADP, 5 mM; $MgSO_4$, 5 mM; glucose, 10 mM; $NADP^+$, 0.2 mM; hexokinase, 10 units, glucose 6-phosphate dehydrogenase, 10 units and extract. Assays were performed at 30 °C and initiated by the addition of ADP.

3.3.3 Aconitase

[E.C.4.2.1.3; citrate (isocitrate) hydro-lyase] was assayed at 30 °C according to Anfinsen (1955) in which the cis-aconitate produced is assayed directly by following the increase in A_{240}

The reaction mixture contained in 1 ml: KH_2PO_4/Na_2HPO_4 buffer, pH 7.6, 30 mM; sodium citrate, 12 mM and extract. Assays were initiated by the addition of sodium citrate and the A_{240} compared with a blank containing all components but sodium citrate.

3.4 Enzymes assayed as possible contaminants of partially-purified NAD^+-dependent isocitrate dehydrogenase preparations

3.4.1 $NADP^+$-dependent isocitrate dehydrogenase

[E.C.1.1.1.42; threo-D_s-isocitrate:$NADP^+$ oxidoreductase] (decarboxylating) was assayed according to Kornberg (1955). The procedure was as described for NAD^+-dependent isocitrate dehydrogenase (3.1.3) but 0.2 mM $NADP^+$ was substituted for NAD^+.

3.4.2 Isocitrate lyase

[E.C.4.1.3.1; threo-D_s-isocitrate glyoxylate-lyase] was assayed by measuring the amount of glyoxylate formed from isocitrate using a colourimetric procedure described by McFadden (1969).

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 min at 30 °C then stopped by the addition of 1 ml 10% (w/v) trichloracetic acid.

To 1 ml reaction mixture, in a 30 ml beaker, was added 6 ml of a mixture of 5 parts 10 mM oxalic acid and 1 part 1% (w/v) phenyl hydrazine hydrochloride. The mixture was heated to boiling then immediately cooled to room temperature. After 5 min the mixture was chilled for 2 min in an ice bath then 4 ml conc. HCl added followed by the addition of 7 ml 5% (w/v) potassium ferricyanide. The A_{520} of the mixture was read against a distilled H_2O blank after a further 7 min.

The yield of glyoxylate (μ moles) was calculated from the formula:

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

3.4.3 Oxoglutarate dehydrogenase complex

[E.C.1.2.4.2; 2-oxoglutarate, lipoate oxidoreductase (decarboxylating and acceptor-succinylating)] was assayed by the method of Reed and Mukherjee (1967) following the reduction of NAD^+ at 340 nm.

The reaction mixture was essentially the same as that described for the assay of pyruvate dehydrogenase (3.1.2) but 1 mM potassium oxoglutarate was substituted for sodium pyruvate.

3.5 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 3 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.

3.6 Acetyl-CoA carboxylase

[E.C.6.4.1.2; acetyl-CoA:carbon dioxide ligase] was assayed by the procedure of Inoue and Lowenstein (1975) following the incorporation of label from [^{14}-C] $NaHCO_3$ into acid-stable malonyl-CoA.

The assay mixture contained in 1 ml: Tris/HCl buffer, pH 7.5, 100 mM; $MgCl_2$, 20 mM; dithiothreitol, 1 mM; ATP, 5 mM; acetyl-CoA, 0.2 mM; $NaH^{14}CO_3$ (1 $\mu Ci \mu mol^{-1}$), 5 mM and extract.

Mixtures, in glass scintillation vials, were incubated at 30 °C for 10 min in a shaking water bath after initiating reactions by the addition of $NaH^{14}CO_3$. Reactions were stopped by the addition of 0.1 ml 4 M HCl and the mixtures evaporated to drynesss in a stream of N_2 to remove unreacted $^{14}CO_2$. Residues were taken up in 0.5 ml distilled H_2O to which was added 10 ml scintillation fluid (2,5-diphenyloxazole, 4 g; naphthalene, 60 g; ethandiol, 20 ml; methanol, 100 ml and dioxan, 880 ml). Samples were stored overnight, in the dark, to avoid transient chemiluminescence prior to counting by liquid scintillation.

4. Experiments with phosphoketolases

4.1 Growth of yeasts

Yeasts were cultivated on nitrogen-limited medium (Medium B, 1.2.1) under batch conditions in 1 *l* vortex-aerated vessels, at 30 °C. Yeasts were harvested in the mid-exponential phase of growth by centrifuging at 2000 *g* for 10 min and 4 °C, and washed by resuspension in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, and centrifuged, as described.

4.2 Preparation of extracts

Washed yeast was suspended in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, containing 1 mM $MgCl_2$ and 0.5 mM dithiothreitol (0.5 g wet wt per ml) and disrupted by a single passage through a pre-cooled French pressure cell at 35 MPa. Whole cells and debris were removed by centrifuging at 45,000 *g* for 30 min and 4 °C and the supernatant filtered through Whatman No. 1 filter paper to remove solidified lipid. The filtrate was retained for assay, as described (3.7 and 3.8).

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

5. Experiments with citrate synthase

5.1 Growth of yeast

Candida 107 was grown in a chemostat, as described (1.4.2). The medium was nitrogen-limiting (Medium B) and the yeast was cultivated at 30 °C and a dilution rate of 0.05 h^{-1}. Cultures were maintained at pH 5.5 by the automatic addition of NaOH.

5.2 Preparation of extracts

Yeast was removed from the chemostat and harvested by centrifuging at 2000 g for 10 min and 4 °C. The pellet was washed by resuspension in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, and centrifuging, as described above. A crude cell-free extract was prepared, in the above buffer, as previously described (4.2).

5.3 Enzyme purification

All procedures were carried out at 4 °C. A 500 ml sample of culture was removed from the chemostat which after harvesting and washing yielded 15 to 20 g wet wt yeast. This was used to prepare a crude cell-free extract, as described.

Solid $(NH_4)_2SO_4$ was added to the stirred extract over 15 min to give 55% saturation. After a further 15 min equilibration period the mixture

was centrifuged at 30,000 *g* for 10 min and the precipitate discarded. The supernatant was taken to 80% 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 7.5.

The $(NH_4)_2SO_4$ fraction was applied to a Sephadex G-150 column (2.5 x 40 cm) equilibrated in 10 m$\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.5. After elution with similar buffer active fractions were combined and applied to a column (3 x 8 cm) of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories, Richmond, California, U.S.A.) equilibrated in 10 m$\underline{\underline{M}}$ KH_2PO_4/Na_2HPO_4 buffer, pH 7.5. The enzyme was eluted with a linear gradient (total volume 200 ml) of KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, from 10 to 200 m$\underline{\underline{M}}$. Active fractions were combined and stored at -20 °C until required.

The protein concentration of extracts was monitored throughout the purification using the dye-binding procedure of Bradford (1976) (see Section 4.3).

5.4 Determination of enzyme purity

A sample of purified citrate synthase was concentrated 10-fold with an Amicon micro-volume ultrafiltration cell, fitted with a PM10 membrane (Amicon Corporation, Lexington, Mass., U.S.A.). Samples of the concentrate, containing approximately 100 µg protein, were developed at 4 °C on 7% (w/v) polyacrylamide gels, according to the procedure of Davis (1964), in 0.04 $\underline{\underline{M}}$ Tris/glycine buffer, pH 8.3, at 2 ma per tube. Protein was detected by staining for 1 h in 1% (w/v) Naphthalene Black dissolved in 7% (v/v) acetic acid. Gels were destained by irrigation in 7% (v/v) acetic acid. Activity was detected by cutting an unfixed gel, which had been rapidly frozen in powdered solid CO_2, into 1 mm slices and immersing the slices in the standard assay mixture. The enzyme was located by the appearance of the yellow mercaptide ion. As

a control, a gel was treated in a similar manner but with oxaloacetate omitted from the assay mixture.

5.5 Kinetic analyses

Kinetic analyses and inhibitor studies were performed as detailed in the relevant Results section using a recording spectrophotometer (Model SP8100 Pye Unicam Co. Ltd., Cambridge, U.K.).

6. Experiments with NAD^+-dependent isocitrate dehydrogenase

6.1 Growth of yeast

Lipomyces starkeyi CBS 1809 was grown in a chemostat, as described (1.4.2) on nitrogen-limiting medium (Medium B) at 30 °C and at a dilution rate of 0.05 h^{-1}.

6.2 Preparation of extracts

Yeast was removed from the chemostat and harvested by centrifuging at 2000 *g* for 10 min and 4 °C. The pellet was washed by resuspension in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, and centrifuging as above. A crude cell-free extract was prepared, in similar buffer, as previously described (4.2).

6.3 Enzyme purification

All procedures were carried out at 4 °C. In a typical purification 250 ml of culture were removed from the chemostat which on harvesting yielded approximately 20 g wet wt yeast. Solid $(NH_4)_2SO_4$ was added to the stirred crude extract (prepared, as described) over a 15 min period, to give 45% saturation. After stirring for a further 15 min the mixture was centrifuged at 30,000 *g* for 10 min and the precipitate discarded. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 70% saturation in a similar manner and the resultant precipitate recovered by centrifuging, as above, then dissolved in a minimum volume of 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5.

The $(NH_4)_2SO_4$ fraction was applied to a Sephadex G-200 column

(2.5 x 40 cm) equilibrated in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5. Following elution in similar buffer active fractions were combined and stored at 4 °C. The protein concentration of extracts was monitored during the purification by the dye-binding method of Bradford (1976) (see Section 4.3).

6.4 Kinetic analyses

Kinetic analyses and inhibitor studies were performed as detailed in the relevant Results section using a recording spectrophotometer (Model SP8100, Pye Unicam Co. Ltd., Cambridge, U.K.).

7. Studies with ATP:citrate lyase

7.1 Survey for the occurrence of ATP:citrate lyase in oleaginous and non-oleaginous yeasts

7.1.1 Cultivation of yeasts

Yeasts were cultivated at 30 °C in 1 l vortex-aerated vessels, as described previously (1.4.1), on both carbon and nitrogen-limiting media (Media A and B, respectively). Yeasts were harvested after 48 h by centrifuging at 2000 *g* for 10 min and washed by resuspension in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM $MgCl_2$ and 10 mM dithiothreitol and centrifuged as described above. Pellets were divided into two equal parts, the first being retained for enzymic analysis and the second lyophilized prior to the determination of lipid content.

7.1.2 Preparation of extracts

French press extracts were prepared in 50 mM Tris/HCl buffer, pH 8.0, containing 1 mM $MgCl_2$ and 10 mM dithiothreitol, as previously described (4.2). In certain experiments the extraction buffer was supplemented with 1 mM ATP and 20 mM tripotassium citrate. The protein concentration of extracts was determined by the dye-binding procedure of Bradford (1976) (see Section 4.3).

7.2 Partial purification of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

ATP:citrate lyase was purified from *Lipomyces starkeyi* CBS 1809 grown in a chemostat at 30 °C at a dilution rate of 0.05 h^{-1} on nitrogen-limiting medium, as previously described (1.4.2). In a typical purification 250 ml of culture was removed from the chemostat which on harvesting and washing yielded approximately 20 g wet wt yeast. All procedures were carried out at 4 °C.

A French press extract was prepared in 20 m$\underline{\underline{M}}$ Tris/HCl buffer, pH 7.5, containing 1 m$\underline{\underline{M}}$ $MgCl_2$, 10 m$\underline{\underline{M}}$ 2-mercaptoethanol, 1 m$\underline{\underline{M}}$ ATP and 20 m$\underline{\underline{M}}$ tripotassium citrate, as previously described (4.2). Solid $(NH_4)_2SO_4$ was added to the stirred extract over a period of 15 min to give 40% saturation. After stirring for a further 15 min the precipitate was sedimented by centrifuging at 30,000 *g* for 10 min. The precipitate was dissolved in a minimum volume of 20 m$\underline{\underline{M}}$ Tris/HCl buffer, pH 7.5, with the additives described above, and applied to a Sepharose 4B column (2.5 x 40 cm) equilibrated in similar buffer. After elution in similar buffer, active fractions were combined and applied to a DEAE-Sephadex (A50) column (2 x 50 cm) also equilibrated in 20 m$\underline{\underline{M}}$ Tris/HCl buffer, pH 7.5, containing the additives. ATP:citrate lyase was recovered in the wash-through peak and was retained at 4 °C for further analysis. The protein concentration of extracts was monitored throughout the purification by the dye-binding procedure of Bradford (1976) (see Section 4.3).

7.3 Calibration of Sephacryl-S300 column for the determination of the molecular weight of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

For the purpose of calibration, the column (2.5 x 90 cm) was equilibrated in 50 m$\underline{\underline{M}}$ Tris/HCl buffer, pH 7.5. The void volume of the column was determined by loading 5 ml 0.2% (w/v) blue dextran (Pharmacia Uppsala, Sweden) and eluting it in similar buffer. The peak of the

eluted dye was determined spectrophotometrically at 630 nm.

The following proteins, in the quantities indicated, were loaded onto the column in a volume of 5 ml and eluted in 50 mM Tris/HCl buffer, pH 7.5: β-galactosidase (Sigma, grade VI), 3 mg; Urease (Sigma, type III), 30 mg; phosphorylase-a (Sigma) and pyruvate kinase (Sigma, type II), 5 mg. Peak activities were detected using the standard assay procedures as described in 'Biochemica Information' (Vol. 2, Boehringer Mannheim GmBH, W. Germany).

On a separate occasion 30 mg thyroglobulin (Sigma, type I) in 5 ml 50 mM Tris/HCl buffer, pH 7.5, was loaded onto the column and eluted in similar buffer. Thyroglobulin was detected spectrophotometrically at 280 nm.

8. Studies with acetyl-CoA carboxylase

8.1 Preparation of extracts

Yeast was harvested by centrifuging at 10,000 *g* for 5 min and then washed by resuspension in 20 mM Tris/Hcl buffer, pH 7.5, containing 1 mM dithiothreitol and centrifuging as above. Washed yeast was suspended in similar buffer (300 mg wet wt per ml) and disrupted by passage through a pre-cooled French pressure cell at 35 MPa. In certain experiments disruption was achieved ultrasonically (3 x 20 s at 5A using a soniprobe, Dawe Instruments Ltd., London, U.K.) or by treatment in a homogenizer (MSE Instruments Ltd., Crawley, Sussex, U.K.).

Debris and whole cells were removed by centrifuging at 45,000 *g* for 30 min and 4 °C and the supernatant filtered through Whatman No. 1 filter paper to remove solidified lipid. The filtrate was centrifuged at 100,000 *g* for 1 h and 4 °C. Endogenous substrates for CO_2-fixation reactions were removed by dialysing extracts against 100 volumes 20 mM Tris/HCl buffer, pH 7.5, containing 1 mM dithiothreitol.

In certain experiments extraction buffers were supplemented with

either glycerol (10% v/v) or bovine serum albumin (0.5% w/v).

8.2 Preparation of toluene-lyzed cells

Toluene-lyzed cells were prepared by the procedure of Serrano *et al.* (1973). Washed yeast was suspended (50 mg wet wt per ml) in 100 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.7, containing 200 mM KCl and 10 mM 2-mercaptoethanol. Toluene/ethanol (1:4, v/v) was added (50 µl/ml yeast suspension) and the mixture subjected to 5 min uninterrupted shaking on a Whirlimixer (Fisons Scientific Apparatus Ltd., Loughborough, U.K.).

8.3 Investigation of the pathway of carbon into lipid in *Lipomyces starkeyi* CBS 1809 and *Saccharomyces cerevisiae*

Exponentially growing yeast was harvested from shake flask cultures, cultivated on nitrogen-limiting medium, by centrifuging at 10,000 *g* for 5 min. The pellet was washed in 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5, by resuspension and centrifuging, as above. Washed yeast cells were permeabilized with toluene as previously described (8.2).

To 1 ml toluene-lyzed yeast suspension (50 mg wet wt per ml) was added 1 ml of reaction mixture (contents indicated in the Results, Table 2). Mixtures were incubated for 1 h at 30 °C. Reactions were stopped with 4 ml chloroform/methanol (2:1 v/v) and lipid extracted overnight. Chloroform/methanol containing extracted lipid was removed and washed twice with 2 ml NaCl (0.5% w/v) then evaporated to dryness in a rotary evaporator. Residues were dissolved in 1 ml toluene and placed in glass scintillation vials to which was also added 10 ml scintillation fluid (4 g 2,5-diphenyloxazole dissolved in 1 l toluene). Radioactivity was counted by liquid scintillation.

RESULTS

1. Studies on physiological changes accompanying lipid accumulation

This section describes experiments carried out on cultures of oleaginous yeasts in which the physiological changes leading to lipid accumulation were investigated. Two yeasts were used, *Candida* 107 and *Lipomyces starkeyi* CBS 1809. The first of these has been frequently used, in this laboratory, as a typical oleaginous yeast and, as its growth characteristics have been well documented, I continued to use it in many of my experiments. However, the use of *Candida* 107 posed certain problems in that some of the key enzymes were apparently labile. Therefore, *L. starkeyi* CBS 1809 which proved more amenable to study was also used.

1.1 Demonstration of lipid accumulation in *L. starkeyi* CBS 1809 and *Candida* 107 grown in batch culture

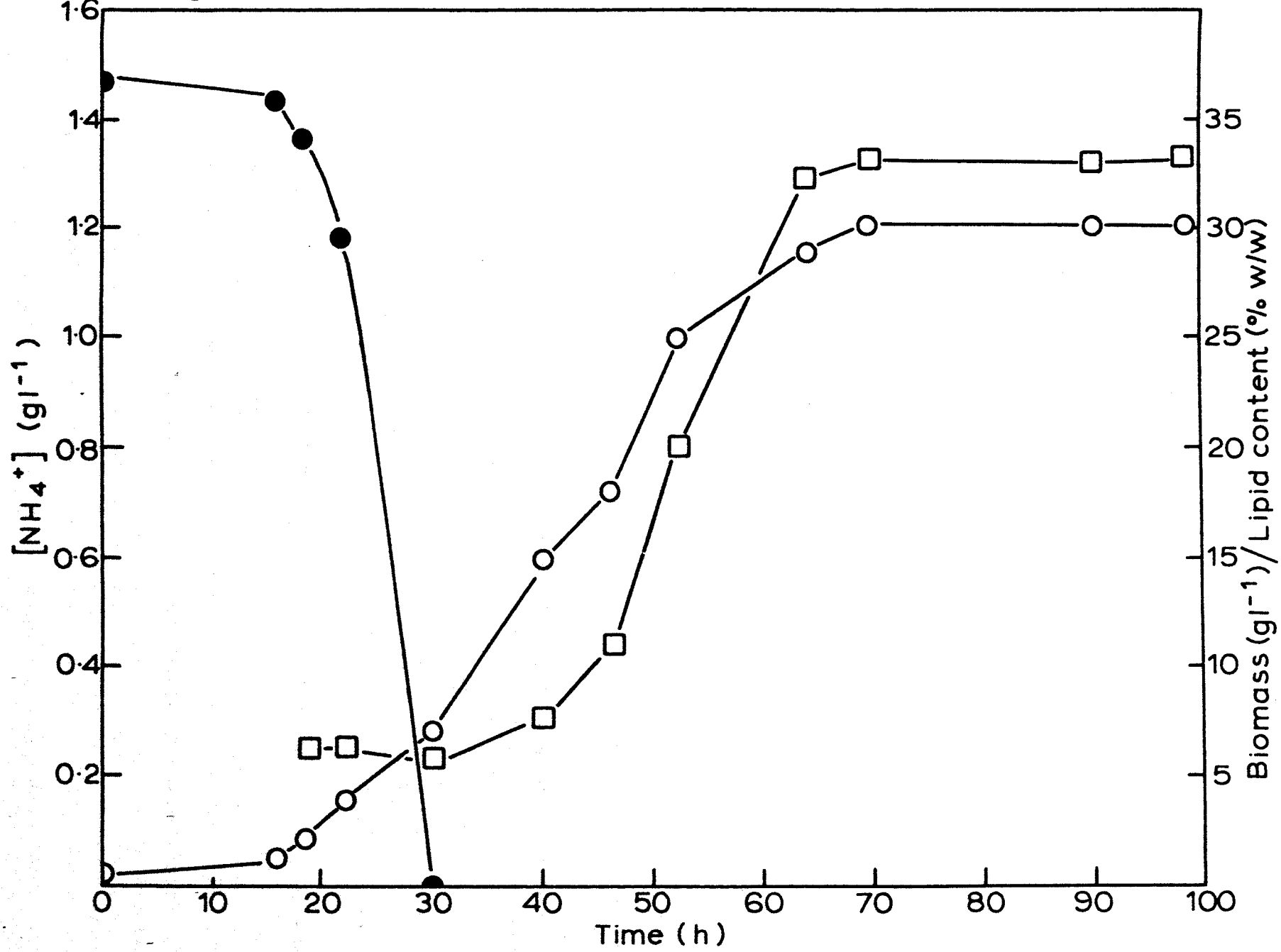
As the pattern of lipid accumulation in *L. starkeyi* CBS 1809 had not been previously studied the growth and lipid content of this yeast were monitored under conditions of batch culture. The experiment was then repeated using *Candida* 107 and the results compared. Cultures were maintained for 100 h and during this time medium samples were removed to determine their NH_4^+ contents. Yeasts were grown at 30 °C and cultures were maintained at pH 5.5 by the automatic addition of NaOH. The medium contained 100 g lit^{-1} glucose (Medium C).

L. starkeyi CBS 1809 did not accumulate lipid in significant quantities until nitrogen became exhausted from the medium (Figure 4). The results for *Candida* 107 are not shown but a similar pattern was observed. Thus, on a superficial level there were no gross differences in the patterns of lipid accumulation in the two organisms.

FIGURE 4

The growth (○) and lipid content (□) of *Lipomyces starkeyi* CBS 1809 cultivated under batch culture conditions. The concentration of NH_4^+ (●) in the medium is also shown.

Fig. 4


1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
[NH4+] (gl-1)
0
10
20
30
40
50
60
70
80
90
100
Time (h)
35
30
25
20
15
10
5
Biomass (gl-1)/Lipid content (% w/w)

1.2 Lipid accumulation in continuous cultures of *L. starkeyi* CBS 1809

It was appreciated at the outset of these studies that batch culture techniques would be of only limited use in the study of oleaginicity because of the uncontrolled nature of cell growth. Since Gill *et al.* (1977) had shown that *Candida* 107 grown on nitrogen-limited medium, in continuous culture, accumulated lipid to a similar extent to that observed in batch cultures, the growth of *L. starkeyi* CBS 1809, in a chemostat, was investigated.

The yeast was cultivated in a chemostat as described in the Materials and Methods (Section 1.4.2). Steady state conditions were established on both carbon and nitrogen-limited media (Media A and C, respectively), at dilution rates between 0.03 h^{-1} and 0.2 h^{-1}. Samples of yeast were removed from the chemostat after each steady state had been established and analyzed for their lipid content. When yeast samples were removed the spent medium was retained and the residual glucose concentration determined.

When the yeast was cultivated on carbon-limited medium the lipid content was low and the specific rate of lipid synthesis (expressed as g lipid synthesized per g fat-free yeast per h) showed little variation with growth rate (Table 2). When growth was nitrogen-limited (Table 3) both the yeast biomass and lipid content were considerably higher, although the biomass yield (g yeast per 100 g glucose consumed) was only approximately half that when growth was carbon-limited. More significantly, yeast cultivated on nitrogen-limited medium had a lipid content that varied inversely with dilution rate whilst the specific rate of lipid synthesis showed an opposite trend. This tends to support the conclusion of Gill *et al.* (1977) that lipid did not accumulate because nitrogen-limitation caused increased rates of lipid biosynthesis. Tables 2 and 3 show that although specific rates of lipid synthesis were

TABLE 2: The biomass and lipid yield of *Lipomyces starkeyi* CBS 1809 grown under carbon-limited conditions

	Dilution rate (h^{-1})				
	0.03	0.06	0.10	0.15	0.2
Biomass ($g l^{-1}$)	5.3	5.0	3.3	1.5	0.9
Lipid content (% w/w)	1.9	2.5	2.7	2.0	1.7
De-fatted biomass ($g l^{-1}$)	5.2	4.9	3.2	1.5	0.9
Residual glucose ($g l^{-1}$)	0	0	0	0	0
Biomass synthesized ($g l^{-1} h^{-1}$)	0.16	0.30	0.33	0.23	0.18
Biomass yield (g yeast per 100 g glucose utilized)	53.0	50.0	33.0	15.0	9.0
Lipid synthesized ($g l^{-1} h^{-1}$)	3×10^{-3}	8×10^{-3}	9×10^{-3}	5×10^{-3}	3×10^{-3}
Lipid yield (g lipid per 100 g glucose utilized)	1.0	1.3	0.9	0.3	0.2
Specific rate lipid synthesis (g lipid.g fat-free $yeast^{-1} h^{-1}$)	5.8×10^{-4}	1.5×10^{-3}	2.8×10^{-3}	3.6×10^{-3}	2.4×10^{-3}

TABLE 3: The biomass and lipid yield of *Lipomyces starkeyi* CBS 1809 grown under nitrogen-limited conditions

	Dilution rate				
	0.03	0.06	0.10	0.15	0.2
Biomass ($g l^{-1}$)	26.3	18.1	15.3	9.5	1.3
Lipid content (% w/w)	42.8	30.0	22.7	19.4	15.3
De-fatted biomass ($g l^{-1}$)	15.0	12.7	11.8	7.7	1.1
Residual glucose ($g l^{-1}$)	1.9	25.0	36.1	64.9	92.9
Biomass synthesized ($g l^{-1} h^{-1}$)	0.8	1.1	1.5	1.4	0.3
Biomass yield (g yeast per 100 g glucose utilized)	26.8	24.1	23.9	27.1	18.5
Lipid synthesized ($g l^{-1} h^{-1}$)	0.34	0.32	0.35	0.28	0.04
Lipid yield (g lipid per 100 g glucose utilized)	11.5	7.3	5.4	5.2	2.8
Specific rate lipid synthesis (g lipid.g fat-free $yeast^{-1} h^{-1}$)	0.023	0.026	0.029	0.036	0.036

higher in yeast grown under conditions of nitrogen-limitation there was no correlation between this parameter and intracellular lipid concentration. Thus, it was the balance between rates of lipid synthesis and the rates of synthesis of other cell constituents, dependent on nitrogen, that determined whether or not lipid accumulated.

1.3 Transition experiments

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 have only been investigated after the establishment of steady state conditions. It was considered that a more fruitful approach would be to investigate the physiological changes that occurred when chemostat cultures of oleaginous yeasts underwent transitions from carbon-limitation to nitrogen-limitation. The intention was to examine a variety of metabolic parameters, during transitions, and establish a sequence of events culminating in lipid accumulation and, possibly identify a 'trigger' event.

Cultural conditions were as described in the Materials and Methods. Carbon-limited medium (Medium A) contained 10 $g l^{-1}$ glucose and, after establishing a steady state, the transition was initiated by replacing the inflowing medium with one containing 100 $g l^{-1}$ glucose (nitrogen-limited medium, Medium C). The chemostat was operated at a dilution rate of 0.06 h^{-1}. This was chosen because it gave a reasonable biomass and lipid content also it resulted in a rapid introduction of excess glucose into the growth vessel. Figure 5 indicates the theoretical increase in glucose concentration in the growth vessel, at a dilution rate of 0.06 h^{-1}, assuming no utilization.

1.3.1 Demonstration of lipid accumulation in *Candida* 107 during a transition from carbon to nitrogen-limitation

Figure 6 shows the results of a transition experiment, using *Candida* 107, in which the biomass and lipid content of the yeast were

FIGURE 5

Theoretical increase in glucose concentration, assuming no utilization, after transition at zero time and a dilution rate of 0.06 h^{-1}.

Fig. 5

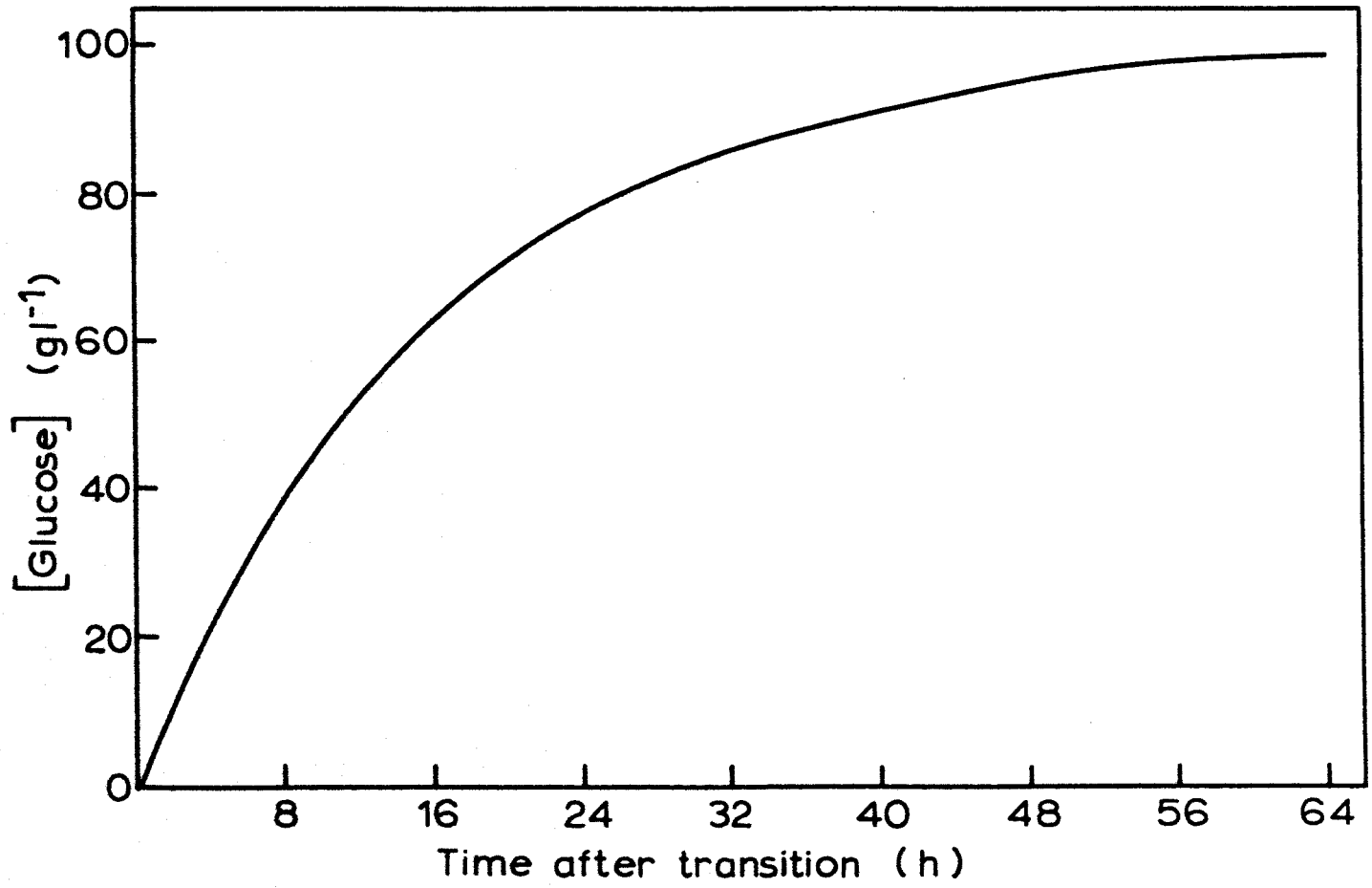

100
80
60
40
20
0
[Glucose] (g l-1)
8
16
24
32
40
48
56
64
Time after transition (h)

FIGURE 6

The growth (○) and lipid content (△) of *Candida* 107 during a transition from carbon-limitation to carbon excess. The concentrations of NH_4^+ (□) and glucose (●) in the medium are also shown.

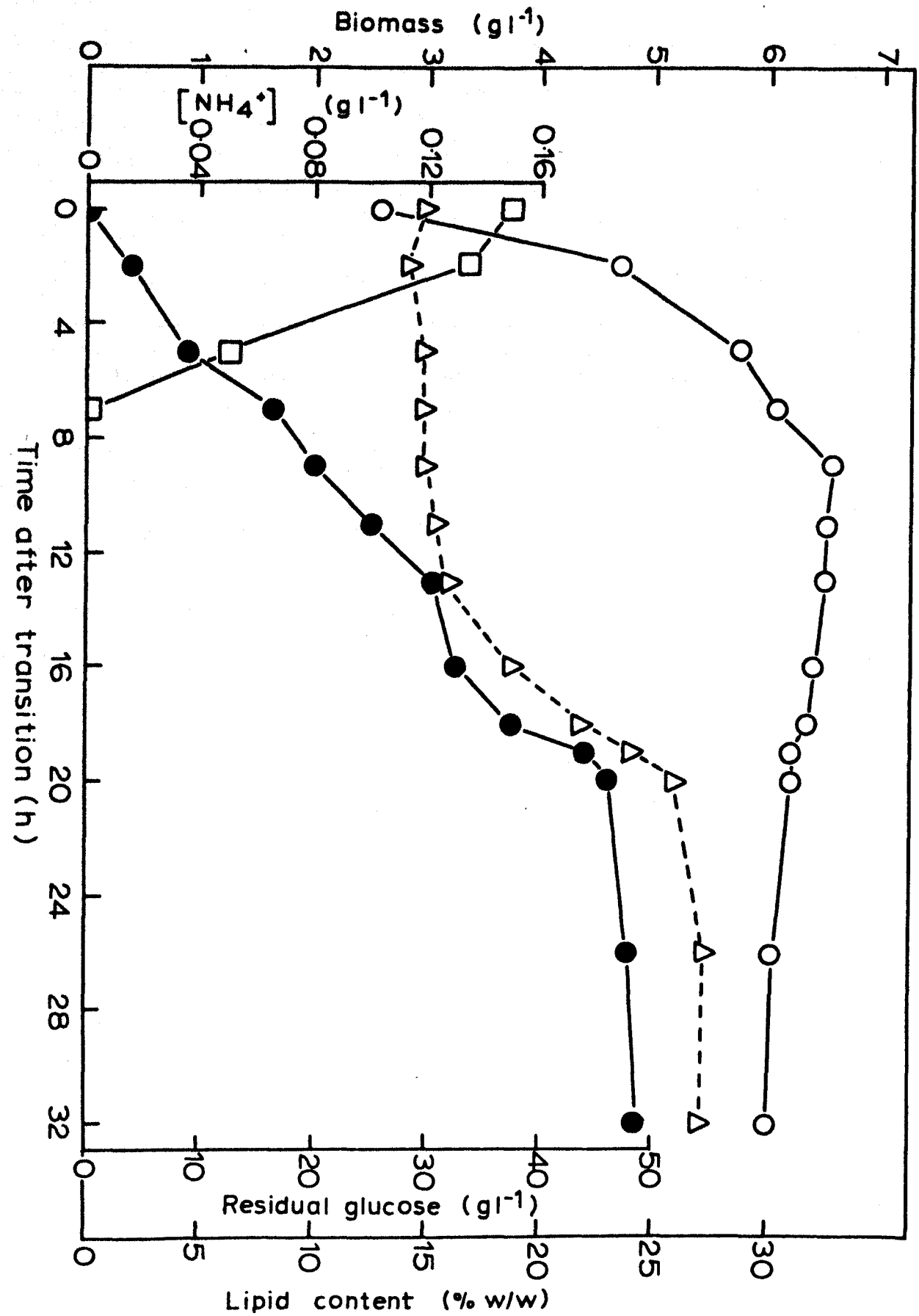

Biomass (g l-1)
[NH4+] (g l-1)
Time after transition (h)
Residual glucose (g l-1)
Lipid content (% w/w)

Fig. 6

monitored for 32 h. In addition, samples of spent medium were retained after the yeast had been removed and analyzed for glucose and NH_4^+ content.

As expected the biomass began to increase as soon as excess glucose became available and this was accompanied by a rapid decrease in the NH_4^+ content of the medium. The culture became nitrogen-limited between 5 and 7 h, however, the lipid content of the yeast did not show any significant increase until approximately 16 h after the initiation of the transition. Thus, the results indicated that there was not a simple causal relationship between the onset of nitrogen-limitation and subsequent lipid accumulation.

1.3.2 Fluctuation in the intracellular amino acid pool and total protein content of *Candida* 107 during a transition from carbon to nitrogen-limitation

Gill *et al.* (1977) concluded that oleaginous yeasts accumulated lipid because nutrient limitation altered the flux of biosynthetic pathways in favour of lipid product at the expense of the production of others. If this were the case it would be expected that nitrogen-limitation would cause a fall in the intracellular levels of metabolites requiring nitrogen for their biosynthesis, such as protein and amino acids. In this experiment the total amino acid pool and the total protein content of *Candida* 107 were monitored during a transition from carbon to nitrogen-limitation and compared with the growth and lipid content of the yeast.

The experimental conditions were as previously described (Results, Section 1.3) but a defined medium (Medium D) was used to reduce interference from extra-cellular organic nitrogen-containing compounds. The sampling of yeast and subsequent handling is described in the Methods (Sections 1.6.4 and 1.6.5).

The results (Figure 7) indicated that both the total amino acid

FIGURE 7

Fluctuation in the intracellular amino acid pool size (●) and protein content (■) of *Candida* 107 during a transition from carbon to nitrogen-limitation. The growth (○) and lipid content (△) of the yeast is shown as is the concentration of NH_4^+ in the medium (□).

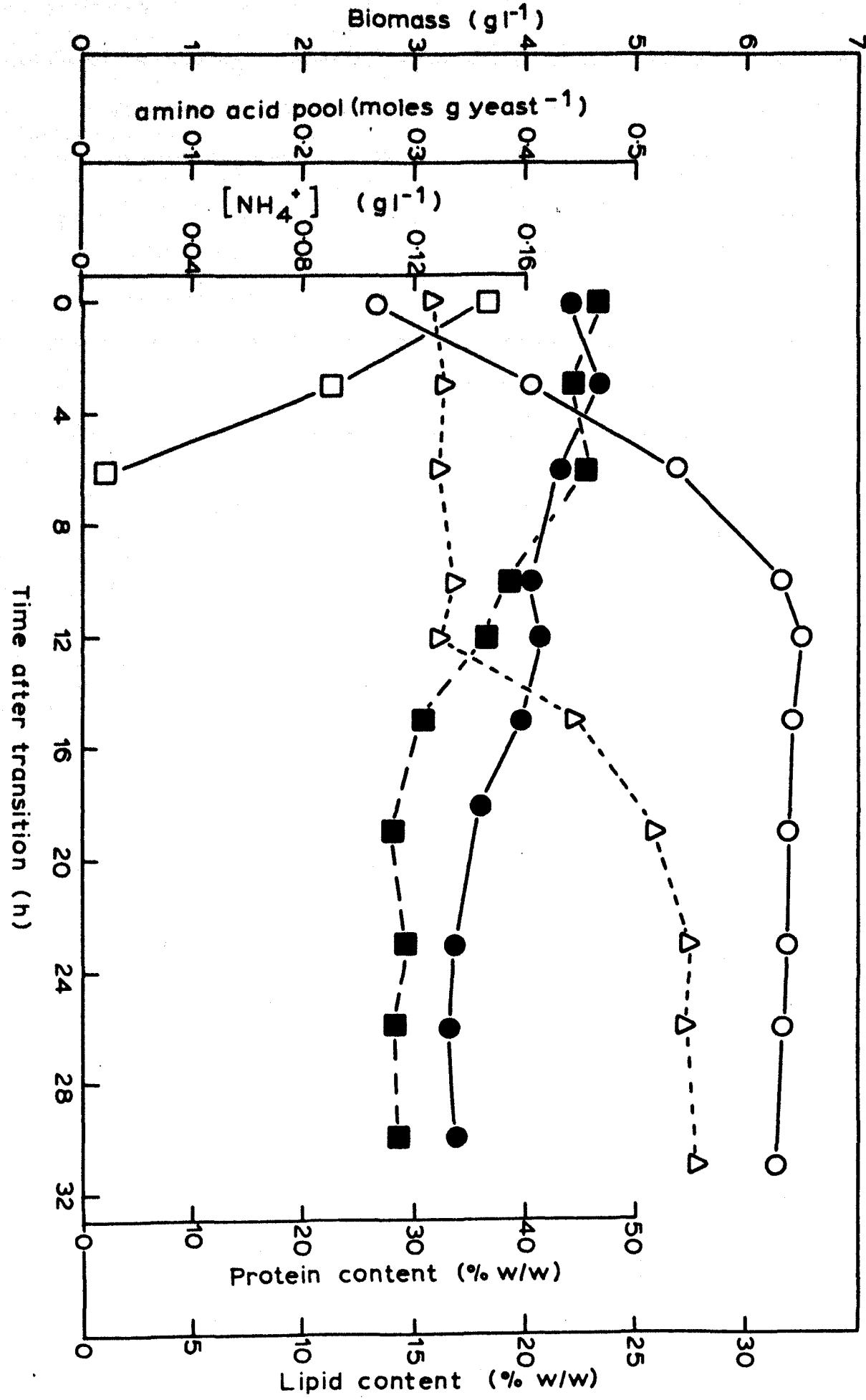

Biomass (g l-1)
amino acid pool (moles g yeast -1)
[NH4+] (g l-1)
Time after transition (h)
Protein content (% w/w)
Lipid content (% w/w)

Fig. 7

pool and the total protein content of *Candida* 107 declined during the transition. In each case the rate of decrease was most marked after the onset of nitrogen-limitation. As the transition progressed the protein content of the yeast fell by approximately 17% and this was accompanied by an increase in the lipid content of the yeast in the region of 11 %. Thus, these results were consistent with the conclusions of Gill *et al.* (1977) that nitrogen depletion resulted in a decline in the biosynthesis of products dependent on this nutrient, whilst allowing lipid biosynthesis to proceed unhindered.

1.3.3 Determination of the *in vivo* adenylate energy charge of *Lipomyces starkeyi* CBS 1809 and its response to changes in nutrient limitation

If the onset of nitrogen-limitation is responsible for the decline of certain biosynthetic pathways and the channelling of excess glucose into lipid production then the mechanisms by which this is accomplished must be sought.

The possibility that the regulation of the flux of carbon flow between lipogenic and other biosynthetic pathways might be accomplished by changes in the *in vivo* energy charge has been discussed (Introduction, Section 2.1.3). In this experiment the changes in adenine nucleotide concentration in *Lipomyces starkeyi* CBS 1809 were monitored during a transition from carbon- to nitrogen-limitation and compared with the time course for lipid accumulation.

Cultural conditions were as previously described (Results, Section 1.3) but a rapid sampling device was fitted to the chemostat, as described (Methods, Section 1.4.2.1). Holms *et al.* (1972) reported that the turn-over of the ATP pool in growing micro-organisms is very rapid, therefore, Knowles (1977) recommends that when cultures are sampled in order to make energy charge measurements the quenching time should be of the order of a few tenths of a second. This condition

was satisfied by the method used. Botham (1978) made extensive studies of the efficacy of various quenching agents and of the subsequent handling of samples prior to the determination of adenine nucleotides. He concluded that 1.5 $\underline{\underline{M}}$ H_2SO_4 was the most suitable quenching agent and, therefore, I adopted this as well as his experimental procedure (Methods, Section 1.6.6).

The results (Figure 8) confirmed those of Botham and Ratledge (1979) that the onset of nitrogen-limitation caused an increase in the *in vivo* energy charge. Furthermore, the observed adenine nucleotide concentrations and computed energy charge were of the same order as the values reported by these authors. Intracellular ADP and ATP levels showed little variation during the transition but there was an eleven-fold decrease in the intracellular AMP concentration (Figure 8). This pattern of fluctuating adenine nucleotide levels was different from that observed in *Candida* 107 by Botham and Ratledge (1979) in that they reported much greater variations in intracellular levels of ADP and ATP.

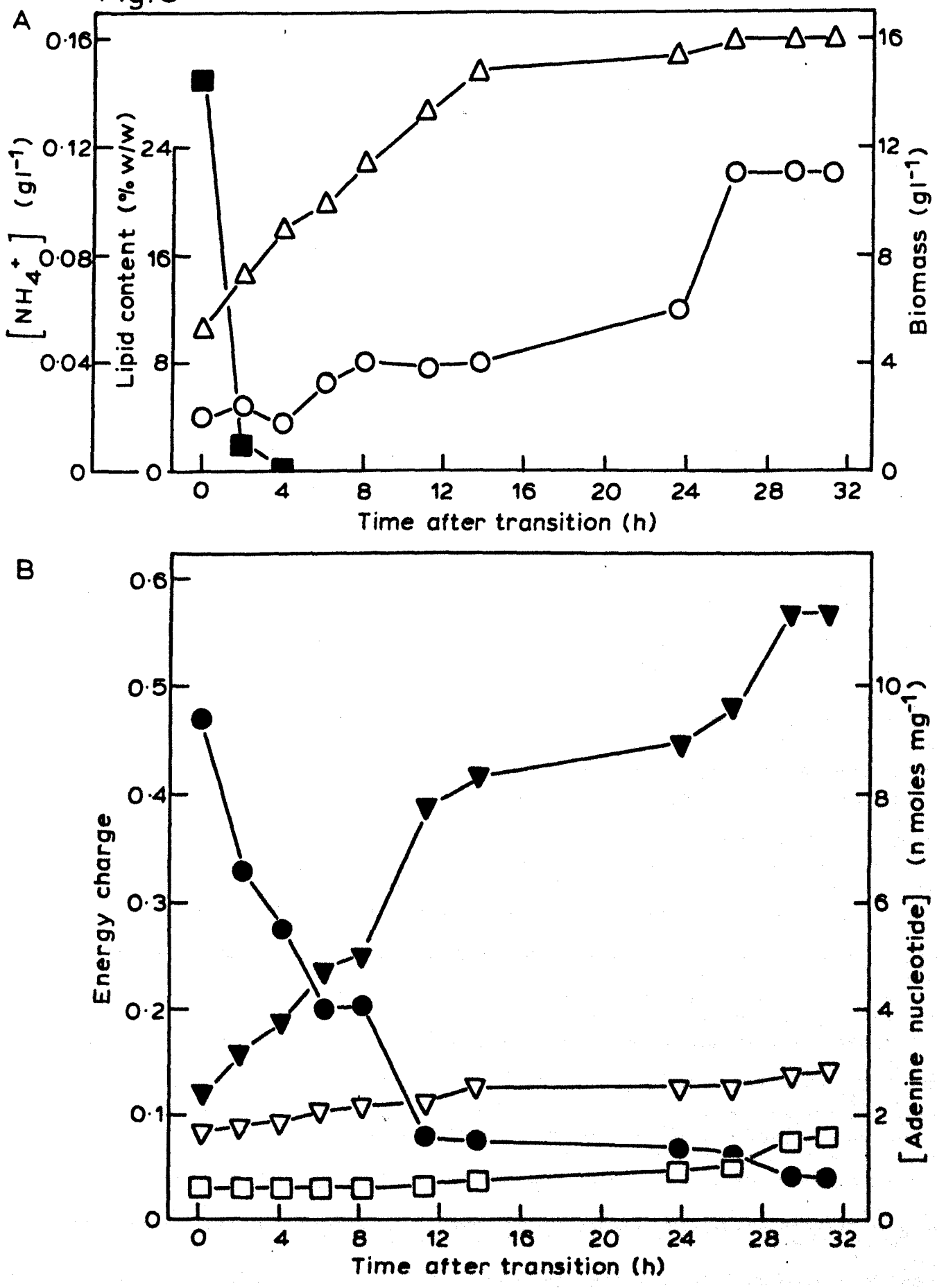
Atkinson (1977) reported that in the vast majority of biological systems examined so far the energy charge was always high and varied between comparatively narrow limits (0.7-0.9). Therefore, the observations of Botham and Ratledge (1979) as well as my own, that the energy charge in oleaginous yeasts varied between 0.1 and 0.6 were clearly atypical. However, Botham (1978) reported that reacidification of the cell debris after the first treatment with H_2SO_4 did not yield further adenine nucleotides, furthermore, the low observed energy charge could not be explained by excretion of adenine nucleotides into the medium and the sampling and quenching procedure was sufficiently rapid to prevent subsequent enzymic degradation of ATP and ADP.

The most striking feature of the results of this transition experiment was that there was little correlation between the time-course

FIGURE 8

The variation in the intracellular concentrations of AMP (●), ADP (▽), ATP (□) and energy charge (▼) [A] compared with the growth (△), lipid content (○) and rate of utilization of NH_4^+ (■) [B] of *Lipomyces starkeyi* CBS 1809 during a transition from carbon to nitrogen limitation.

Fig. 8


A
0·16
0·12
0·08
0·04
0
$[NH_4^+]$ (gl^{-1})
24
16
8
0
Lipid content (% w/w)
16
12
8
4
0
Biomass (gl^{-1})
0
4
8
12
16
20
24
28
32
Time after transition (h)
B
0·6
0·5
0·4
0·3
0·2
0·1
0
Energy charge
10
8
6
4
2
0
[Adenine nucleotide] (n moles mg^{-1})
0
4
8
12
16
20
24
28
32
Time after transition (h)

of increase in energy charge and the onset of lipid accumulation. The more significant parameter would seem to have been the intracellular AMP concentration, as it was only when this had declined to a basal level that the lipid content of the yeast showed a marked increase. However, there was still a considerable time-lag between these two events, therefore, investigations of the intervening physiological changes remained to be made.

1.3.4 Determination of the intracellular citrate concentration of *Lipomyces starkeyi* CBS 1809 and its response to changes in nutrient limitation

It was shown in the previous experiment (1.3.3) that the onset of nitrogen-limitation resulted in a dramatic decrease in the intracellular AMP concentration of *Lipomyces starkeyi* CBS 1809. This was in keeping with the hypothesis of Botham and Ratledge (1979) that depletion of AMP inactivates NAD^+-dependent isocitrate dehydrogenase thereby diverting carbon-flow into lipid production. Mitsushima *et al.* (1978) reported that a similar mechanism was responsible for the accumulation of citric acid in nitrogen-limited cultures of the yeast, *Candida lipolytica.*

The intracellular citrate concentration may be important in determining the rate and extent of lipogenesis by supplying precursor acetyl-CoA and as an activator of acetyl-CoA carboxylase.

There have been no investigations of the intracellular citrate concentrations in oleaginous yeasts, therefore, this parameter was monitored in *Lipomyces starkeyi* CBS 1809 during a transition from carbon- to nitrogen-limitation. Experimental conditions were as described previously (Results, Section 1.3) but the chemostat was fitted with a rapid sampling device (Methods, Section 1.4.2.1). Citrate was determined by an enzymic method (Methods, Section 1.6.7). The concentration of this metabolite in quenched samples was compared with the citrate concentration in the spent medium, thus, the intracellular

citrate concentration could be calculated.

When *Lipomyces starkeyi* CBS 1809 was cultivated under conditions of carbon-limitation, the citrate concentration of the medium under steady-state conditions was 12 $\mu\underline{\underline{M}}$ and during the transition this gradually increased to 38 $\mu\underline{\underline{M}}$ (Figure 9B). The intracellular citrate content of yeast grown under steady-state carbon-limited conditions was 3.8 nmoles per mg dry weight and this rapidly fell when the transition was initiated. When the culture became nitrogen-limited, however, the intracellular citrate concentration increased rapidly to a value of 6.5 nmoles per mg dry weight (Figure 9A, B). Knowles (1977) made the assumption that the intracellular water space of microorganisms was in the range 2 to 4 µl per mg dry weight. Using these values the intracellular citrate content may be expressed in concentration terms. Thus, the citrate content of carbon-limited yeast was 0.95 to 1.9 $m\underline{\underline{M}}$ and this increased to 1.6 to 3.2 $m\underline{\underline{M}}$ during the transition.

The significance of the increase in citrate concentration remains in doubt. Gill and Ratledge (1973) reported that 50 $m\underline{\underline{M}}$ citrate was required to fully activate acetyl-CoA carboxylase from *Candida* 107, i.e. an order of magnitude higher than the concentrations reported here. However, only the average intracellular concentration of citrate has been determined here, no account being taken of possible compartmentalization of this metabolite.

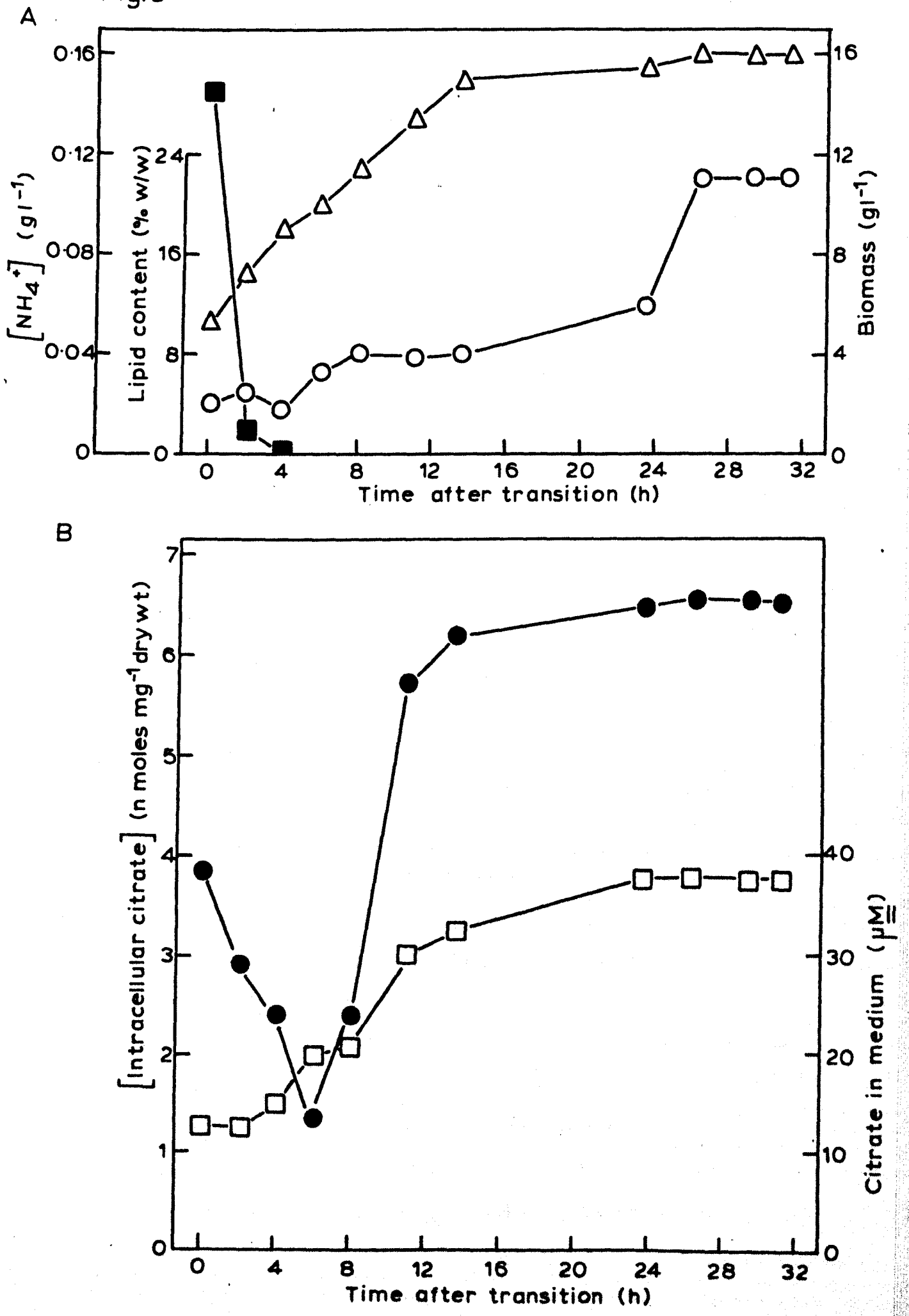
The sequence of events that occurred during transitions from carbon- to nitrogen-limited growth in *Lipomyces starkeyi* CBS 1809 may be divided into three phases:

1. A rapid fall in the intracellular AMP concentration, a basal level being achieved soon after the disappearance of NH_4^+ from the medium.
2. The intracellular citrate concentration initially fell, when growth was neither limited by carbon nor nitrogen, but then rose sharply

FIGURE 9

Fluctuation in the intracellular (●) and extracellular (□) citrate concentrations [B] compared with the growth (△), lipid content (○) and rate of utilization of NH_4^+ (■) [A] of *Lipomyces starkeyi* CBS 1809 during a transition from carbon- to nitrogen-limitation.

Fig.9


A
0·16
0·12
0·08
0·04
0
[NH4+] (g l-1)
24
16
8
0
Lipid content (% w/w)
16
12
8
4
0
Biomass (g l-1)
0
4
8
12
16
20
24
28
32
Time after transition (h)
B
7
6
5
4
3
2
1
0
[Intracellular citrate] (n moles mg-1 dry wt)
40
30
20
10
0
Citrate in medium (μM)
0
4
8
12
16
20
24
28
32
Time after transition (h)

as the AMP concentration declined.

3. A gradual increase in the lipid content of the yeast during the initial stages of the transition, followed by a rapid increase in lipid content after the establsihment of constant AMP and citrate levels.

These observations were consistent with the hypothesis of Botham and Ratledge (1979) that the AMP content of the yeast is the primary determinant in controlling the flow of carbon into citrate production, which itself may then be utilized as a lipid precursor and possibly act as an activator of acetyl-CoA carboxylase.

2. Attempts to isolate mitochondria from oleaginous yeasts

Clearly intracellular compartmentalization plays an important role in oleaginous yeasts in determining the sequence of events leading from the utilization of glucose to the production of acetyl-CoA, in the cytosol, and its subsequent conversion to lipid. It was considered, therefore, that valuable information might be gained if intact mitochondria could be isolated from these microorganisms. The aims of this part of the investigation were three-fold.

1. A demonstration that the key enzymes of carbohydrate metabolism and lipogenesis did occupy the expected locations. This was necessary in order to eliminate the possibility that cytosolic acetyl-CoA might arise directly as a result of an unusual enzyme distribution such as the provision of a cytosolic pyruvate dehydrogenase.
2. Development of techniques for the investigation of the size and location of intracellular metabolite pools.
3. An investigation of the transport of citrate and other related metabolites between mitochondria and the cytosol. This could be a regulated process and thus, could control

the amount of lipid synthesized by regulating the supply of substrate.

2.1 Assessment of mitochondrial preparations

If the aims of this investigation were to be achieved it was appreciated that mitochondrial preparations had to be of good quality. Thus, methods were required to show that mechanical damage and contamination of preparations with other cellular components were minimal. Mitochondrial integrity was assessed by attempts to demonstrate the respiratory control ratio (Methods, Section 2.1.2) and by assaying the cytosolic fraction for enzymes of the mitochondrial matrix (Methods, Section 2.1.1).

2.2 Attempts to produce sphaeroplasts from oleaginous yeasts

Mitochondria are isolated from yeasts by disrupting cells in isotonic medium and then separating the organelles from other components by differential centrifugation. The gentlest and hence most favoured methods are those in which yeasts are suspended in isotonic medium then treated with cell wall-hydrolyzing enzymes to produce sphaeroplasts. Sphaeroplasts may then be disrupted by mild mechanical agitation, thereby allowing recovery of intact mitochondria. The enzyme preparations used most often are those derived from the gut juice of the edible snail, *Helix pomatia*, the active components of which are reported to be endo-β-(1 → 3) and endo-β-(1 → 6) glucanases (Anderson and Millbank, 1966)

Attempts to produce sphaeroplasts (as judged by osmotic fragility or by direct microscopic examination) from a number of oleaginous yeasts by the method of Ohnishi *et al.* (1966) (Methods, Section 2.2) were unsuccessful. The procedure did work, however, when applied to a control non-oleaginous yeast, *Saccharomyces carlsbergensis*. Various enzyme preparations, including one prepared myself from live *Helix pomatia* (see Methods, Section 2.2.3), had no effect on the oleaginous

yeasts tested.

It has been reported that pre-treatment of yeasts with thiol-containing reagents increased the efficacy of snail gut-juice enzyme. However, when sodium thioglycollate (Methods, Section 2.2.1) or mercaptoethylamine (Methods, Section 2.2.2) was included in reaction mixtures and tested against *Candida* 107 and *Lipomyces starkeyi* CBS 1809, no sphaeroplasts were produced.

The results of experiments designed to produce sphaeroplasts from oleaginous yeasts, including details of the various strains tested, are summarized in Table 4.

2.3 Attempts to isolate mitochondria from oleaginous yeasts using mechanical cell disruption methods

Since oleaginous yeasts were not susceptible to enzymic methods of cell wall degradation, attempts were made to effect the initial cell disruption step by mechanical means. Various methods for cell disruption were used, experimental details are given in the Methods (Section 2.3). In each case yeasts were susepnded in isotonic buffered medium and after cell disruption mitochondria were isolated by differential centrifugation.

The results (Table 5) illustrated the difficulties encountered when mechanical cell disruption techniques were employed in attempts to isolate mitochondria from oleaginous yeasts. In all of these methods there had to be a compromise betwen using sufficient force to disrupt the yeast cell wall whilst minimising mitochondrial damage. Thus, ultrasonic disruption and the agitator ball mill produced very efficient cell breakage but the presence of mitochondrial matrix enzymes in the cytosolic fraction and the lack of respiratory control indicated considerable mitochondrial damage. Conversely, the cell disruption bomb effected cell breakage in a gentle manner but the yield of mitochondria was too low to be of practical use.

TABLE 4: Summary of the methods tested and results obtained in attempts to produce sphaeroplasts from various oleaginous yeasts as compared with a control non-oleaginous yeast, *Saccharomyces carlsbergensis*

Procedure	Test yeast	Enzyme Preparation	Sphaeroplast formation
Standard procedure (see Methods, Section 2.2)	*Candida* 107	β-glucuronidase from *Helix pomatia* (Sigma type H-2)	-
	Lipomyces starkeyi CBS 1809		-
	L. starkeyi CBS 6047		-
	L. lipofer CBS 5482		-
	Rhodosporidium toruloides CBS 14		-
	Saccharomyces carlsbergensis		+
	Candida 107	Helicase (L'Industrie Biologique Francaise)	-
	S. carlsbergensis		+
	Candida 107	Helicase prepared from live *Helix pomatia*	-
	Lipomyces starkeyi CBS 1809		-
	S. carlsbergensis		+
Pre-treatment with sodium thioglycollate (see Methods, Section 2.2.1)	*Candida*	Helicase (L'Industrie Biologique Francaise)	-
	Lipomyces starkeyi CBS 1809		-
	S. carlsbergensis		+
Pre-treatment with mercaptoethylamine (see Methods, Section 2.2.2)	*Candida* 107	Helicase (L'Industrie Biologique Francaise)	-
	Lipomyces starkeyi CBS 1809		-
	S. carlsbergensis		+

TABLE 5: Summary of the results obtained when mechanical methods of cell disruption were employed in procedures for the isolation of mitochondria from oleaginous yeasts

Apparatus	Test yeast	Degree of Cell Breakage	Assessment of Mitochondrial Preparation
Chaikoff press (Methods, Section 2.3.1)	*Candida* 107	None	-
Cell disruption bomb (Methods, Section 2.3.2)	*Candida* 107	Limited cell disruption	Yield too small to proceed with
Agitator ball mill (Methods, Section 2.3.3)	*Lipomyces starkeyi* CBS 1809	Total disruption	No respiratory control and considerable mitochondrial damage indicated by heavy contamination of cytosolic fraction by citrate synthase and NAD^+-dependent isocitrate dehydrogenase
Braun disintegrator (Methods, Section 2.3.4)	*Candida* 107	Moderate disruption	Low yield because of limited disruption and small working volume, no respiratory control
Motorized homogenizer (Methods, Section 2.3.5)	*Candida* 107	Moderate disruption	Low yield of mitochondria, no respiratory control
Ultrasonic disruption (Methods, Section 2.3.6	*Candida* 107	Total disruption	No respiratory control, considerable mitochondrial damage
French pressure cell (Methods, Section 2.3.7)	*Lipomyces starkeyi* CBS 1809	Total disruption	Reasonable yield but considerable damage as indicated by citrate synthase contamination of cytosolic fraction. No respiratory control

The most satisfactory method was that in which the initial cell disruption step was achieved by passage through a French pressure cell, even so no respiratory control could be demonstrated in the mitochondria that were subsequently isolated. This was possibly not surprising in that Chappell and Hansford (1972) reported that fatty acids were potent uncouplers of mitochondrial respiration, and in crude cell-free extracts of oleaginous yeasts these compounds are likely to be present in considerable amounts.

In view of the fact that the integrity of mitochondrial preparations could not be guaranteed it was reluctantly concluded that it was not practicable to proceed with investigations of citrate transport or the size of intracellular metabolite pools.

2.4 The location of pyruvate dehydrogenase and ATP:citrate lyase in *Lipomyces starkeyi* CBS 1809

Although it did not prove possible to isolate mitochondria from oleaginous yeasts for the intended purposes of investigating transport phenomena and the size of intracellular metabolite pools it was considered that this did not preclude investigations into the location of key enzymes of intermediate metabolism. This was necessary in order to eliminate the possibility that acetyl-CoA might arise in the cytosol as a consequence of an unusual enzyme distribution such as the provision of a cytosolic pyruvate dehydrogenase.

Mitochondrial and cytosolic fractions were isolated from a crude cell-free extract of *Lipomyces starkeyi* CBS 1809 by the French pressure cell method (Methods, Section 2.4). Each fraction was then assayed for pyruvate dehydrogenase and ATP:citrate lyase activity as well as marker enzymes of known location (Table 6).

Allowing for some degree of mitochondrial damage during the preparation, the results confirmed the proposed locations of

TABLE 6: Activities of ATP:citrate lyase and pyruvate dehydrogenase and other marker enzymes in cytosolic and mitochondrial fractions of *Lipomyces starkeyi* CBS 1809

Enzyme	Expected location	Specific activity [nmoles min^{-1} (mg protein)$^{-1}$]	
		Mitochondrial fraction	Cytosolic fraction
NAD^+-dependent isocitrate dehydrogenase	Mitochondrial	38.4	20.1
Citrate synthase	Mitochondrial	940.1	87.0
Pyruvate dehydrogenase	Mitochondrial	85.1	0
Glucose 6-phosphate dehydrogenase	Cytosolic	49.2	720.1
ATP:citrate lyase	Cytosolic	0	34.2

ATP:citrate lyase and pyruvate dehydrogenase as being respectively, cytosolic and mitochondrial.

3. Phosphoketolase activity in oleaginous yeasts

Phosphoketolases have been detected in two species of oleaginous yeasts, *Candida* 107 and *Rhodotorula graminis* (Whitworth and Ratledge, 1977). The presence of these activities would result in the production of cytosolic acetyl-CoA directly from glucose, as indicated below, and thus, cast some doubt on the necessity for the ATP:citrate lyase pathway.

xylulose 5-phosphate $\xrightarrow{\text{xylulose 5-phosphate phosphoketolase}}$ glyceraldehyde 3-phosphate + C_2 unit

fructose 6-phosphate $\xrightarrow{\text{fructose 6-phosphate phosphoketolase}}$ erythrose 4-phosphate + C_2 unit

Fructose 6-phosphate phosphoketolase was detected in extracts of *Candida* 107 and *Rhodotorula graminis* NCYC 50 at activities of 9.2 and 7.8 nmol min^{-1}(mg protein)$^{-1}$, respectively. However extracts of *Hansenula saturnus* CBS 5761 and *Lipomyces starkeyi* CBS 1807 and 1809 showed no activity with either xylulose 5-phosphate or fructose 6-phosphate as substrate.

Clearly, therefore, phosphoketolases are not essential for lipid accumulation in oleaginous yeasts, although they may augment the supply of acetyl-CoA derived by the ATP:citrate lyase pathway in those oleaginous yeasts possessing them, to an unknown degree.

4. Studies with citrate synthase from *Candida* 107

The suggestion that the activity of citrate synthase may be regulated by changes in the intracellular adenylate energy charge such that activity becomes restricted under conditions of high energy

charge has been discussed (Introduction, Section 2.1.3). It is an essential prerequisite of the hypothesis proposed by Botham and Ratledge (1979) to explain the phenomenon of lipid accumulation in oleaginous yeasts that there be no restriction in the flow of carbon from the principal growth substrate, to acetyl-CoA in the cytosol. However, in the same report these authors demonstrated that when cultural conditions favoured lipid accumulation the adenylate energy charge was considerably higher than that recorded for cultures where the conditions did not favour lipogenesis. These observations had been confirmed in cultures of *Lipomyces starkeyi* CBS 1809 (Results, Section 1.3.3) and there was no reason to suppose that the major flux of carbon flow from the principal growth substrate, glucose, to acetyl-CoA was not via the TCA cycle. Therefore, the kinetic properties of citrate synthase from *Candida* 107 were analyzed in some detail in order to resolve the apparent paradox that citrate synthase activity might be restricted during lipogenesis.

4.1 Purification of citrate synthase from *Candida* 107

Citrate synthase was purified from *Candida* 107 cultivated in a chemostat under nitrogen-limiting conditions, as described (Methods, Section 5.3). A concentrated sample of the purified enzyme was subjected to polyacrylamide disc gel electrophoresis (Methods, Section 5.4) and estimated to be 10-15% pure. Although this was a modest purification as judged by other achievements (e.g. Srere, 1969; Parvin, 1969) the preparation was free from contaminating activities of acetyl-CoA deacylase, malate dehydrogenase, aconitase, ATP:citrate lyase and adenylate kinase and was, therefore, considered suitable for subsequent work. The progress of a typical purification is shown in Table 7. This preparation was used in all subsequent work.

TABLE 7: Results of a typical purification of citrate synthase from *Candida* 107

Fraction	Volume (ml)	Total Units (μmol min^{-1})	Total protein (mg)	Specific activity [μmol min^{-1} (mg protein)$^{-1}$]	Purification factor	Yield (%)
Crude	45.0	239.8	364.5	0.66	1.0	100
55-80% $(NH_4)_2SO_4$	2.9	184.6	44.1	4.19	6.4	77.0
Pooled Sephadex G150 eluate	43.0	150.2	21.1	7.11	10.8	62.6
Pooled hydroxyapatite eluate	46.0	132.0	5.8	22.6	34.3	55.0

4.2 Variation in activity of citrate synthase from *Candida* 107 in response to changes in pH

The reaction velocity varied only slightly between pH 7.4 and pH 9.0 when the enzyme was assayed in 0.1 M Tris/HCl buffer by the procedure of Srere *et al.* (1963). Consequently, it was concluded that pH 8.0 was appropriate for subsequent assays.

4.3 Determination of the kinetic parameters of citrate synthase from *Candida* 107

The initial velocity of reaction was measured whilst varying the acetyl-CoA concentration at six fixed oxaloacetate concentrations. A primary Lineweaver-Burk plot (Figure 10) in which the lines were fitted by linear regression analysis using a Hewlett-Packard Model 9810 A calculator indicated that the usual Michaelis-Menten relationship was valid.

The linear regression analysis programme used gave the slopes and ordinate intercepts of the primary lines as output and these values were used to construct the two secondary plots shown in Figure 11. From the secondary plots the ϕ parameters (Dalziel, 1957) were calculated and from these the true K_m values for oxaloacetate and acetyl-CoA, as shown

ϕ_1 = slope Figure 11A = 2.89×10^{-4}

ϕ_0 = ordinate intercept Figure 11A = 0.54

ϕ_2 = ordinate intercept Figure 11B = 1.86×10^{-3}

ϕ_{12} = slope Figure 11B = 6.1×10^{-5}

K_m oxaloacetate = ϕ_1/ϕ_0 = 0.54 μM

K_m acetyl-CoA = ϕ_2/ϕ_0 = 3.47 μM

4.4 Inhibition of citrate synthase from *Candida* 107 by adenine nucleotides

Adenine nucleotides inhibited the activity of citrate synthase from *Candida* 107 when assayed with non-saturating concentrations of either oxaloacetate or acetyl-CoA (Figure 12). The order of efficacy was

FIGURE 10

Variation in initial velocity of citrate synthase from *Candida* 107 with varying acetyl-CoA concentration. Oxaloacetate concentrations were: 10 μM, □ ; 12.5 μM, ● ; 20 μM, ○ ; 30 μM, △ ; 40 μM, ■ and 50 μM, ▲ .

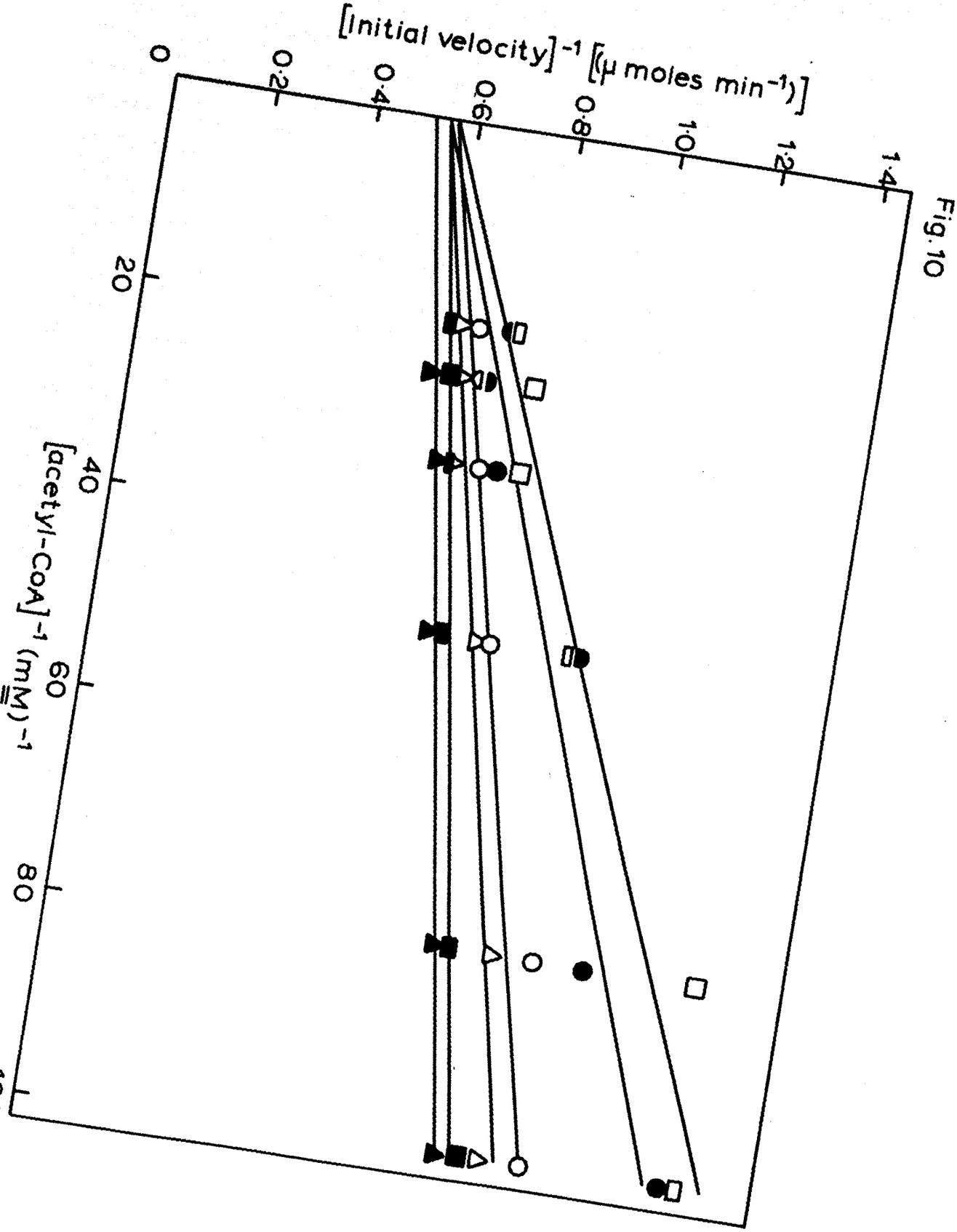

[Initial velocity]$^{-1}$ [(μ moles min^{-1})]
0
0·2
0·4
0·6
0·8
1·0
1·2
1·4
20
40
60
80
100
[acetyl-CoA]$^{-1}$ (mM)$^{-1}$

Fig. 10

FIGURE 11

Secondary plots of the primary data shown in Figure 10. In A the ordinate intercepts are plotted against the corresponding values of 1/[oxaloacetate]. In B the slopes of the primary lines are plotted against the corresponding values of 1/[oxaloacetate].

Fig.11

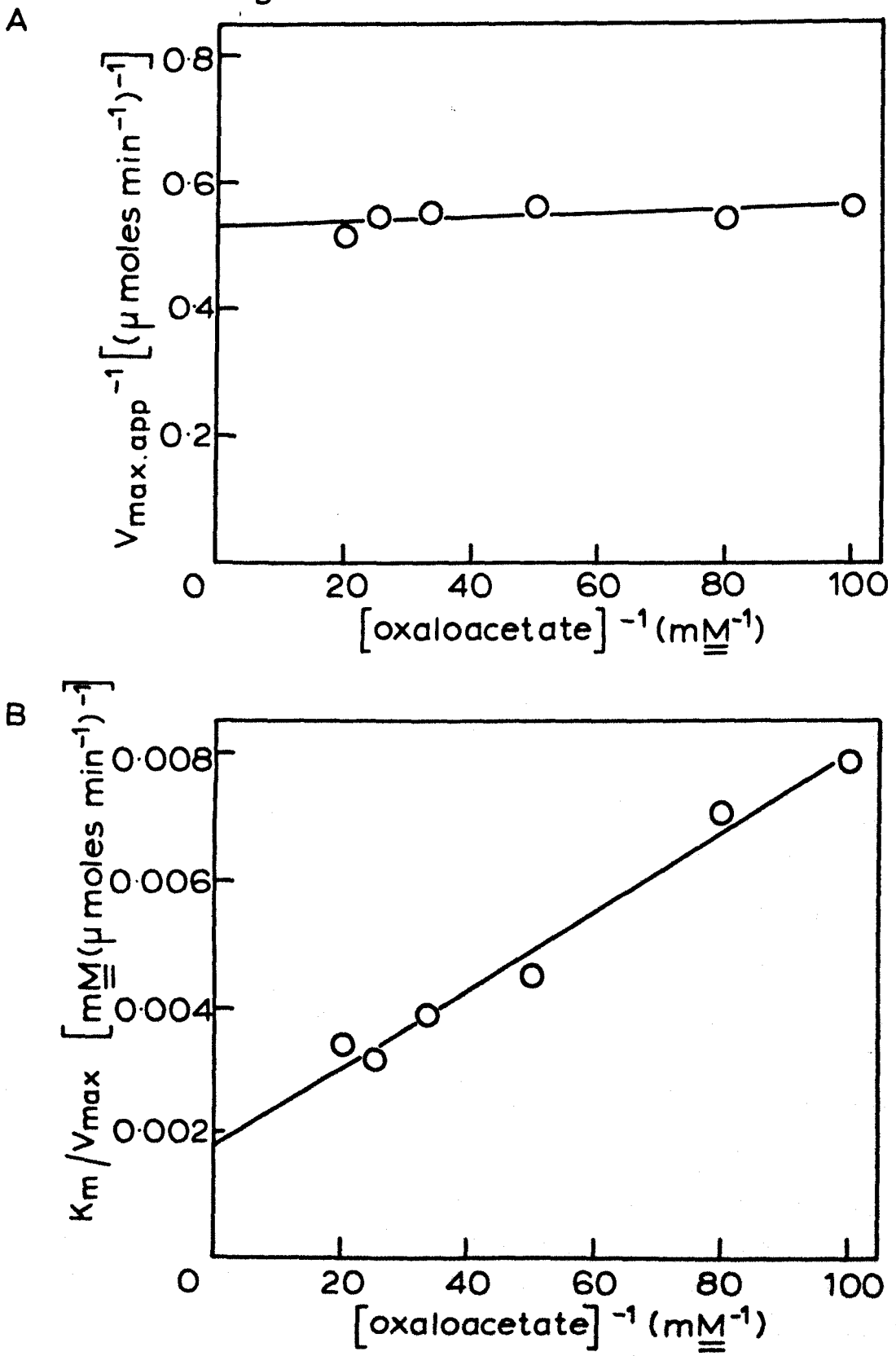
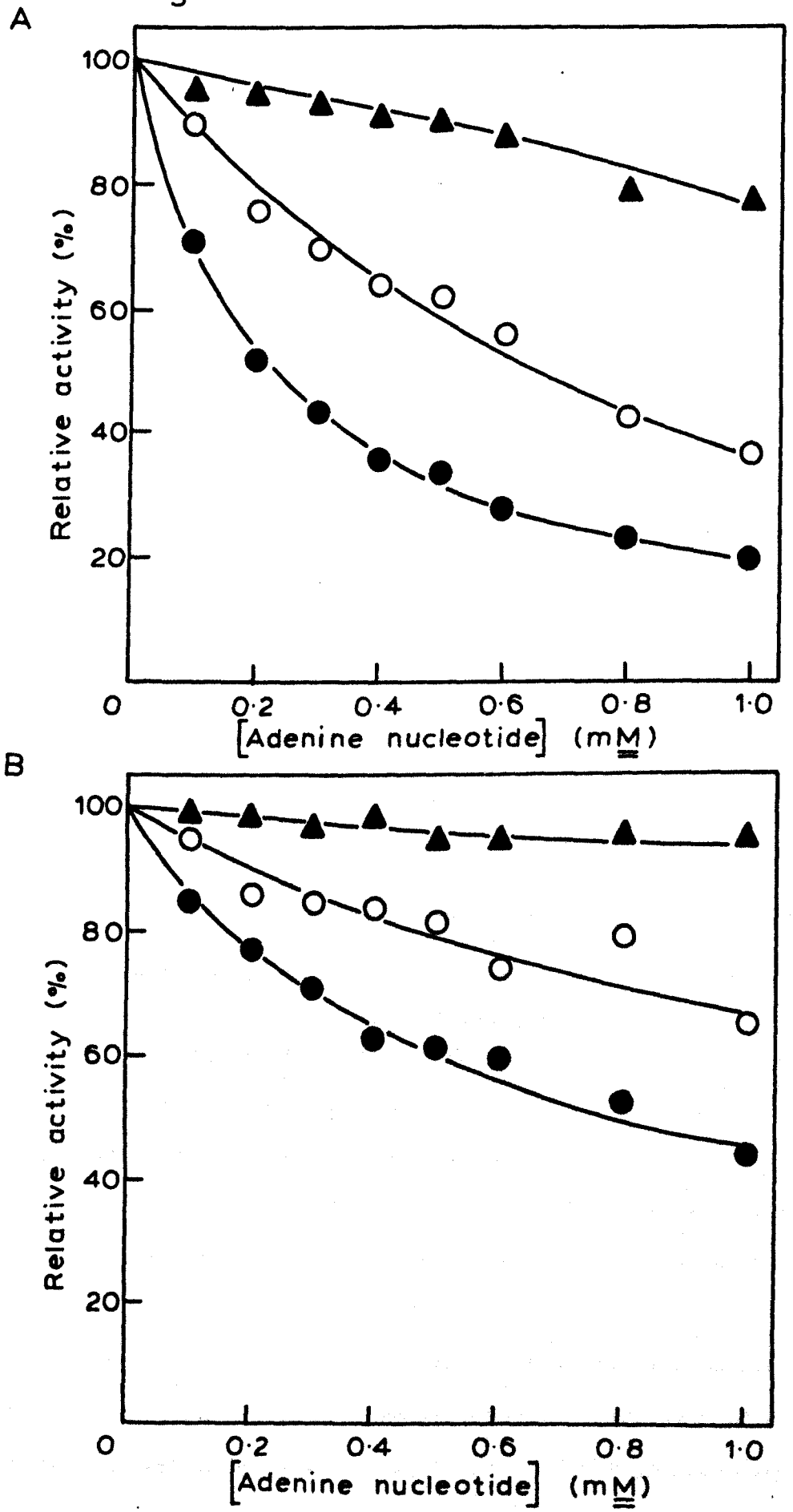

A
$V_{max.app}^{-1}$ [(μ moles min^{-1})$^{-1}$]
0·8
0·6
0·4
0·2
0
20
40
60
80
100
[oxaloacetate] $^{-1}$ (mM^{-1})
B
K_m/V_{max} [mM(μ moles min^{-1})$^{-1}$]
0·008
0·006
0·004
0·002
0
20
40
60
80
100
[oxaloacetate] $^{-1}$ (mM^{-1})

FIGURE 12

Effect of adenine nucleotides (ATP, ●; ADP, ○; AMP, ▲) on the activity of citrate synthase from *Candida* 107. Substrate concentrations were: 50 μM oxaloacetate and 5 μM acetyl-CoA (A) and 50 μM acetyl-CoA and 5 μM oxaloacetate (B). Activities are shown relative to the activity observed in the absence of added adenine nucleotide [0.16 and 0.12 μmol min^{-1} $(mg\ protein)^{-1}$ in (A) and (B), respectively].

Fig. 12


A
100
80
60
40
20
Relative activity (%)
0
0·2
0·4
0·6
0·8
1·0
[Adenine nucleotide] (mM)
B
100
80
60
40
20
Relative activity (%)
0
0·2
0·4
0·6
0·8
1·0
[Adenine nucleotide] (mM)

ATP > ADP > AMP and the degree of inhibition was greater at non-saturating concentrations of acetyl-CoA than with non-saturating concentrations of oxaloacetate. This pattern of inhibition was similar to that reported for adenine nucleotide inhibition of citrate synthase from *Saccharomyces cerevisiae* (Hathaway and Atkinson, 1965).

4.4.1 The kinetics of ATP inhibition of citrate synthase from *Candida* 107

As ATP was the most potent inhibitor of the three adenine nucleotides tested the kinetics of the inhibition with respect to acetyl-CoA and oxaloacetate was investigated.

4.4.1.1 The kinetics of ATP inhibition of citrate synthase from *Candida* 107 at non-saturating acetyl-CoA concentrations

Inhibition of citrate synthase from *Candida* 107 by ATP was competetive with respect to acetyl-CoA (Figure 13). From the secondary plot of the primary data (Figure 14) the K_i for ATP was calculated to be 0.15 mM.

4.4.1.2 The kinetics of ATP inhibition of citrate synthase from *Candida* 107 at non-saturating oxaloacetate concentrations

The kinetics of ATP inhibition of citrate synthase from *Candida* 107 at non-saturating concentrations of oxaloacetate were mixed (competitive, non-competitive) [Figure 15].

4.4.2 Response of citrate synthase from *Candida* 107 to mixtures of adenine nucleotides simulating various energy charge values

Initial velocity measurements were made with combinations of adenine nucleotides simulating various energy charge values. The relative proportions of each nucleotide, at each energy charge value, were calculated according to Bomsel and Pradet (1968) assuming an equilibrium constant for adenylate kinase of 1.2.

Citrate synthase activity responded to mixtures of adenine nucleotides simulating various energy charge values as predicted by Hathaway and Atkinson (1965). As the energy charge increased enzyme

FIGURE 13

Double reciprocal plot of initial velocity of citrate synthase from *Candida* 107 at varying concentrations of acetyl-CoA, at the following concentrations of ATP: zero, ●; 0.2 mM, □; 0.4 mM, Δ and 0.8 mM, ○. Oxaloacetate concentration was 50 μM.

Fig.13

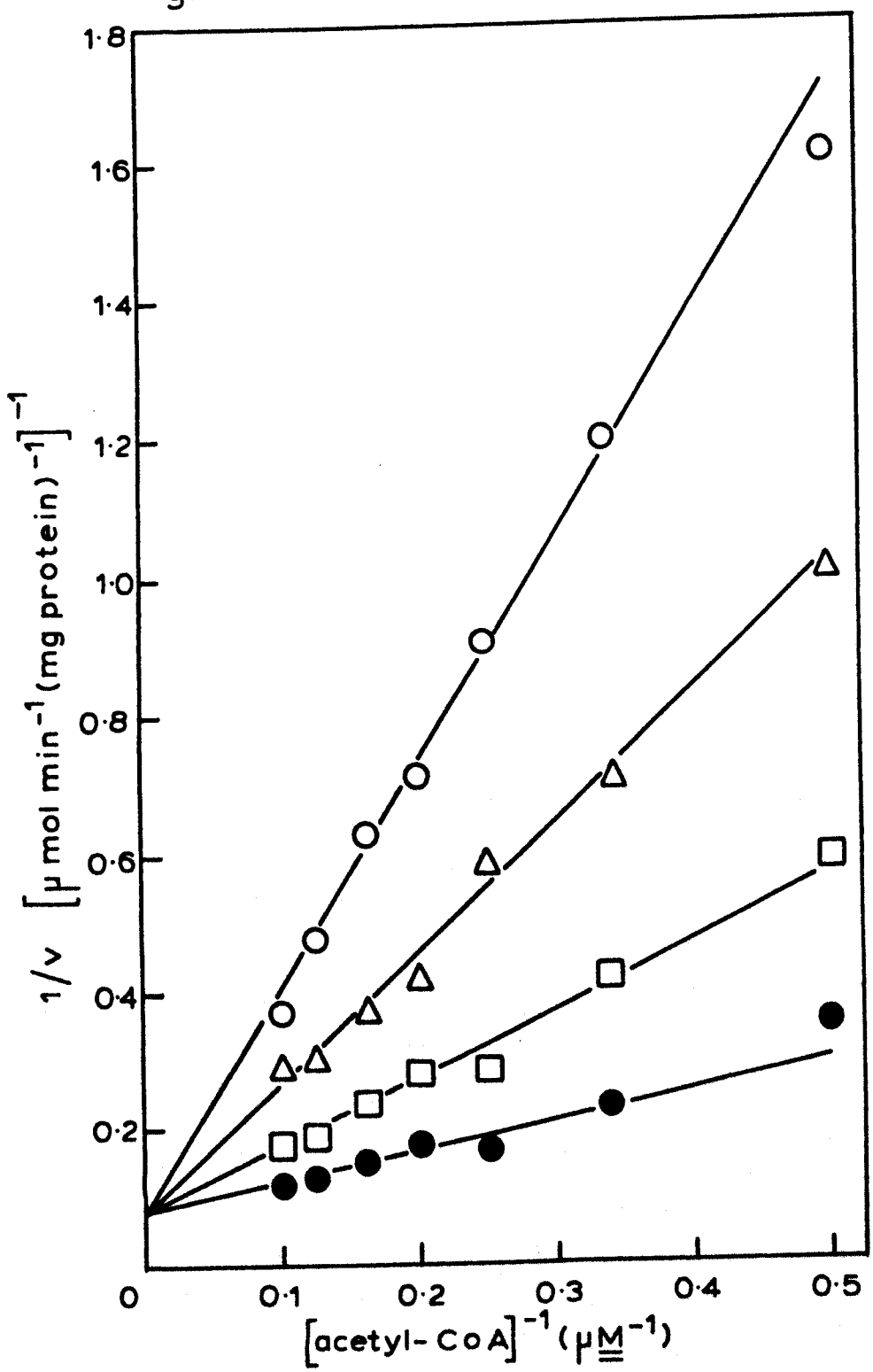

1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
0·1
0·2
0·3
0·4
0·5
1/v [μ mol min⁻¹ (mg protein)⁻¹]⁻¹
[acetyl-CoA]⁻¹ (μM⁻¹)

FIGURE 14

Secondary plot of the slopes of the lines shown in Figure 13 plotted against the corresponding concentration of ATP.

Fig.14

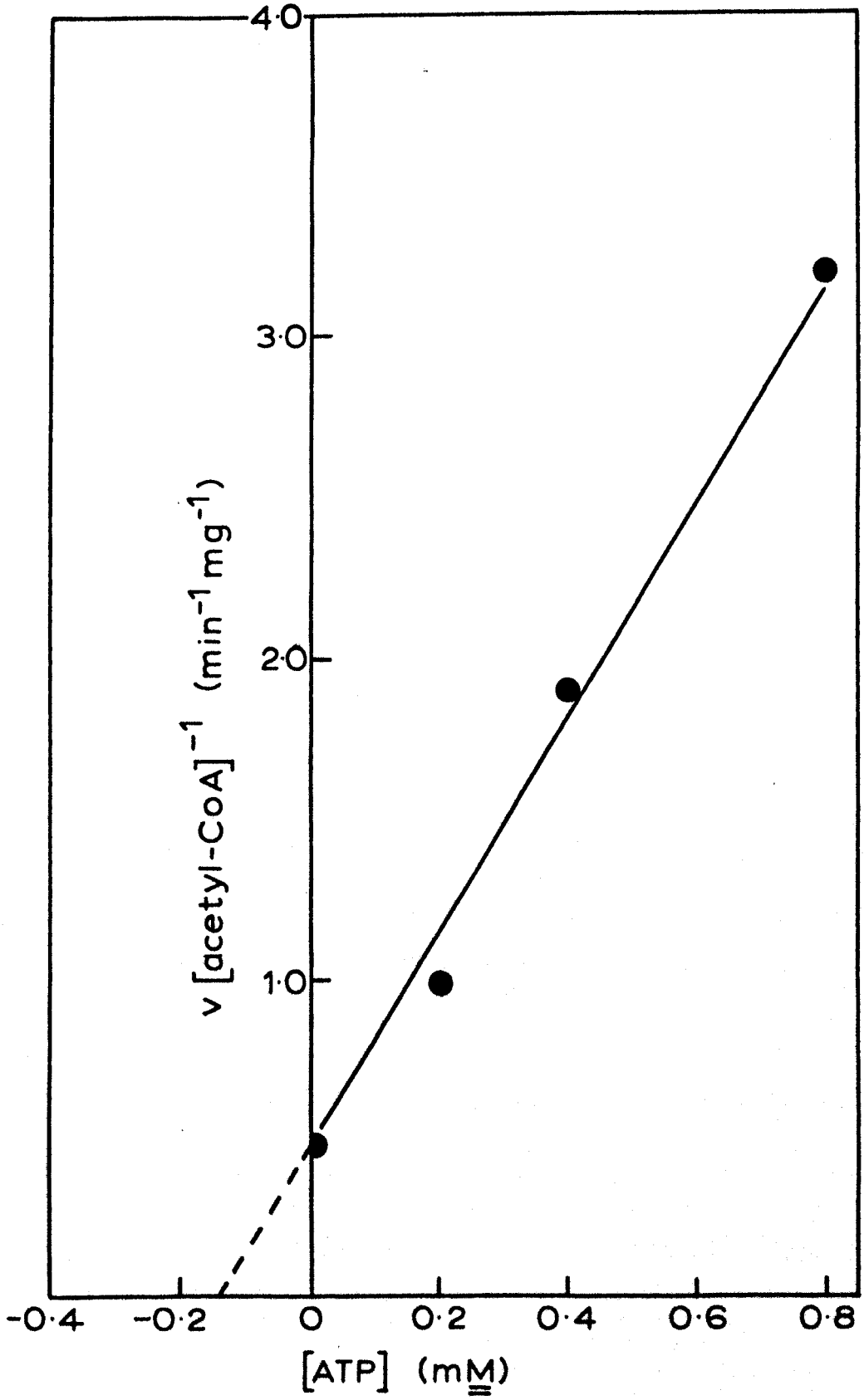
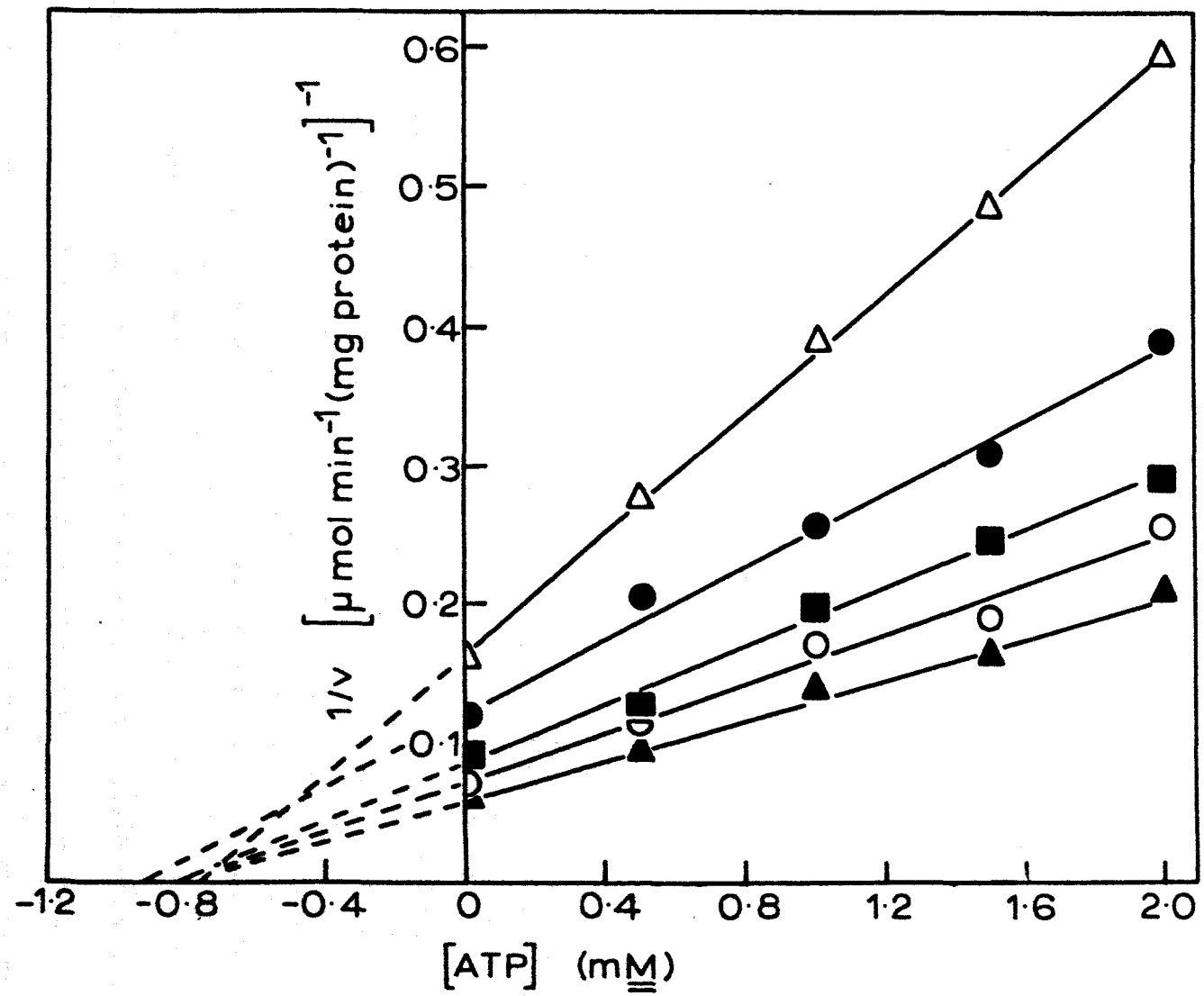


FIGURE 15

Plot of reciprocal activity of citrate synthase from *Candida* 107 at varying concentrations of ATP, at the following non-saturating concentrations of oxaloacetate: 2 $\mu\underline{M}$, Δ; 3 $\mu\underline{M}$, ●; 4 $\mu\underline{M}$, ■; 6 $\mu\underline{M}$, O and 10 $\mu\underline{M}$, ▲. Acetyl-CoA concentration was 50 $\mu\underline{M}$.

Fig.15


0·6
0·5
0·4
0·3
0·2
0·1
1/v [μ mol min⁻¹(mg protein)⁻¹]⁻¹
-1·2
-0·8
-0·4
0
0·4
0·8
1·2
1·6
2·0
[ATP] (mM)

activity decreased (Figure 16). However, the total adenine nucleotide concentration was as important as the relative concentrations of each in determining the degree of inhibition observed. The pattern of inhibition was similar whether acetyl-CoA or oxaloacetate was the non-saturating substrate.

4.4.3 The effect of Mg^{2+} on the pattern of ATP inhibition of citrate synthase from *Candida* 107

Kosicki and Lee (1966) reported that the inhibition of pig heart citrate synthase by adenine nucleotides was relieved by divalent metal ions. In their opinion this was probably due to the chelation of the metal ion with the polyphosphate moiety of the adenine nucleotide, thereby, reducing the affinity of these molecules for the active site of the enzyme.

Inhibition of citrate synthase from *Candida* 107 by ATP was considerably alleviated when Mg^{2+} was included at saturating concentrations (Figure 17). Mg^{2+} ions themselves were inhibitory to the reaction, the degree of inhibition being proportional to the concentration of Mg^{2+} added. However, with the highest concentration of Mg^{2+} used (2.5 m$\underline{\underline{M}}$) only minimal inhibition was produced even by the highest concentration of ATP tested (2.0 m$\underline{\underline{M}}$).

Thus, the observations of Kosicki and Lee (1966) were confirmed for citrate synthase from *Candida* 107. In this context it is noteworthy that in the report of Hathaway and Atkinson (1965) regarding the inhibition by ATP of citrate synthase from *Saccharomyces cerevisiae* no account was taken of the ameliorating effect of divalent metal ions.

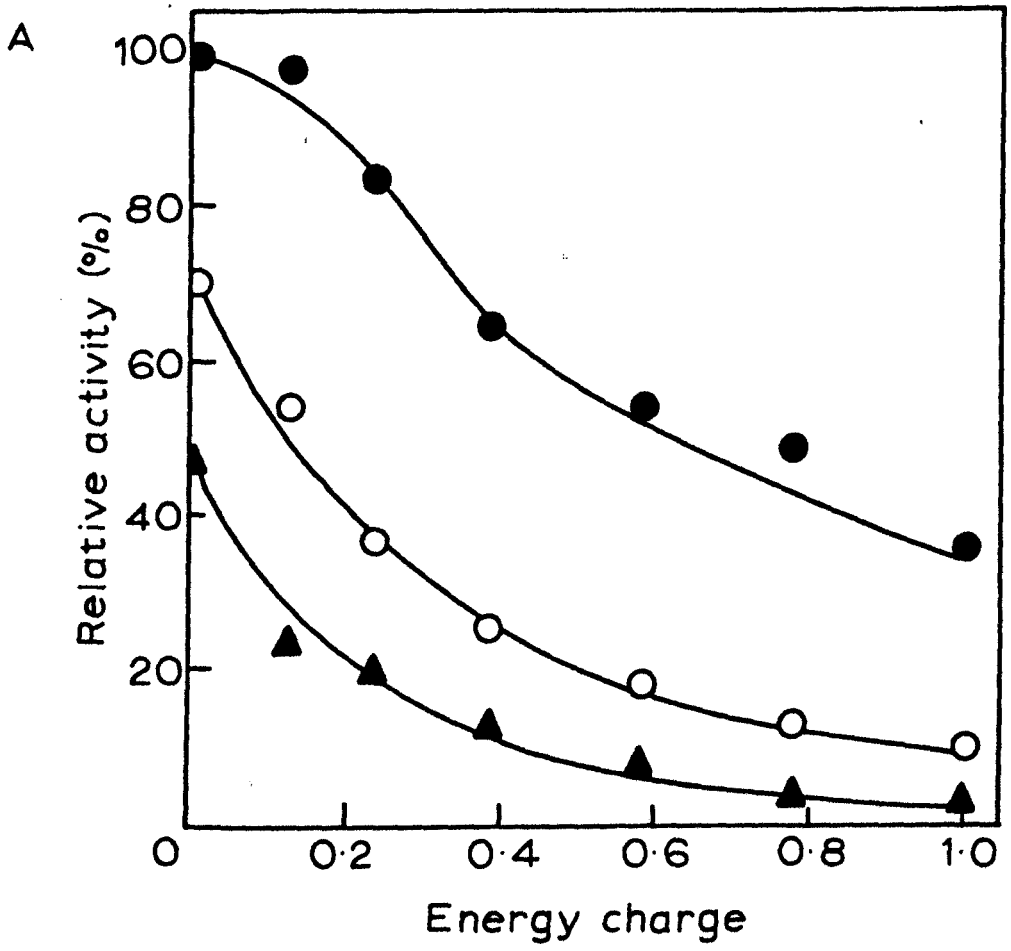
4.4.4 The response of citrate synthase from *Candida* 107 to mixtures of adenine nucleotides simulating various energy charge values in the presence of Mg^{2+}

When Mg^{2+} was added to assay mixtures which also included combinations of adenine nucleotides simulating various energy charge values, the alleviatory effect of Mg^{2+} on adenine nucleotide was also

FIGURE 16

Activity of citrate synthase from *Candida* 107 at various simulated energy charge values [(ATP + $\frac{1}{2}$ADP)(ATP + ADP + AMP)$^{-1}$] using total adenine nucleotide concentrations of 0.5 m$\underline{\underline{M}}$, ●; 2.5 m$\underline{\underline{M}}$, ○ and 5.0 m$\underline{\underline{M}}$, ▲. Activities shown relative to that obtained with 0.5 m$\underline{\underline{M}}$ AMP [0.14 and 0.11 μmol min^{-1}(mg protein)$^{-1}$ in (A) and (B), respectively]. Substrate concentrations were: oxaloacetate, 50 μ$\underline{\underline{M}}$ and acetyl-CoA, 5 μ$\underline{\underline{M}}$ in (A) and the reverse in (B).

Fig. 16


A
100
80
60
40
20
Relative activity (%)
0
0·2
0·4
0·6
0·8
1·0
Energy charge

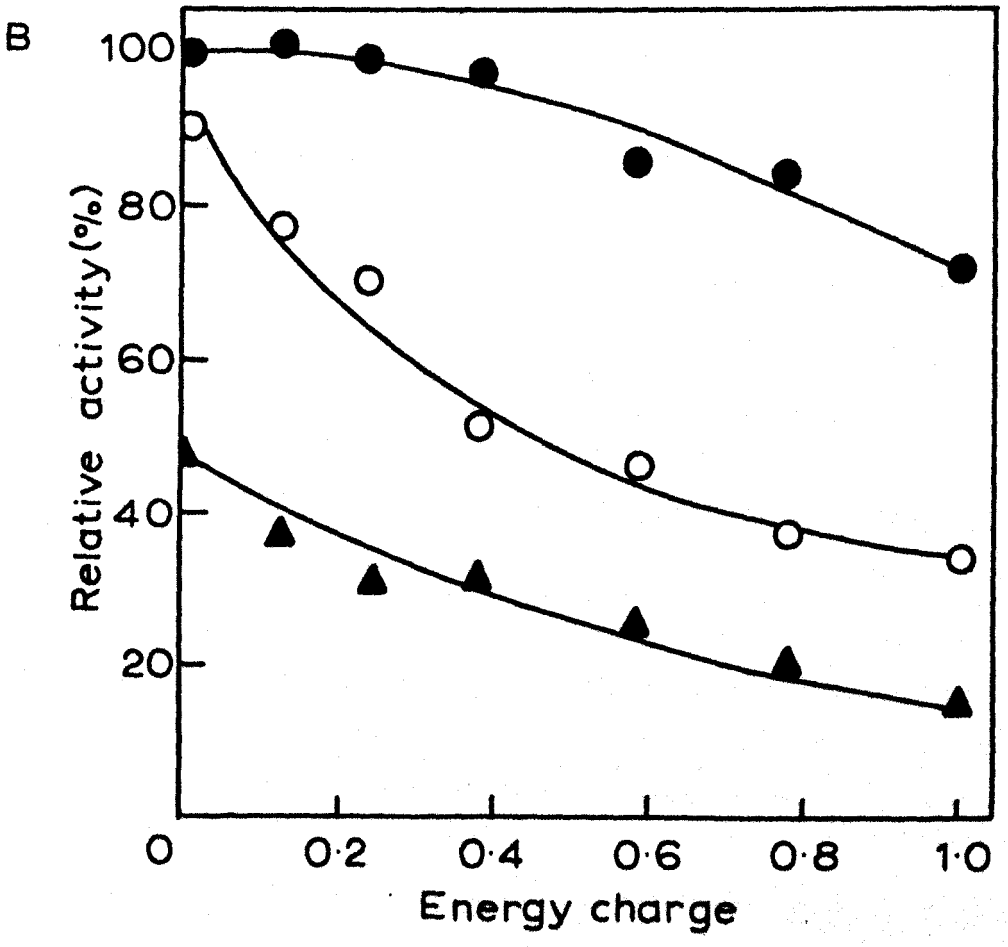
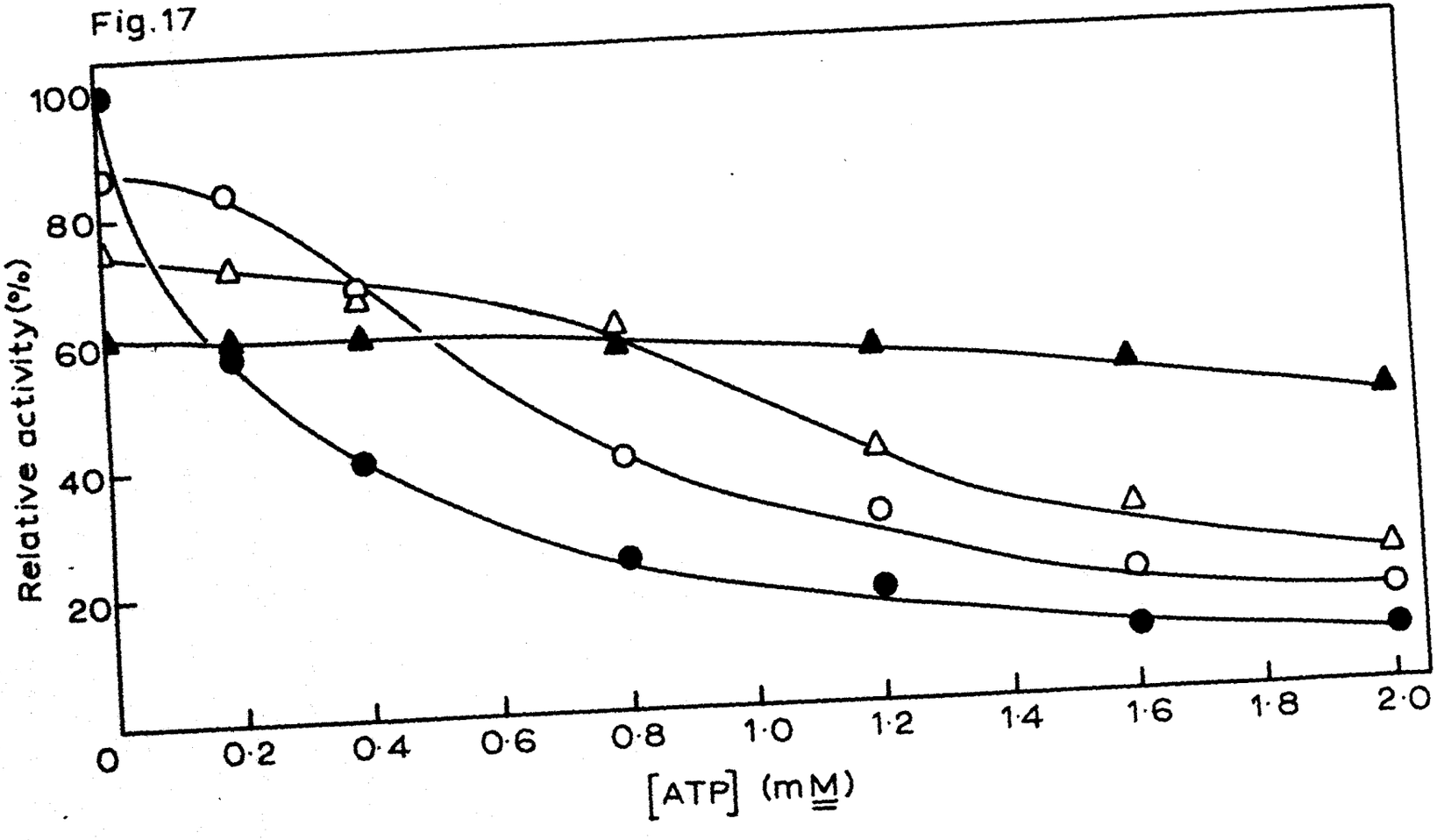

B
100
80
60
40
20
Relative activity (%)
0
0·2
0·4
0·6
0·8
1·0
Energy charge

FIGURE 17

Inhibition of citrate synthase from *Candida* 107 by ATP in the presence of Mg^{2+}. Mg^{2+} concentrations were: zero, ●; 0.5 mM, ○; 1.0 mM, △ and 2.5 mM, ▲. Substrate concentrations were: acetyl-CoA, 5 μM and oxaloacetate, 50 μM. Activities shown relative to that observed in the absence of ATP and Mg^{2+} [0.16 μmol min^{-1}(mg protein)$^{-1}$].

Fig.17


100
80
60
40
20
0
Relative activity (%)
0·2
0·4
0·6
0·8
1·0
1·2
1·4
1·6
1·8
2·0
[ATP] (mM)

noted (Figure 18). The data clearly indicated that energy charge did not exert a controlling influence on enzyme activity when Mg^{2+} was present. As noted previously (Figure 16) the total adenine nucleotide concentration was equally as important as the relative proportions of each in determining the degree of inhibition observed.

4.5 The response of citrate synthase from *Candida* 107 to other metabolites

Various metabolites were included in the standard assay mixture to ascertain whether they had any effect on the activity of citrate synthase from *Candida* 107 (Table 8). No effects were noted with the exception of nicotinamide adenine nucleotides which were mildly inhibitory. The most effective was NADPH when tested in the presence of non-saturating concentrations of acetyl-CoA.

4.6 The effect of fatty acyl-CoA esters on the activity of citrate synthase from *Candida* 107

Citrate synthases from many sources have been reported to be potently inhibited by fatty acyl-CoA esters (Tubbs, 1963; Wieland and Weiss, 1963; Srere, 1975). Hsu and Powell (1975) and Caggiano and Powell (1979) both working with mammalian citrate synthases provided evidence that there was a specific binding site for the molecules and that the inhibition was not merely a non-specific detergent effect. Therefore, they concluded that there was strong evidence to support the view that the inhibitory effect was of regulatory significance in the control of fatty acid biosynthesis by a feed-back control mechanism.

Long chain fatty acyl-CoA esters were found to be potent inhibitors of citrate synthase from *Candida* 107 (Table 9). However, the degree of inhibition was markedly and progressively reduced with reduction in the number of carbon atoms in the fatty acid moiety of the ester. Since oleoyl-CoA was such a potent inhibitor it was decided to further investigate its action on citrate synthase from *Candida* 107.

FIGURE 18

Relative activity of citrate synthase from *Candida* 107 at various simulated energy charge values in the presence of Mg^{2+}. Total adenine nucleotide concentrations were: 0.5 m$\underline{\underline{M}}$ in (A), 2.5 m$\underline{\underline{M}}$ in (B) and 5.0 m$\underline{\underline{M}}$ in (C). Mg^{2+} concentrations were: zero, ●; 2.5 m$\underline{\underline{M}}$, ○ and 5.0 m$\underline{\underline{M}}$, △. Substrate concentrations were: oxaloacetate, 50 $\mu\underline{\underline{M}}$ and acetyl-CoA, 5 $\mu\underline{\underline{M}}$ and activities were compared to that observed in the absence of Mg^{2+} and in the presence of 0.5 m$\underline{\underline{M}}$ AMP [0.14 μmol min^{-1}(mg protein)$^{-1}$].

Fig.18

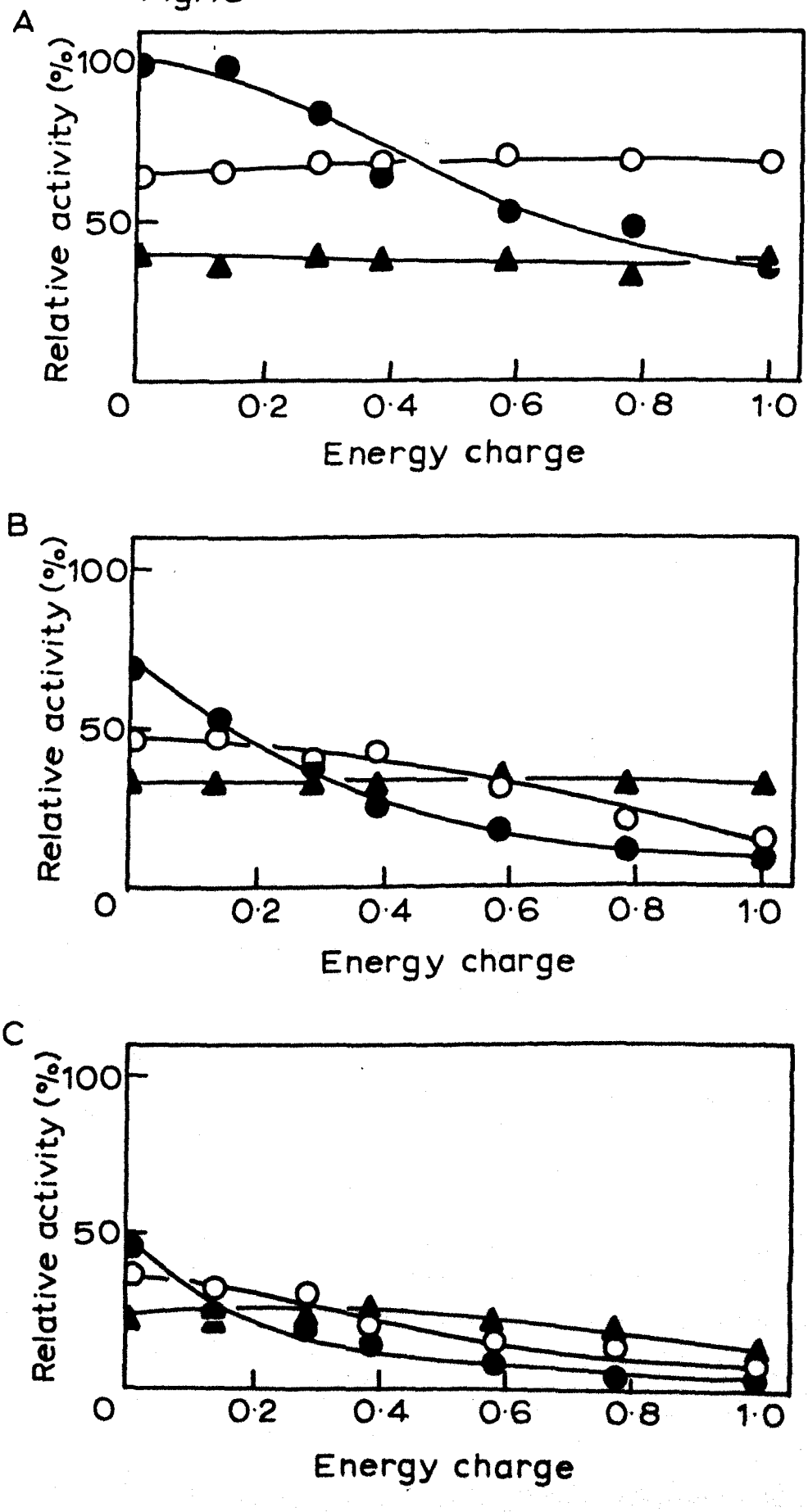

A
Relative activity (%)
100
50
0
0·2
0·4
0·6
0·8
1·0
Energy charge
B
Relative activity (%)
100
50
0
0·2
0·4
0·6
0·8
1·0
Energy charge
C
Relative activity (%)
100
50
0
0·2
0·4
0·6
0·8
1·0
Energy charge

TABLE 8: The effect of various metabolites on the activity of citrate synthase from *Candida* 107 when tested at a final concentration of 1 mM, with either non-saturating oxaloacetate (5 μM) and saturating acetyl-CoA (50 μM) or non-saturating acetyl-CoA (5 μM) and saturating oxaloacetate (50 μM). Activities expressed relative to that observed in the absence of added metabolite [0.21 μmol min^{-1} $(mg\ protein)^{-1}$].

Metabolite	Relative activity (%)	
	non-saturating oxaloacetate	non-saturating acetyl-CoA
NAD^+	101.7	95.1
NADH	81.7	79.0
$NADP^+$	90.0	88.3
NADPH	66.4	48.8
Citrate	85.1	81.0
DL-isocitrate	87.2	89.3
Pyruvate	101.7	111.2
α-Ketoglutarate	83.0	91.2
L-malate	101.0	100
Phospho(enol)pyruvate	101.7	69.3
Acetyl-phosphate	94.8	88.8
Fructose-1,6 bisphosphate	84.7	96.9
Glutamate	98.3	108.7
DL-glutamine	91.9	102.0
Aspartate	97.7	105.1

TABLE 9: Effect of fatty acyl-CoA esters on the activity of citrate synthase from *Candida* 107. Activities are shown relative to the control rate [0.21 μmol min^{-1}(mg protein)$^{-1}$]. The standard assay mixture was used but the enzyme was preincubated for 1 min at 30 °C, with the test compound at a final concentration of 10 μM, prior to initiating reactions by the addition of oxaloacetate.

Test compound	Relative activity (%)
Malonyl -CoA	108.1
Lauroyl -CoA ($C_{12:0}$)	104.6
Myristoyl-CoA ($C_{14:0}$)	95.9
Palmitoyl-CoA ($C_{16:0}$)	53.7
Stearoyl -CoA ($C_{18:0}$)	5.8
Oleoyl -CoA ($C_{18:1}$)	7.6

4.6.1 Time-dependency of oleoyl-CoA inhibition of citrate synthase from *Candida* 107

Hsu and Powell (1975) reported that the inhibition of pig heart citrate synthase by oleoyl-CoA was time dependent, maximum inhibition being observed after 5 min. A similar effect with oleoyl-CoA inhibition of citrate synthase from *Candida* 107 was observed but the time scale was considerably reduced, only 30 sec being required to effect maximum inhibition (Figure 19).

4.6.2 The pattern of oleoyl-CoA inhibition of citrate synthase from *Candida* 107

The degree of inhibition of citrate synthase from *Candida* 107 increased in a sigmoidal manner, with increase in the concentration of oleoyl-CoA (Figures 20 and 21). Thus, when the inhibitor was included in assays at concentrations lower than 4-5 $\mu\underline{M}$ there was little effect on enzyme activity. However, when the oleoyl-CoA concentration was increased from 5 to 7 $\mu\underline{M}$ there was a dramatic increase in the degree of inhibition observed. This effect was most pronounced when the enzyme was preincubated with oleoyl-CoA and oxaloacetate, prior to initiating reactions by the addition of acetyl-CoA (Figure 20).

This observation was of key significance in that Hsu and Powell (1975) reported that the critical micellar concentration (CMC) of oleoyl-CoA is 4.7 $\mu\underline{M}$. Thus, oleoyl-CoA did not inhibit citrate synthase from *Candida* 107 when tested at concentrations below the CMC. However, when tested at concentrations above the CMC the inhibition was virtually total (Figures 20 and 21). This provided strong evidence, therefore, that the inhibition was a non-specific detergent effect and was not of any regulatory significance.

4.6.3 The effect of Mg^{2+} on oleoyl-CoA inhibition of citrate synthase from *Candida* 107

Kosicki and Lee (1966) reported that divalent metal ions relieved adenine nucleotide inhibition of pig heart citrate synthase. They also

FIGURE 19

Time dependency of oleoyl-CoA inhibition of citrate synthase from *Candida* 107. Oleoyl-CoA (10 μM) was preincubated with the enzyme at 30 °C in the standard assay mixture, for the time indicated, prior to initiating reactions by the addition of oxaloacetate. Activities shown relative to that observed in the absence of inhibitor [0.19 μmol min^{-1}(mg protein)$^{-1}$].

Fig.19

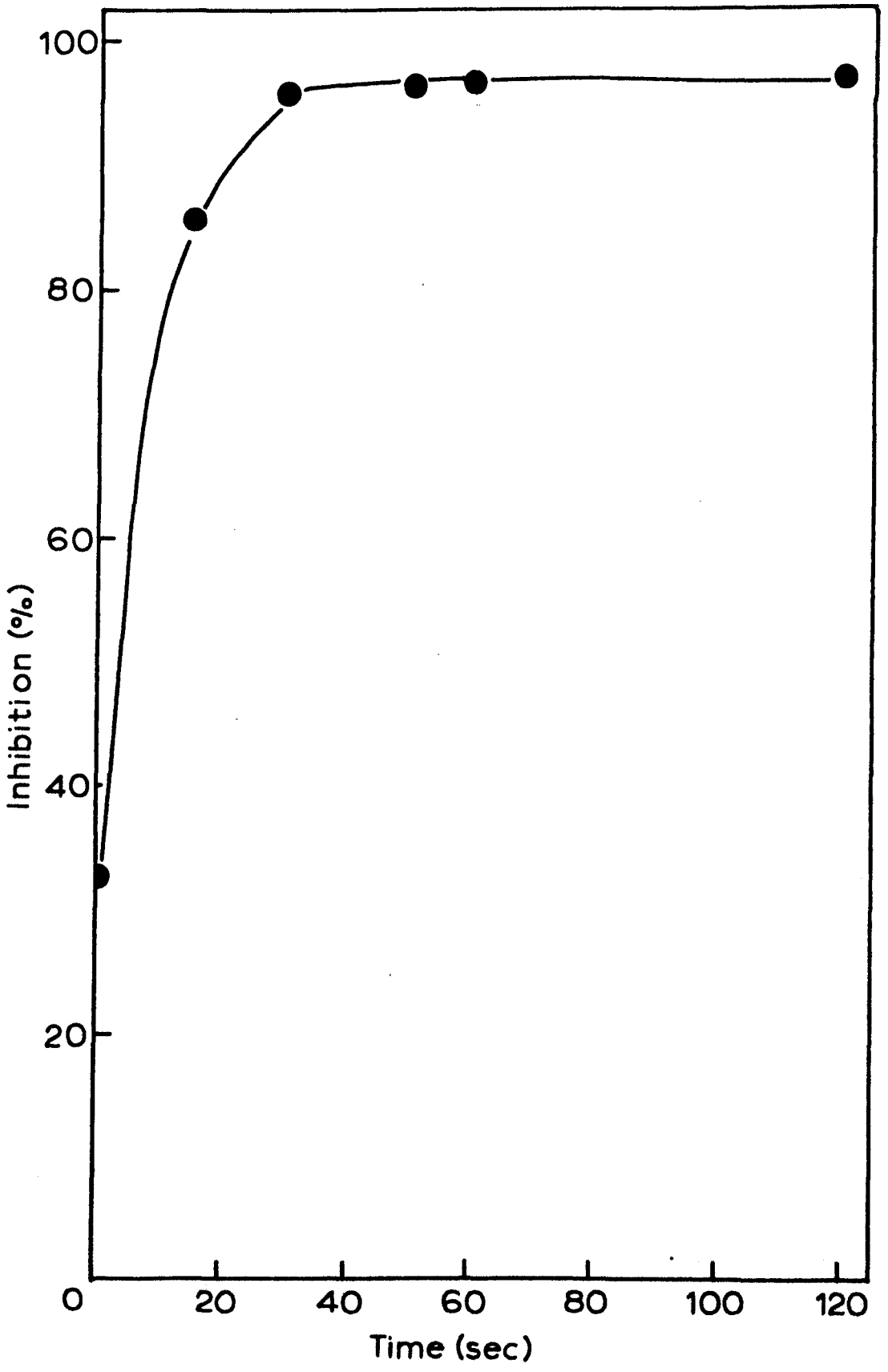


FIGURE 20

Inhibition of citrate synthase from *Candida* 107 by oleoyl-CoA when preincubated for 2 min at 30 °C in the standard assay mixture, at the concentrations indicated, prior to initiating reactions by the addition of acetyl-CoA. Activities expressed relative to that obtained in the absence of oleoyl-CoA [0.21 μmol min^{-1}(mg protein)$^{-1}$].

Fig. 20

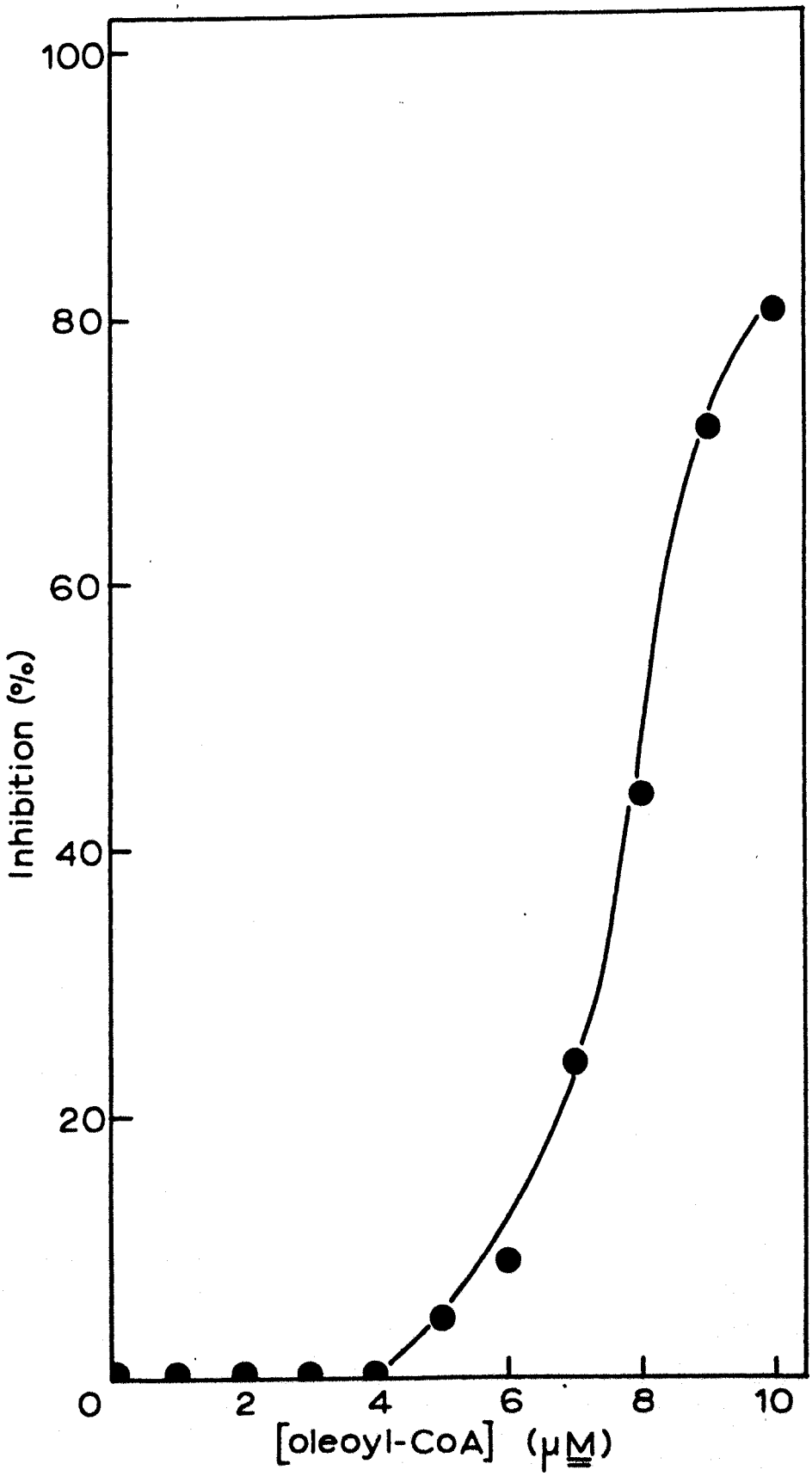
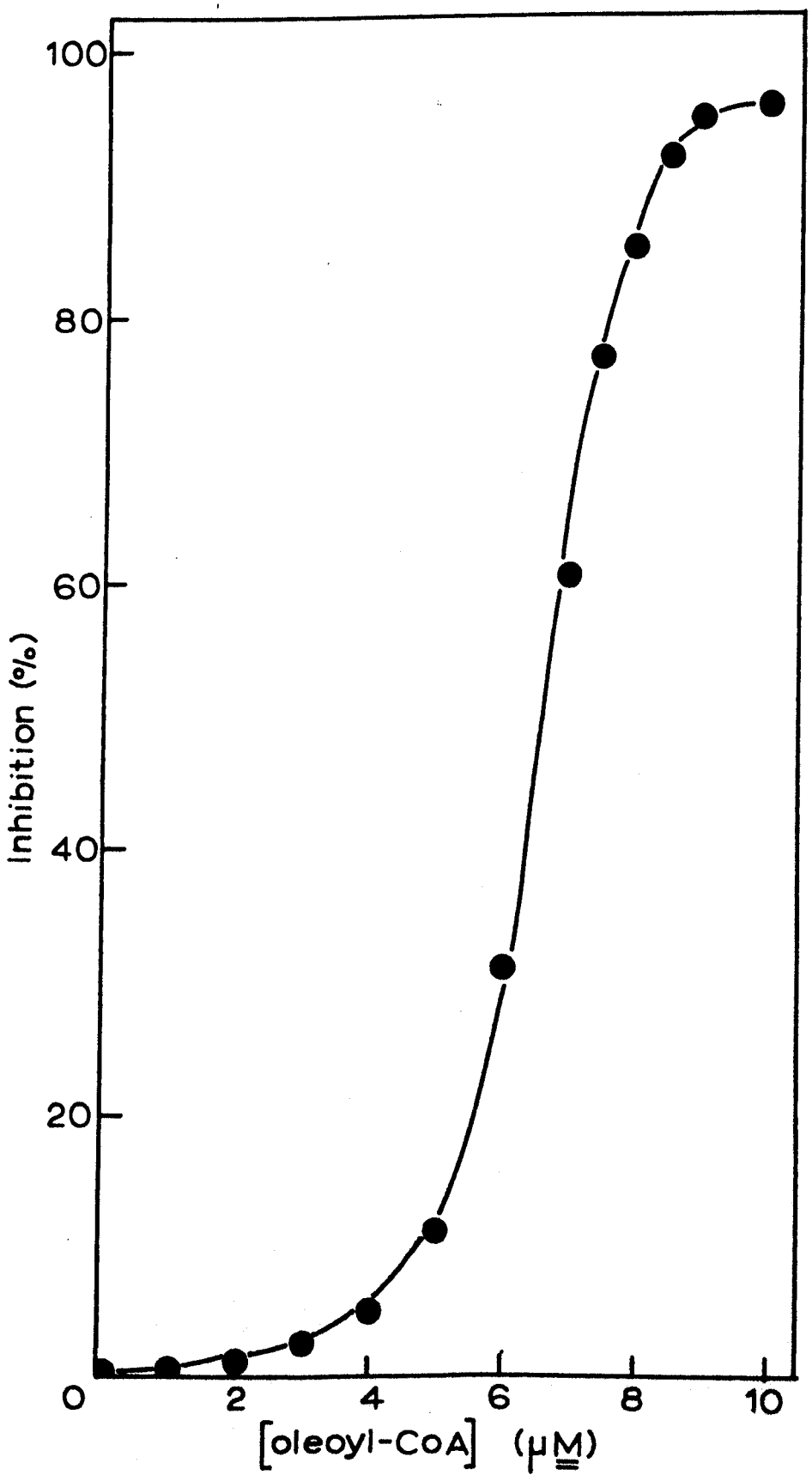


FIGURE 21

Inhibition of citrate synthase from *Candida* 107 by oleoyl-CoA when preincubated for 2 min at 30 °C in the standard assay mixture, at the concentrations indicated, prior to initiating reactions by the addition of oxaloacetate. Activities expressed relative to that observed in the absence of oleoyl-CoA [0.21 μmol min^{-1}(mg protein)$^{-1}$].

Fig.21



observed a similar ameliorating effect on the inhibition of the enzyme by palmitoyl-CoA.

In much the same way Mg^{2+} also relieved oleoyl-CoA inhibition of citrate synthase from *Candida* 107 (Figure 22), thereby, possibly casting even further doubt on the *in vivo* significance of acyl-CoA ester inhibition of this enzyme.

4.6.4 Effect of protein concentration on the inhibition of citrate synthase from *Candida* 107 by oleoyl-CoA

Acyl-CoA esters will bind non-specifically to proteins. If the inhibition of citrate synthase from *Candida* 107 by oleoyl-CoA was caused by non-specific denaturation, due to the molecule acting as a detergent, then the degree of inhibition should be dependent on protein concentration.

In fact, no inhibition was observed in the presence of 10 $\mu\underline{\underline{M}}$ oleoyl-CoA when 1 mg of bovine serum albumin was included in the assay mixture. Furthermore, when the enzyme was inhibited by 10 $\mu\underline{\underline{M}}$ oleoyl-CoA the inhibition could not be relieved by the subsequent addition of bovine serum albumin (Table 10). These results were contrary to those of Goodridge (1972) who observed a reversal of the inhibition of chick liver acetyl-CoA carboxylase by palmitoyl-CoA, on the addition of bovine serum albumin.

4.6.5 Effect of lipid intermediates and detergents on the activity of citrate synthase from *Candida* 107

Various lipid intermediates and detergents did not inhibit the activity of citrate synthase from *Candida* 107 when tested in the standard assay (Table 11).

5. Studies with NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809

It was originally intended to study this enzyme from *Candida* 107 as some preliminary work had already been performed (Botham and Ratledge, 1979). However, the NAD^+-dependent isocitrate dehydrogenase from

FIGURE 22

The effect of Mg^{2+} on the inhibition of citrate synthase from *Candida* 107 by oleoyl-CoA. Oleoyl-CoA (10 $\mu\underline{M}$) was preincubated for 2 min with enzyme and Mg^{2+}, at the concentration indicated, in the standard assay mixture prior to initiating reactions by the addition of oxaloacetate. Activities are expressed relative to that observed in the absence of oleoyl-CoA [0.19 μmol min^{-1}(mg protein)$^{-1}$].

Fig. 22

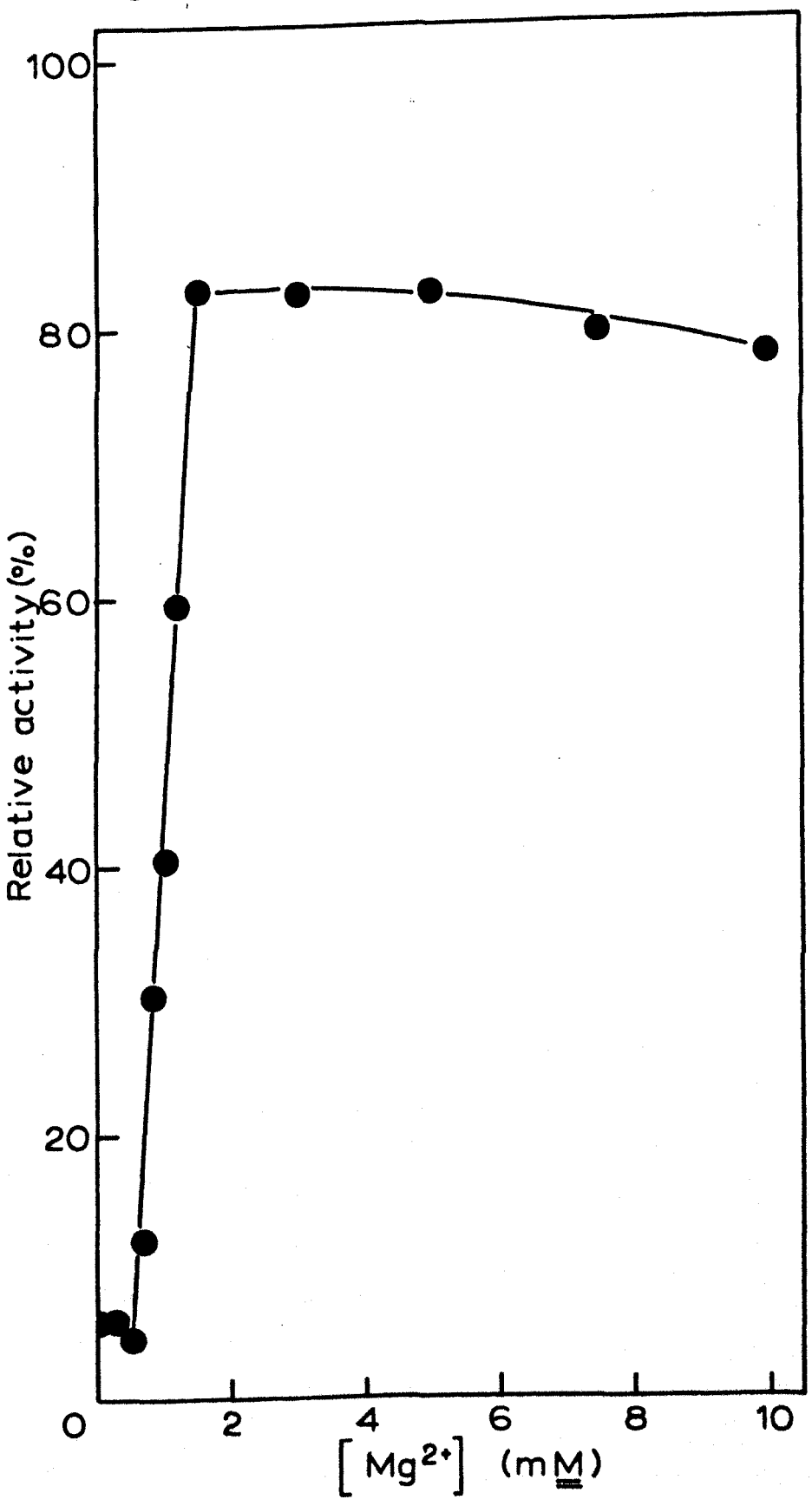


TABLE 10: The effect of bovine serum albumin on the inhibition of citrate synthase from *Candida* 107 by oleoyl-CoA. Initial rates were measured using the standard assay mixture under the conditions described. Activities are expressed relative to that observed in the absence of added oleoyl-CoA [0.21 μmol min^{-1}(mg protein)$^{-1}$].

	Conditions	Relative Activity (%)
1.	Enzyme preincubated for 1 min at 30 °C with 10 μM oleoyl-CoA prior to initiation of reaction by addition of oxaloacetate	8.6
2.	Enzyme preincubated for 1 min at 30 °C with 10 μM oleoyl-CoA and 1 mg BSA prior to initiation of reaction by addition of oxaloacetate	98.5
3.	As 1	7.0
	1 mg BSA added to inhibited enzyme	7.0
	10 mg BSA added to inhibited enzyme	7.0

TABLE 11: The effect of lipid intermediates and detergents on the activity of citrate synthase from *Candida* 107. Compounds were preincubated with the enzyme for 1 min at 30 °C, in the standard assay mixture, prior to initiating reactions by the addition of oxaloacetate. Activities expressed relative to that observed in the absence of added test compound [0.21 μmol min^{-1}(mg protein)$^{-1}$].

Test compound	Concentration (μM)	Relative activity (%)
Palmitoyl acetate	100	91.9
Palmitic acid ethyl ester	100	90.4
Palmityl DL carnitine chloride	100	100
Palmitic acid methyl ester	100	90.7
Oleoyl alcohol	100	93.6
Oleoyl laurate	100	88.0
Oleic acid methyl ester	100	99.1
Myristic acid palmityl ester	100	91.3
Sodium lauryl sulphate	10	94.6
Sodium lauryl sulphate	100	71.4
Triton X-100	0.01% (w/v)	100
Triton X-100	0.1% (w/v)	98.5

Candida 107 was extremely labile, such that attempts to purify the enzyme resulted in unacceptable losses of units. Attempts to stabilize the enzyme by the addition to extracts, singly or in combination, of substrates, adenine nucleotides, reducing agents, divalent metal ions, proteinase inhibitors and glycerol were unsuccessful. Consequently, attention was diverted to the NAD^+-dependent isocitrate dehydrogenase of *Lipomyces starkeyi* CBS 1809 which proved more amenable to study.

5.1 Partial purification of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809

NAD^+-dependent isocitrate dehydrogenase was partially purified from crude cell-free extracts of *Lipomyces starkeyi* CBS 1809 using standard techniques (Table 12). Attempts to improve the purification by the use of further treatments resulted in unacceptable losses of units. Therefore, the pooled Sephadex eluate which was free from contaminating activities of $NADP^+$-dependent isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, aconitase, isocitrate lyase, and adenylate kinase was used in the experiments described subsequently.

5.2 Variation in activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 with changes in pH

The activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 varied substantially with changes in pH (Figure 23). Optimal activity was observed between pH 6.8 and pH 7.2, therefore, pH 7.0 was used in subsequent assays.

5.3 Variation in activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 with changes in substrate concentration

The usual Michaelis-Menten relationship was valid when the initial velocity was measured as a function of D-isocitrate and NAD^+ concentrations (Figures 24 and 25). Apparent K_m values were calculated to be 89 $\mu\underline{M}$ and 115 $\mu\underline{M}$ for D-isocitrate and NAD^+, respectively.

TABLE 12: Flow diagram of a typical partial purification of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809

Fraction	Volume (ml)	Units (μmol $min^{-1} ml^{-1}$)	Protein (mg ml^{-1})	Specific activity [μmol min^{-1} (mg protein)$^{-1}$]	Purification	Yield (%)
Crude	62.0	0.71	8.2	0.087	1	100
45-70% $(NH_4)_2SO_4$	5.4	6.80	39.1	0.174	2.0	83.4
Sephadex G200 pooled eluate	51.0	0.50	0.58	0.870	10.2	58.3

FIGURE 23

Variation in activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 with pH. The standard assay mixture was used with $KH_2PO_4/NaHPO_4$ buffer at a final concentration of 50 mM.

Fig.23

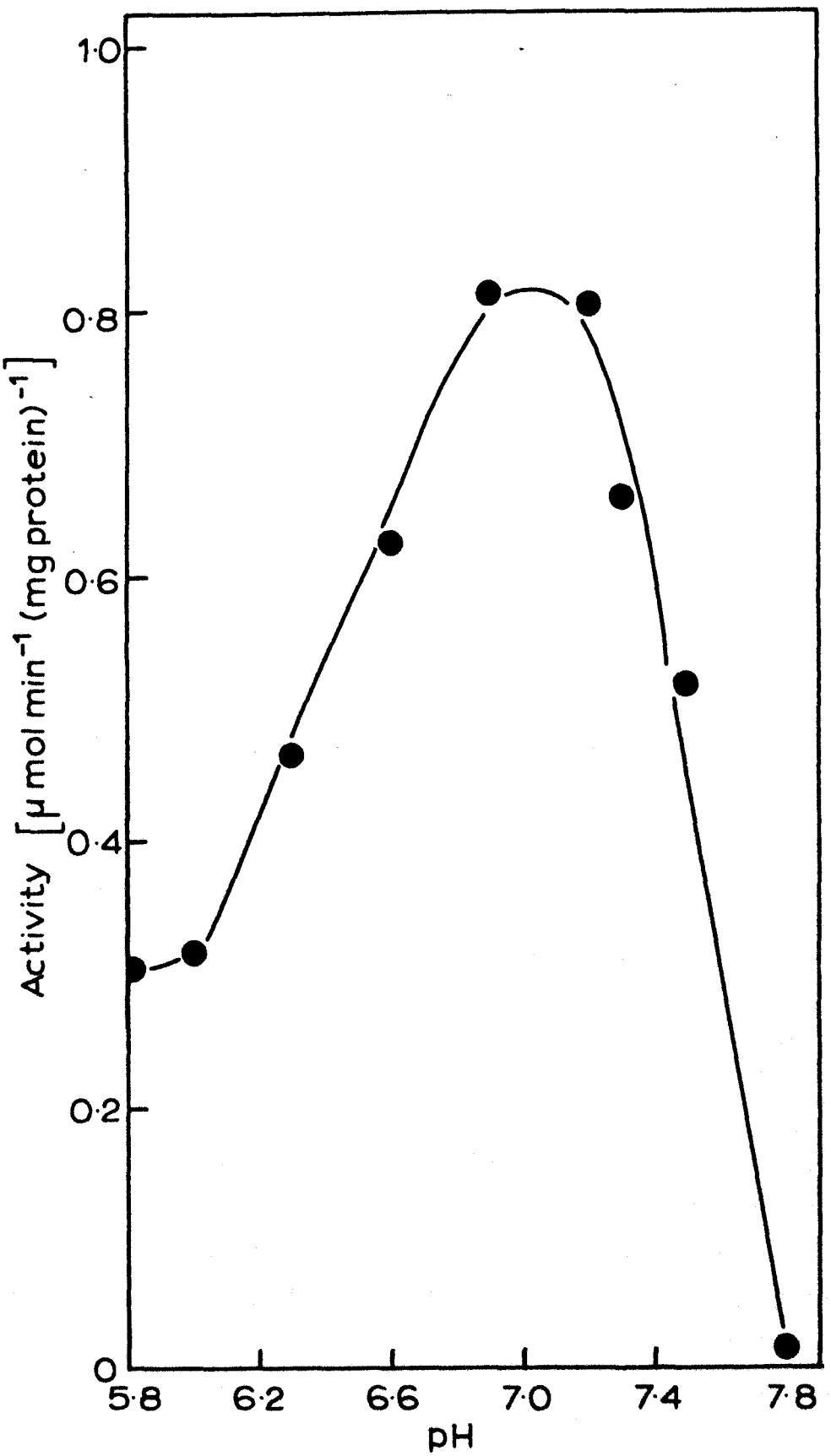

1·0
0·8
0·6
0·4
0·2
0
Activity [μ mol min⁻¹ (mg protein)⁻¹]
5·8
6·2
6·6
7·0
7·4
7·8
pH

FIGURE 24

Lineweaver-Burk plot showing the variation in initial velocity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 with varying concentrations of NAD^+. The concentration of DL-isocitrate was 2 mM and assay mixtures contained 0.5 mM AMP.

Fig. 24

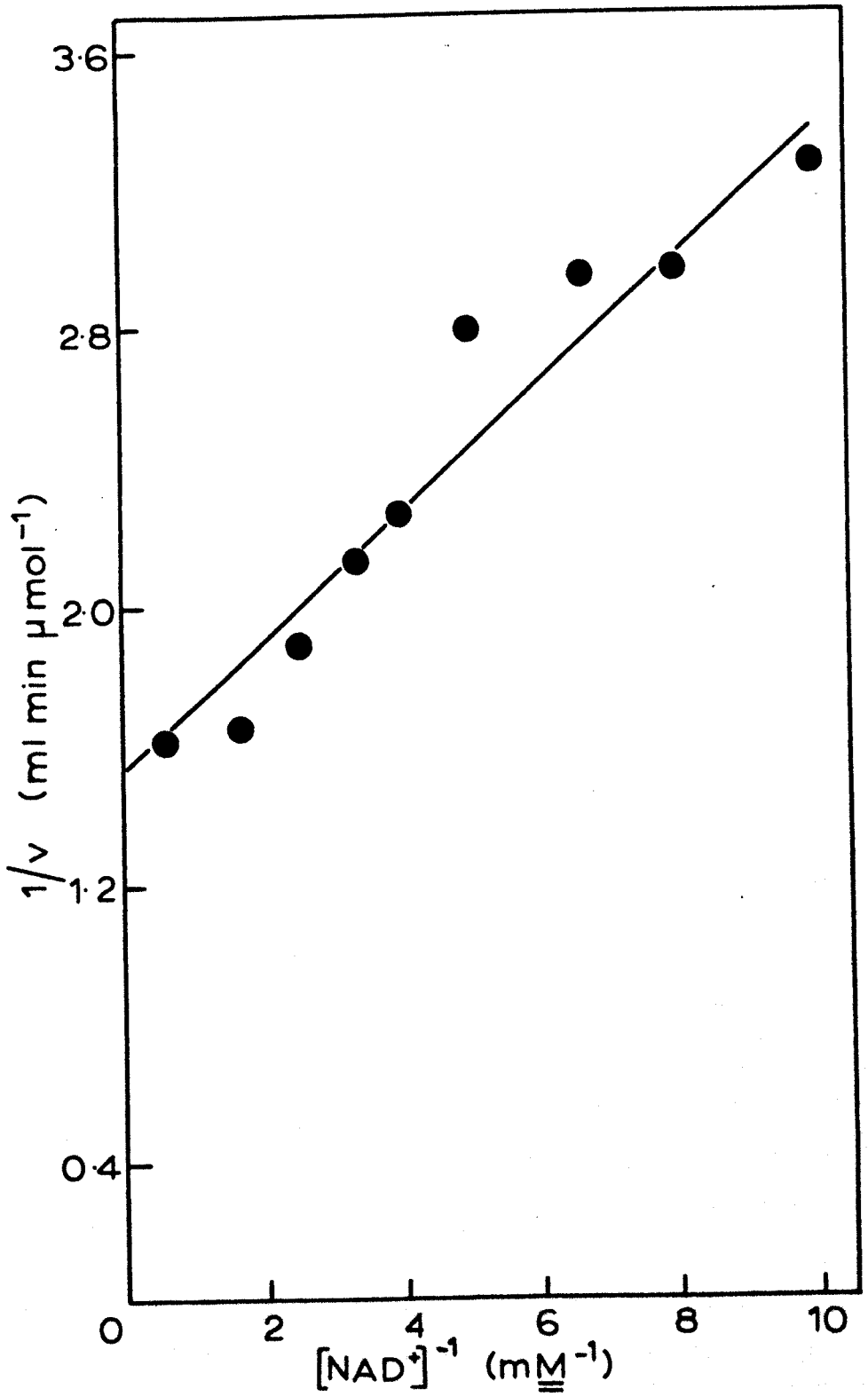
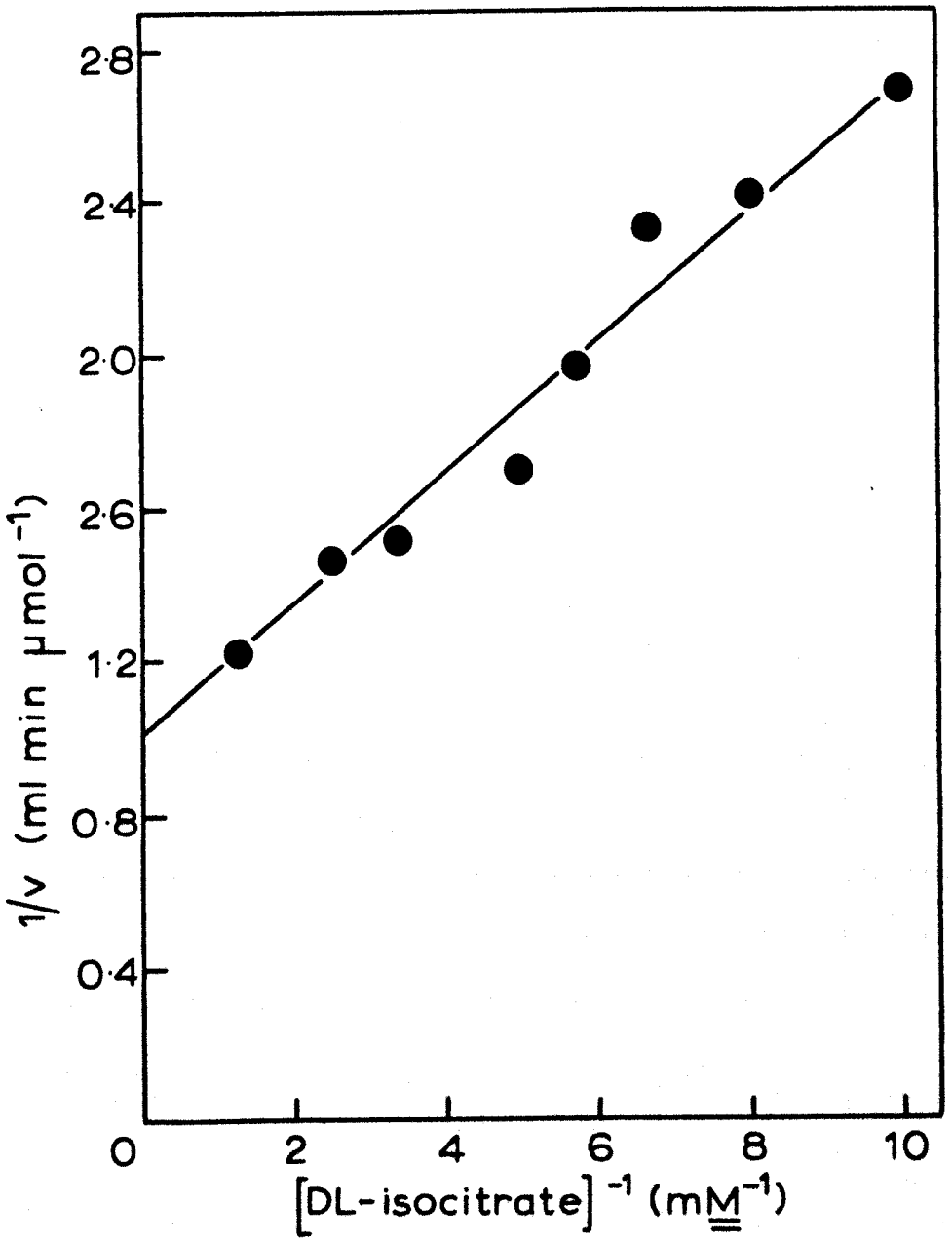


FIGURE 25

Lineweaver-Burk plot showing the variation in initial velocity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 with varying concentrations of DL-isocitrate. The concentration of NAD^+ was 1 mM and assay mixtures contained 0.5 mM AMP.

Fig.25



5.4 The effect of adenine nucleotides on the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809

Botham and Ratledge (1979) reported that NAD^+-dependent isocitrate dehydrogenase from *Candida* 107 showed little activity unless AMP was added to assay mixtures. The addition of 0.5 mM AMP resulted in a 30-fold stimulation of activity. In the same report these authors observed that AMP did not stimulate this enzyme from a non-oleaginous yeast, *Candida utilis* and that the enzyme was active in the absence of added AMP. Since Botham and Ratledge (1979) had observed that when cultural conditions favoured lipogenesis, the intracellular concentration of AMP, in *Candida* 107, was low they considered that this might inactivate NAD^+-dependent isocitrate dehydrogenase and thereby, restrict the oxidative role of the TCA cycle and divert the flow of carbon into the production of cytosolic citrate.

As this phenomenon had not been investigated in *Lipomyces starkeyi* CBS 1809 the effect of adenine nucleotides on the NAD^+-dependent isocitrate dehydrogenase from this oleaginous yeast was studied.

5.4.1 The effect of AMP on the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809

AMP at concentrations up to 10 mM did not stimulate the activity of the enzyme when tested in the standard assay mixture. This lack of response was not due to the presence of endogenous AMP as dialysis of extracts did not restore AMP sensitivity.

AMP did stimulate activity, however, when the D-isocitrate concentration was reduced to a non-saturating level (50 μM). Maximum stimulation (3.5 fold) was observed with 50 μM AMP (Figure 26).

5.4.2 The effect of ATP on the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809

In the absence of added AMP, ATP was a potent inhibitor of enzyme activity when tested at non-saturating concentrations of D-isocitrate (Figure 27).

FIGURE 26

The effect of AMP on the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 at a non-saturating concentration of D-isocitrate (50 $\mu\underline{\underline{M}}$). NAD^+ concentration was 1 $m\underline{\underline{M}}$.

Fig. 26

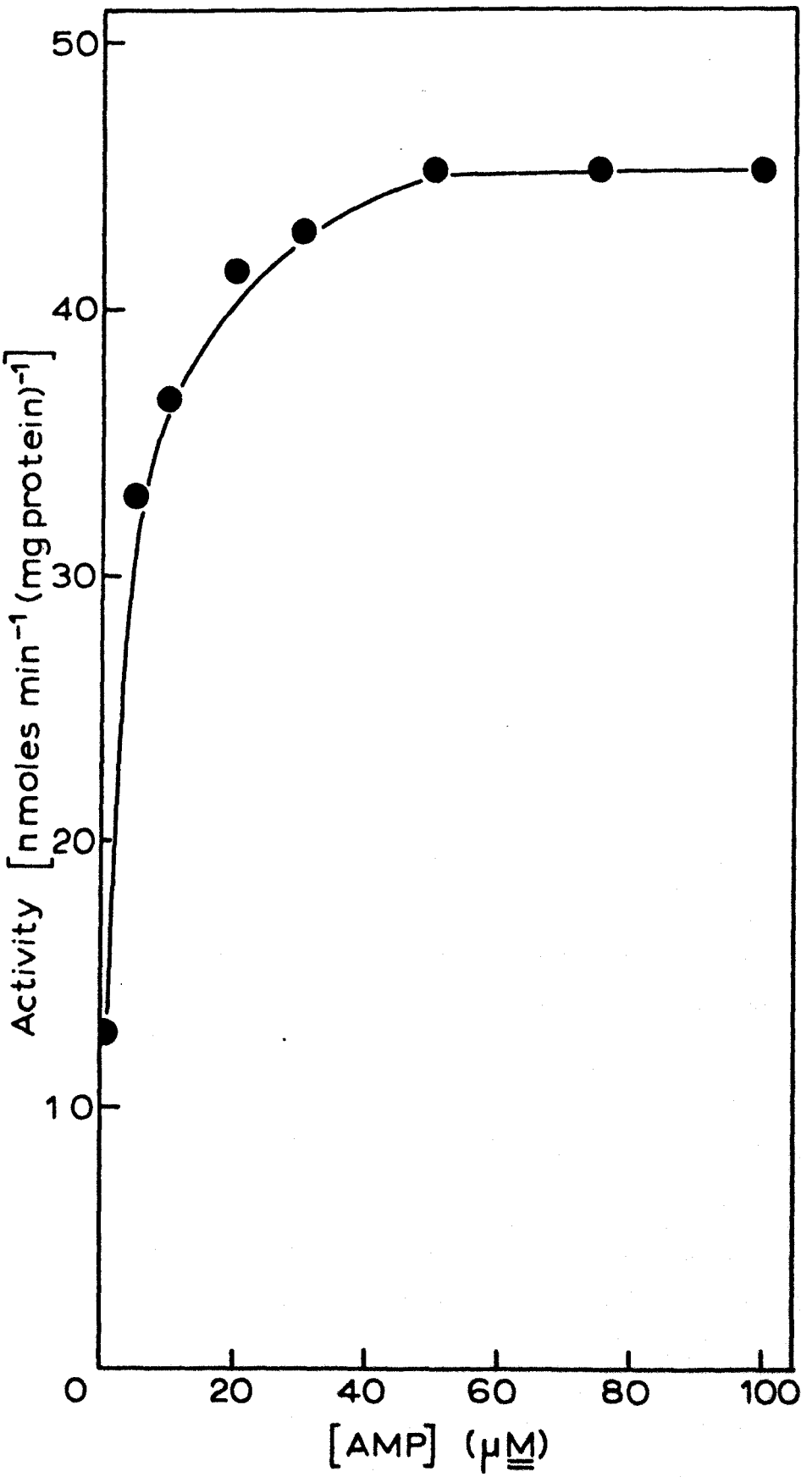
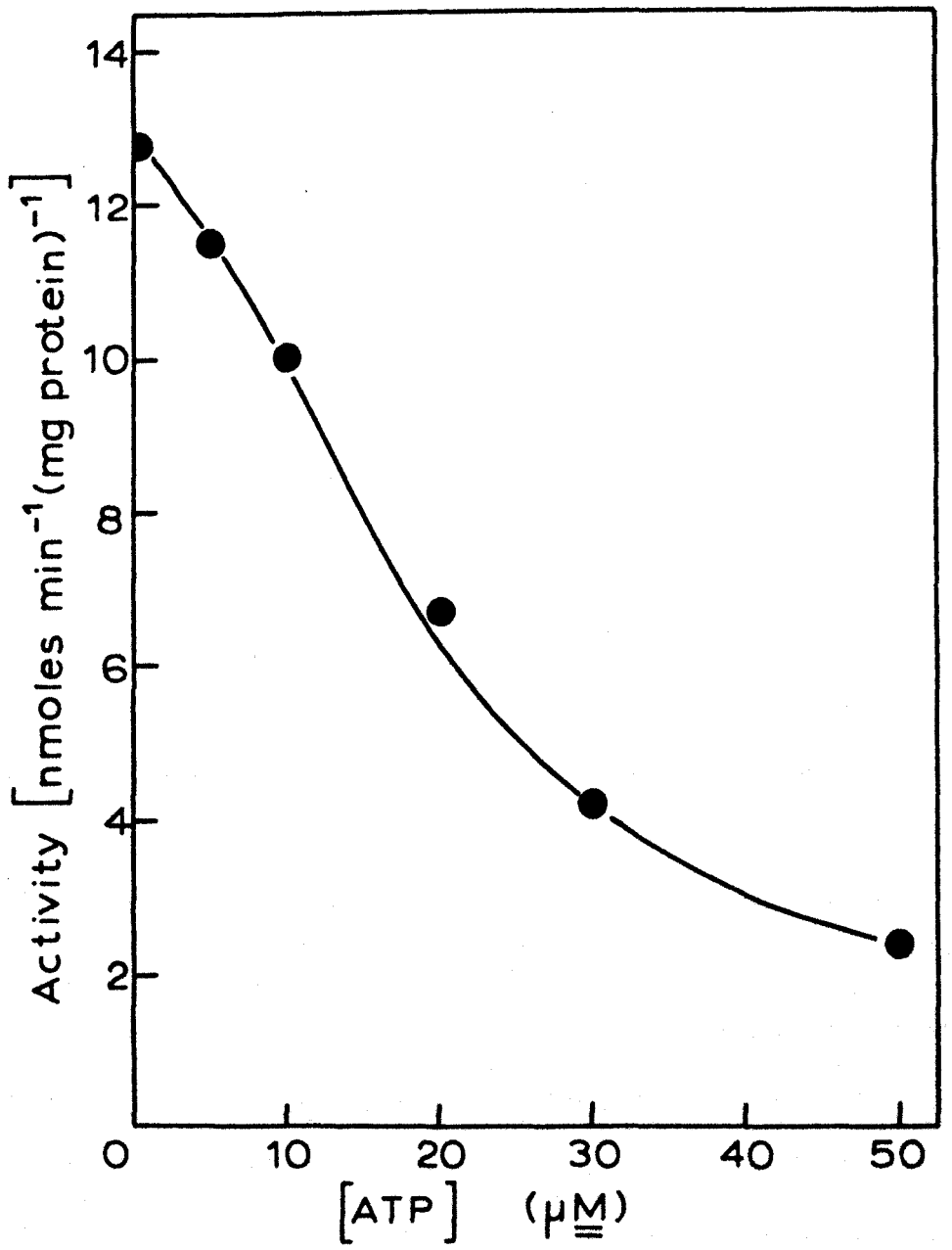

50
40
30
20
10
0
20
40
60
80
100
Activity [n moles min⁻¹ (mg protein)⁻¹]
[AMP] (μM)

FIGURE 27

The effect of ATP on the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 in the absence of added AMP and at a non-saturating concentration of D-isocitrate (50 $\mu\underline{M}$). NAD^+ concentration was 1 $m\underline{M}$.

Fig. 27


Activity [nmoles min^{-1}(mg protein)$^{-1}$]
14
12
10
8
6
4
2
0
10
20
30
40
50
[ATP] (μM)

When AMP was included in assay mixtures not only was there a stimulation of overall activity but the concentration of ATP required to effect considerable inhibition was markedly increased (Figure 28). Thus, 50 $\mu\underline{M}$ ATP reduced activity by 83% in the absence of added AMP (Figure 27), whereas, when 100 $\mu\underline{M}$ AMP was included in assay mixtures, 6.8 $m\underline{M}$ ATP (138-fold higher) was required to achieve the same degree of inhibition (Figure 28).

5.4.3 The response of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 to mixtures of adenine nucleotides simulating various energy charge values

The activity of the enzyme responded to mixtures of adenine nucleotides simulating various energy charge values as predicted by Atkinson (1968) for those enzymes involved in sequences leading to the regeneration of ATP (Figure 29). Thus, enzyme activity decreased with increase in energy charge.

In previous studies reported here (Results, Section 4.7) it was noted that Mg^{2+} exerted a considerable alleviatory effect on adenine nucleotide inhibition of citrate synthase from *Candida* 107. In the case of NAD^+-dependent isocitrate dehydrogenase, however, the involvement of divalent metal ions cannot be questioned as saturating concentrations of Mg^{2+} (4 $m\underline{M}$) were included in all assays.

The total adenine nucleotide concentration did not appear to be a significant determinant of the activity of NAD^+-dependent isocitrate dehydrogenase as a ten-fold increase from 0.1 to 1 $m\underline{M}$ produced curves of activity against energy charge that were virtually superimposable (Figure 29).

Thus, the results were in accord with the premise of Atkinson (1968) that NAD^+-dependent isocitrate dehydrogenase is an enzyme whose activity is controlled by changes in adenylate energy charge. Furthermore, there is every likelihood that when cultural conditions

FIGURE 28

The effect of ATP on the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 in the presence of AMP (100 $\mu\underline{\underline{M}}$). Concentrations of D-isocitrate and NAD^+ were 50 $\mu\underline{\underline{M}}$ and 1 $m\underline{\underline{M}}$, respectively.

Fig. 28

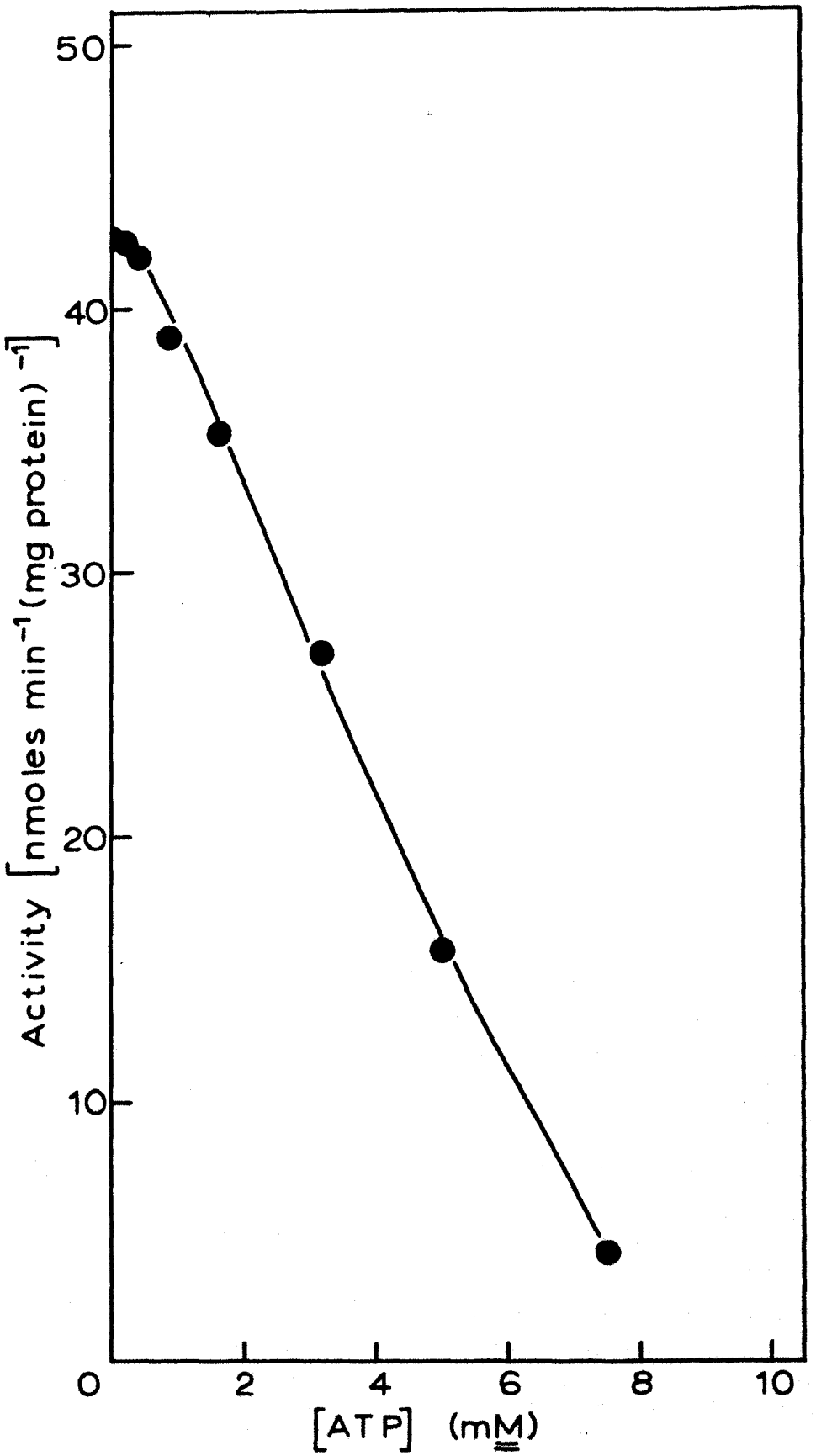
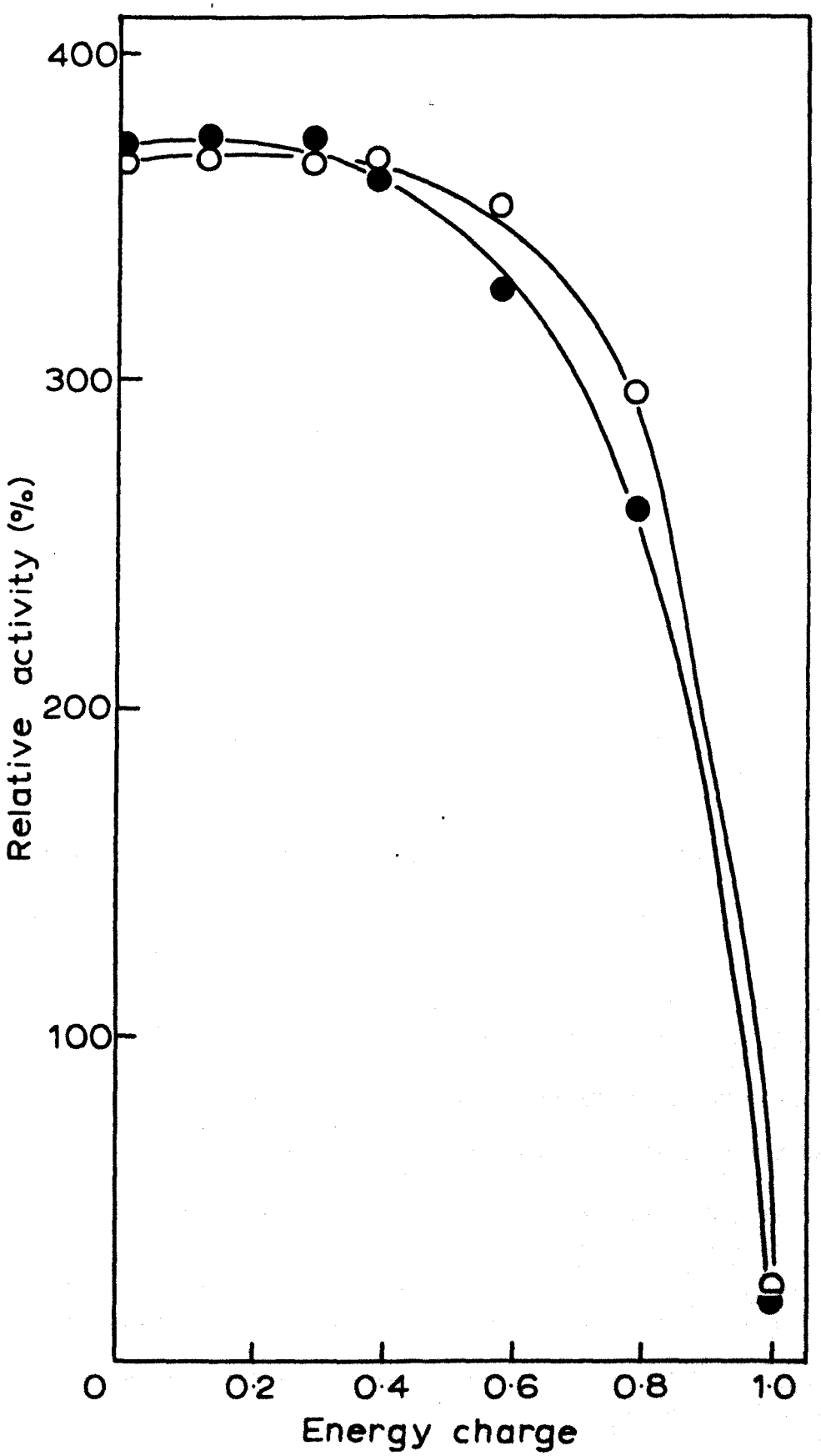

50
40
30
20
10
0
2
4
6
8
10
Activity [nmoles min^{-1}(mg protein)$^{-1}$]
[ATP] (mM)

FIGURE 29

The response of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 to mixtures of adenine nucleotides simulating various energy charge values. Total adenine nucleotide concentrations were: 0.1 mM, ● and 1 mM, ○. Concentrations of D-isocitrate and NAD^+ were 50 μM and 1 mM, respectively. Activities expressed relative to that observed in the absence of added adenine nucleotide [12.8 nmol min^{-1}(mg protein)$^{-1}$].

Fig. 29


400
300
200
100
Relative activity (%)
0
0·2
0·4
0·6
0·8
1·0
Energy charge

favour lipogenesis the resultant high adenylate energy charge observed in cultures of *Lipomyces starkeyi* CBS 1809 (Results, Section 1.3.3) would be sufficient to restrict NAD^+-dependent isocitrate dehydrogenase activity.

6. Studies with ATP:citrate lyase from oleaginous yeasts

ATP:citrate lyase is considered to occupy a central role in lipogenesis in many tissues by providing precursor acetyl-CoA units which are themselves derived from intramitochondrial citrate (see Introduction, Section 2.2.1). With the exception of our own laboratory there have been very few reports where the enzyme has been detected in microorganisms. Those reports that have appeared describe microorganisms that exhibit a propensity to accumulate lipid, or lipid-related products, in large quantities, for example, the yeast *Rhodotorula gracilis* (Guerritore and Hanozet, 1970) and the fungi, *Mortierella* spp. (Attwood, 1973) and *Penicillium spiculisporum* (Mahlén, 1973).

There have been no indications that these initial reports of ATP:citrate lyase activity from micro-organisms have been further developed. Therefore, it was considered that a study of the enzyme from oleaginous yeasts was merited and in particular to ascertain whether any regulatory function could be ascribed.

6.1 Survey for the occurrence of ATP:citrate lyase in oleaginous and non-oleaginous yeasts compared with their lipid content

Botham and Ratledge (1979) reported that *Candida* 107 possessed an active ATP:citrate lyase but the enzyme could not be detected in the non-oleaginous yeast, *Candida utilis*. Similarly, oleaginous strains of *Rhodotorula glutinis* and *Mucor circinelloides* contained the enzyme but it was absent from non-oleaginous representatives of these species.

I examined 23 yeasts both oleaginous and non-oleaginous, cultivated in 1*l* vortex-aerated batch cultures (Methods, Section 1.4.1)

for 48 h on both carbon- and nitrogen-limiting media, for the occurrence of ATP:citrate lyase as compared with their lipid content (Table 13).

The enzyme was consistently present in those yeasts capable of accumulating lipid to 20% (w/w), or greater, of their biomasses. Maximum lipid accumulation occurred when nitrogen-limiting medium was used. When carbon-limiting medium was used the lipid content of the yeasts was usually substantially lower but there was no decrease in ATP:citrate lyase activity. Thus, the possession of ATP:citrate lyase indicated that any particular yeast was potentially oleaginous but was not necessarily correlated with the prevailing lipid content.

In those cases where ATP:citrate lyase activity could not be detected assays were repeated at pH 6.5 using 0.1 M Na_2HPO_4/KH_2PO_4 buffer and at pH 7.5 using 0.1 M Tris/HCl buffer, however, the original observations were not reversed. Dialysis of crude cell-free extracts of *Saccharomyces cerevisiae* and *Rhodosporidium toruloides* CBS 6016 against 0.05 M Tris/HCl, pH 7.5 containing 1 mM $MgCl_2$ and 10 mM dithiothreitol also failed to elicit activity, as did fractionation of the extracts by ammonium sulphate precipitation. When cell-free extracts of ATP:citrate lyase-negative yeasts were mixed with an equal volume of an active preparation derived from *Lipomyces starkeyi* CBS 1809 no reduction in activity was observed, thus, indicating that the activity of the enzyme was not being masked by an inhibitor. Preparation of cell-free extracts from *Saccharomyces cerevisiae*, *S. carlsbergensis* and *Rhodosporidium toruloides* CBS 6016 in buffers containing 1 mM ATP and 20 mM tripotassium citrate, which stabilized the enzyme from *Lipomyces starkeyi* CBS 1809 (see below), also failed to produce any detectable activity.

6.2 The response of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 to changes in nutrient limitation and growth rate

It has been known for some time that the activity of rat hepatic

TABLE 13: Specific activity of ATP:citrate lyase in crude cell-free extracts of the yeasts indicated compared with their lipid content. Enzyme assays were performed at pH 8.4 according to Srere (1959).

Yeast		Carbon-limiting medium		Nitrogen-limiting medium	
		Specific activity [nmoles min^{-1} (mg protein)$^{-1}$]	Lipid content (% w/w)	Specific activity [nmoles min^{-1} (mg protein)$^{-1}$]	Lipid content (% w/w)
Candida curvata D		7.1	29.0	7.2	33.7
Candida curvata R		4.8	21.5	5.2	33.9
Candida tropicalis		0	2.3	0	4.0
Candida utilis		0	1.7	0	3.6
Candida 107		6.1	18.9	7.8	25.1
Cryptococcus albidus	NCYC 602	ND	ND	0	2.0
Hansenula ciferii	CBS 111	0	2.6	0	7.2
Hansenula saturnus	CBS 5761	ND	ND	11.0	24.5
Lipomyces lipofer	NCYC 944	ND	ND	50.0	36.2
Lipomyces lipofer	CBS 5842	34.2	15.4	38.0	27.2
Lipomyces starkeyi	CBS 1809	37.2	21.2	54.0	36.5
Lipomyces starkeyi	CBS 1807	ND	ND	19.0	29.8

Lipomyces starkeyi	CBS 6132	0	2.8	0	6.4
Lipomyces starkeyi	CBS 6047	37.0	38.5	51.0	42.5
Rhodosporidium toruloides	CBS 5490	0	2.9	0	8.0
Rhodosporidium toruloides	CBS 5490	48.6	3.9	41.8	25.9
Rhodotorula glutinis	NCYC 59	9.0	15.8	12.0	23.7
Rhodotorula graminis	NCYC 502	ND	ND	41.2	24.2
Saccharomyces carlsbergensis	NCYC 502	0	1.9	0	6.8
Saccharomyces cerevisiae	NCYC 33	0	3.1	0	6.0
Saccharomyces uvarum	NCYC 74	ND	ND	0	8.4
Trichosporon cutaneum	40	15.2	9.1	13.8	24.4

ND - not determined

ATP:citrate lyase is dependent on the nutritional and hormonal state of the animal. Thus, starvation of rats for several days results in a decrease in ATP:citrate lyase activity, whereas, refeeding fasted animals on a high carbohydrate diet causes a dramatic increase in enzyme activity (Kornacker and Lowenstein, 1963, 1964, 1965a, 1965b). Recently, Yen and Mack (1980) have reported that the increase in activity is an induction process such that additional copies of the ATP:citrate lyase protein are synthesized during conditions of active lipogenesis.

Whitworth and Ratledge (1975a) determined the activities of a number of enzymes of potential importance in lipogenesis in cultures of *Candida* 107 cultivated on both carbon- and nitrogen-limited media. In the case of citrate synthase, NAD^+-dependent isocitrate dehydrogenase, malate dehydrogenase and malic enzyme no differences were detected. However, their data on ATP:citrate lyase was incomplete. Since preliminary evidence had been obtained here that the activity of ATP:citrate lyase in oleaginous yeasts did not necessarily correlate with the prevailing lipid content (Results, Section 6.1) the response of the enzyme's activity from *Lipomyces starkeyi* CBS 1809 to changes in nutrient limitation and growth rate was investigated.

When the yeast was cultivated under conditions of nitrogen-limitation at various dilution rates there was a positive correlation between the activity of ATP:citrate lyase and the specific rate of lipid synthesis (g lipid synthesized per g fat-free yeast per h) but neither of these parameters correlated with the actual lipid content (Figure 30A). When the yeast was cultivated under carbon-limiting conditions, however, the lipid content was low but there was no reduction in ATP:citrate lyase activity (Figure 30B). This result was subsequently confirmed when ATP:citrate lyase activity from *Lipomyces starkeyi* CBS 1809 was monitored during a transition from carbon- to nitrogen-limited growth. Once again

FIGURE 30

The effect of dilution rate on lipid content and specific activity of ATP:citrate lyase in *Lipomyces starkeyi* CBS 1809 cultivated in a chemostat under nitrogen-limiting conditions (A) and under carbon-limiting conditions (B). Specific activity of ATP:citrate lyase, ○; lipid content, △; specific rate of lipid biosynthesis, ▲; biomass, ●.

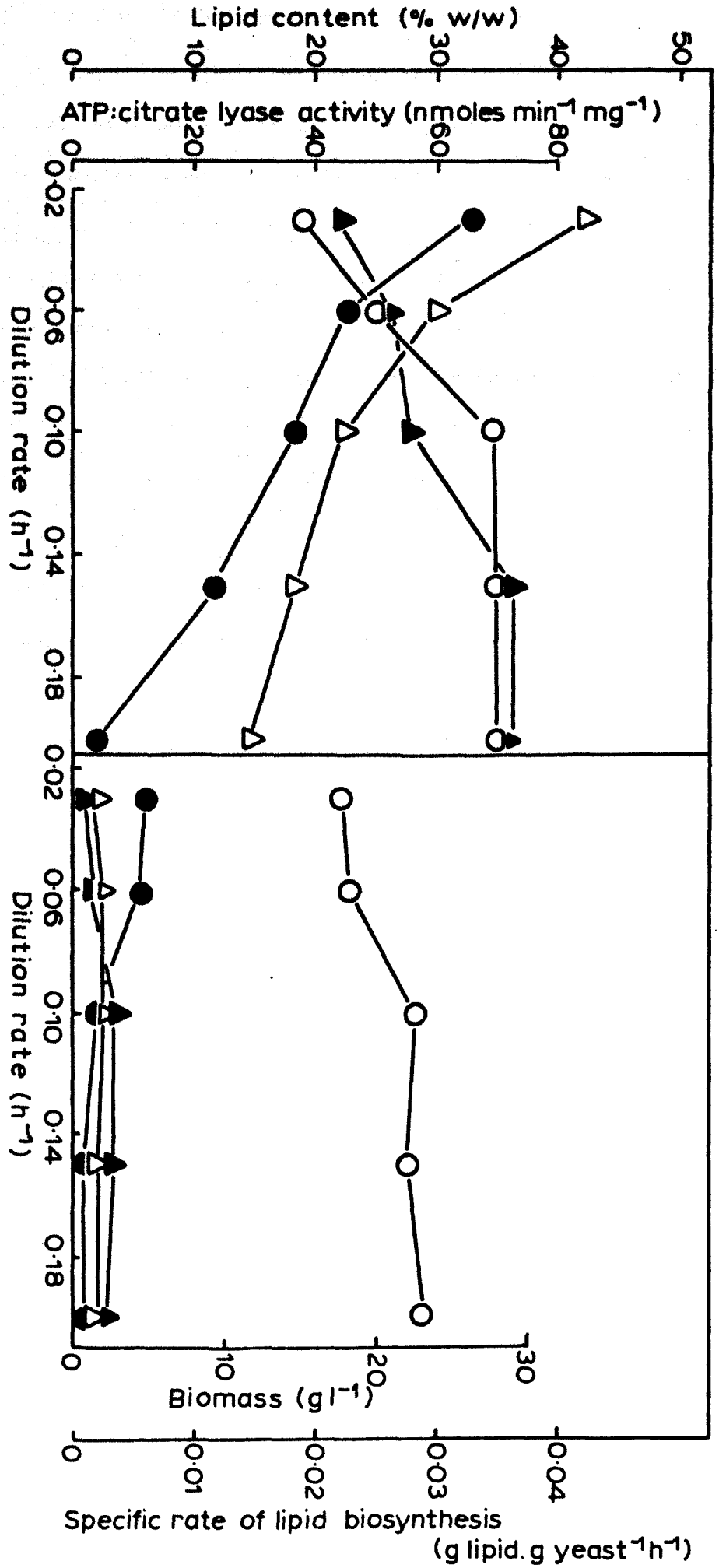

Lipid content (% w/w)
0
10
20
30
40
50
ATP:citrate lyase activity (nmoles min⁻¹ mg⁻¹)
0
20
40
60
80
Dilution rate (h⁻¹)
0·02
0·06
0·10
0·14
0·18
Dilution rate (h⁻¹)
0·02
0·06
0·10
0·14
0·18
0
10
20
30
Biomass (g l⁻¹)
0
0·01
0·02
0·03
0·04
Specific rate of lipid biosynthesis
(g lipid. g yeast⁻¹h⁻¹)

Fig. 30

no alteration in activity was observed (Figure 31).

Thus, it appeared that ATP:citrate lyase from oleaginous yeasts differs from that in mammalian tissues in that it is not subject to control by induction or repression. This, of course, does not preclude the possibility that the activity of the enzyme may not be subject to control by effector molecules and or the availability of substrates. It would not be expected that this type of control would be detected in an experiment of this type as the preparation of extracts results in a massive dilution and disruption of cell contents such that observed enzyme activities probably bear little resemblance to their actual *in vivo* reaction velocities.

6.3 Attempts to stabilize ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

In order to evaluate the role of ATP:citrate lyase in lipogenesis attempts were made to purify the enzyme from *Lipomyces starkeyi* CBS 1809. These attempts were considerably hindered by the apparent instability of the enzyme such that even the simplest fractionation procedures resulted in unacceptable losses of units. Extraction of the enzyme in the presence of Mg^{2+} and 2-mercaptoethanol which Inoue *et al.* (1966) reported to stabilize ATP:citrate lyase from rat liver did not alleviate the problem. The addition of ATP and tripotassium citrate to extraction buffers at final concentrations of 1 m$\underline{\underline{M}}$ and 20 m$\underline{\underline{M}}$, respectively, did exert a stabilizing effect on the ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809. Tripotassium citrate alone was more effective than ATP but the maximum effect was observed when the two substrates were used in combination (Table 14). The potential stabilizing effect of coenzyme A was not tested for reasons of economy. However, ATP and tripotassium citrate were included, at the concentrations indicated, in buffers used for the purification and subsequent storage of ATP: citrate lyase from *Lipomyces starkeyi* CBS 1809.

FIGURE 31

Fluctuation in the specific activity of ATP:citrate lyase (●) and lipid content (○) in *Lipomyces starkeyi* CBS 1809 during a transition from carbon to nitrogen-limitation. The growth of the yeast (△) and the rate of NH^+ utilization from the medium (■) are also shown.

Fig. 31

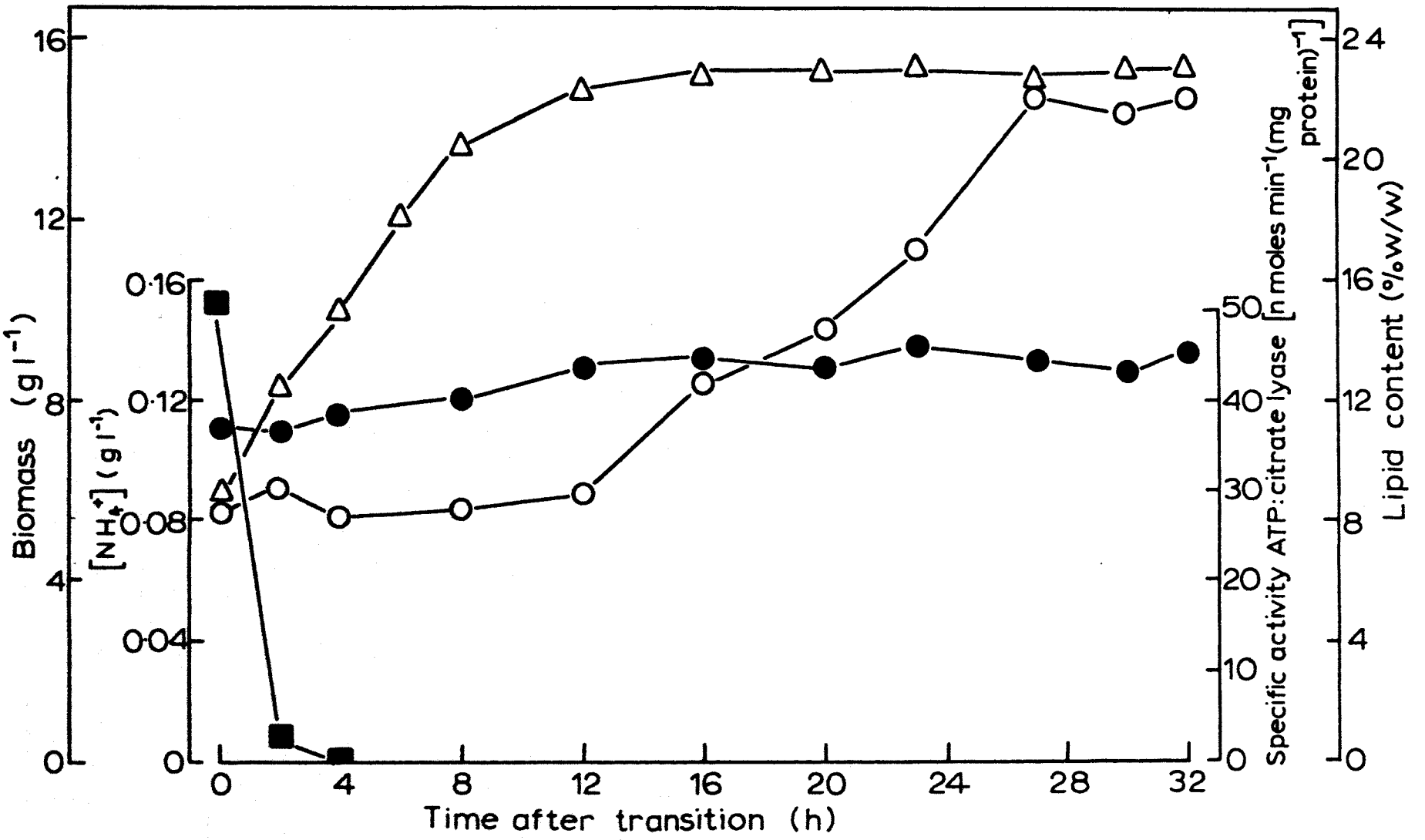

Biomass (g l⁻¹)
[NH₄⁺] (g l⁻¹)
Specific activity ATP:citrate lyase [n moles min⁻¹(mg protein)⁻¹]
Lipid content (%w/w)
Time after transition (h)
0
4
8
12
16
20
24
28
32
0·04
0·08
0·12
0·16
10
30
40
50

TABLE 14: Activity of ATP:citrate lyase in crude cell-free extracts from *Lipomyces starkeyi* CBS 1809 stored at 4 °C in the presence and absence of tripotassium citrate and ATP. In addition extracts contained 50 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$ and 10 mM 2-mercapto-ethanol. Activities expressed relative to that obtained at zero time in the absence of substrates [61 nmoles min^{-1} (mg protein)$^{-1}$].

Additives	Relative activity (%) Storage time (h)	
	24	48
None	45.2	17.2
1 mM ATP	62.4	48.0
20 mM tripotassium citrate	74.2	69.7
1 mM ATP + 20 mM tripotassium citrate	96.8	91.2

6.4 Partial purification of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

ATP:citrate lyase was partially purified from *Lipomyces starkeyi* CBS 1809 cultivated in a chemostat under nitrogen-limiting conditions using standard procedures (Methods, Section 7.2). The preparation (Table 15) was free from contaminating activities of citrate synthase, adenylate kinase, acetyl-CoA deacylase and acetyl-CoA synthetase, although it did contain malate dehydrogenase [specific activity 0.5 μmol min^{-1}(mg protein)$^{-1}$]. As this latter enzyme was a constituent of the assay system it was not considered to be a contaminant of serious consequence, therefore, this preparation was used in subsequent experiments.

6.5 Variation in activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 with changes in pH

Maximum activity was observed between pH 8.2 and pH 8.8 (Figure 32) and pH 8.4 was judged suitable for subsequent assays.

6.6 The effect of divalent metal ions on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

The activity of the enzyme was absolutely dependent on the presence of Mg^{2+}. Mn^{2+} and Co^{2+} could partially substitute giving rates of 22.6% and 37.1%, respectively, of that observed in the presence of Mg^{2+}, when tested in the standard assay at a final concentration of 10 m$\underline{\underline{M}}$.

6.7 Variation in initial velocity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 with substrate concentration

The usual Michaelis-Menten relationship was valid when initial velocity was measured as a function of ATP and citrate concentration (Figures 33 and 34). Apparent K_m values for ATP and citrate were calculated to be 135 μ$\underline{\underline{M}}$ and 70 μ$\underline{\underline{M}}$, respectively. It was not possible to calculate the apparent K_m for CoA because non-saturating concentrations of this substrate were so low that rates rapidly became non-linear, thus, making initial velocity measurements unreliable.

TABLE 15: Partial-purification of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

Fraction	Volume (ml)	Total units (μmol min^{-1})	Total protein (mg)	Specific activity [μmol min^{-1} (mg protein)$^{-1}$]	Purification factor	Yield (%)
Crude	27.5	11.0	236.5	0.047	1	100
0-40% $(NH_4)_2SO_4$	4.8	9.0	68.2	0.132	2.8	90.0
Pooled Sepharose 4B eluate	60	8.4	29.4	0.286	6.1	76.4
Pooled DEAE-Sephadex eluate	48.2	6.3	6.8	0.922	19.6	57.3

FIGURE 32

Variation in activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 with changes in pH. The standard assay mixture of Srere (1959) was used with Tris/HCl at a final concentration of 250 mM and reactions were initiated by the addition of coenzyme A.

Fig. 32

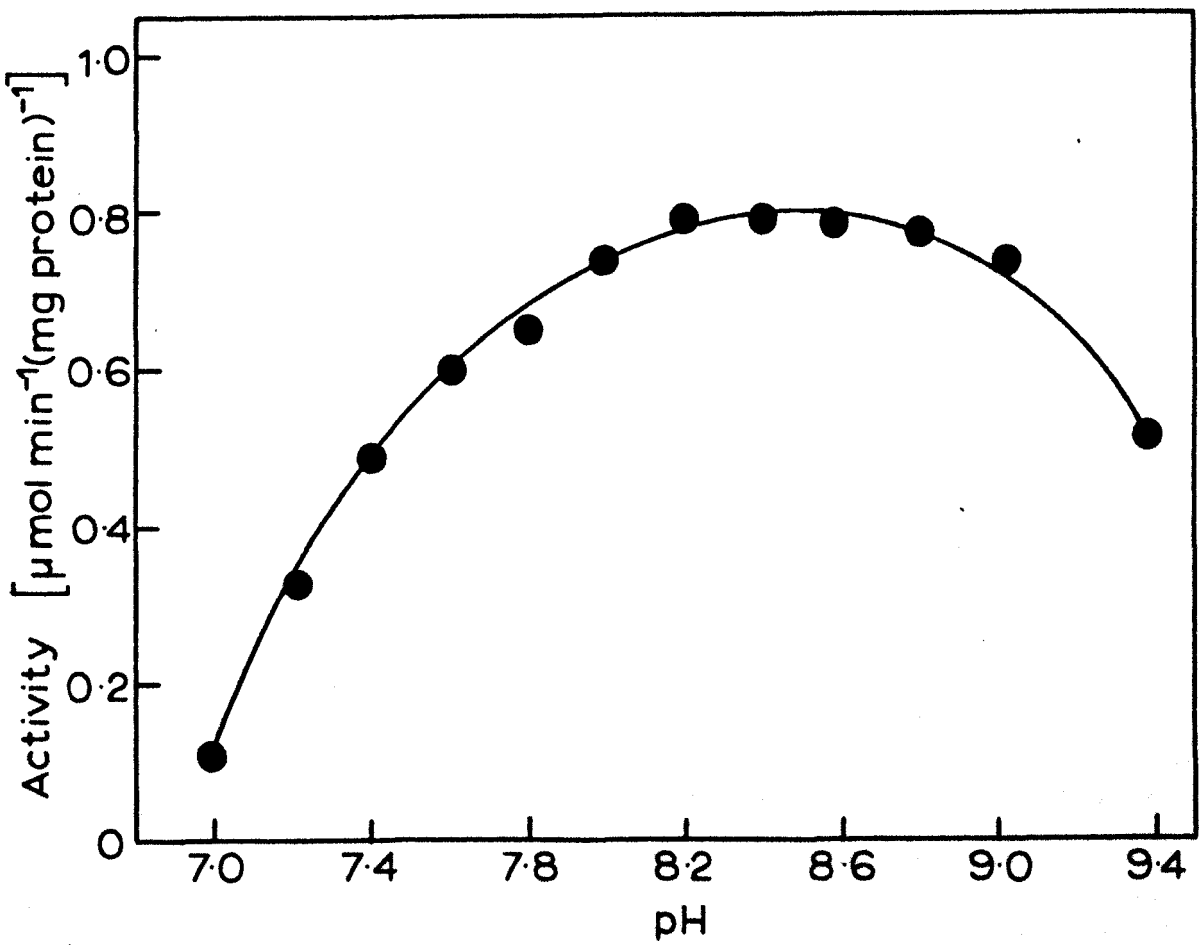

Activity [μ mol min⁻¹(mg protein)⁻¹]
1·0
0·8
0·6
0·4
0·2
0
7·0
7·4
7·8
8·2
8·6
9·0
9·4
pH

FIGURE 33

Lineweaver-Burk plot showing variations in initial velocity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 with variation in ATP concentration. Citrate and coenzyme A concentrations were 20 m$\underline{\underline{M}}$ and 0.2 m$\underline{\underline{M}}$, respectively.

Fig. 33

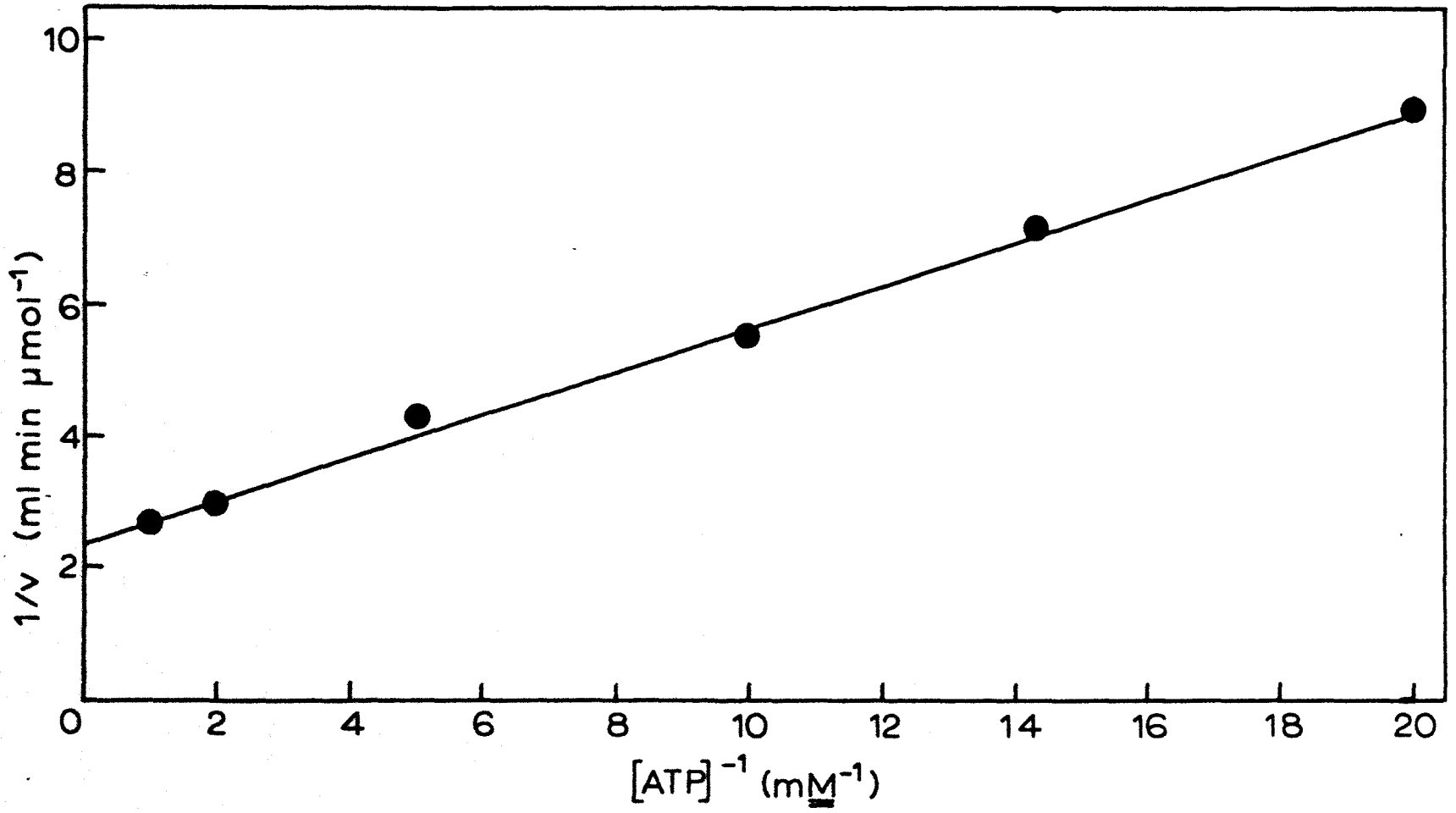
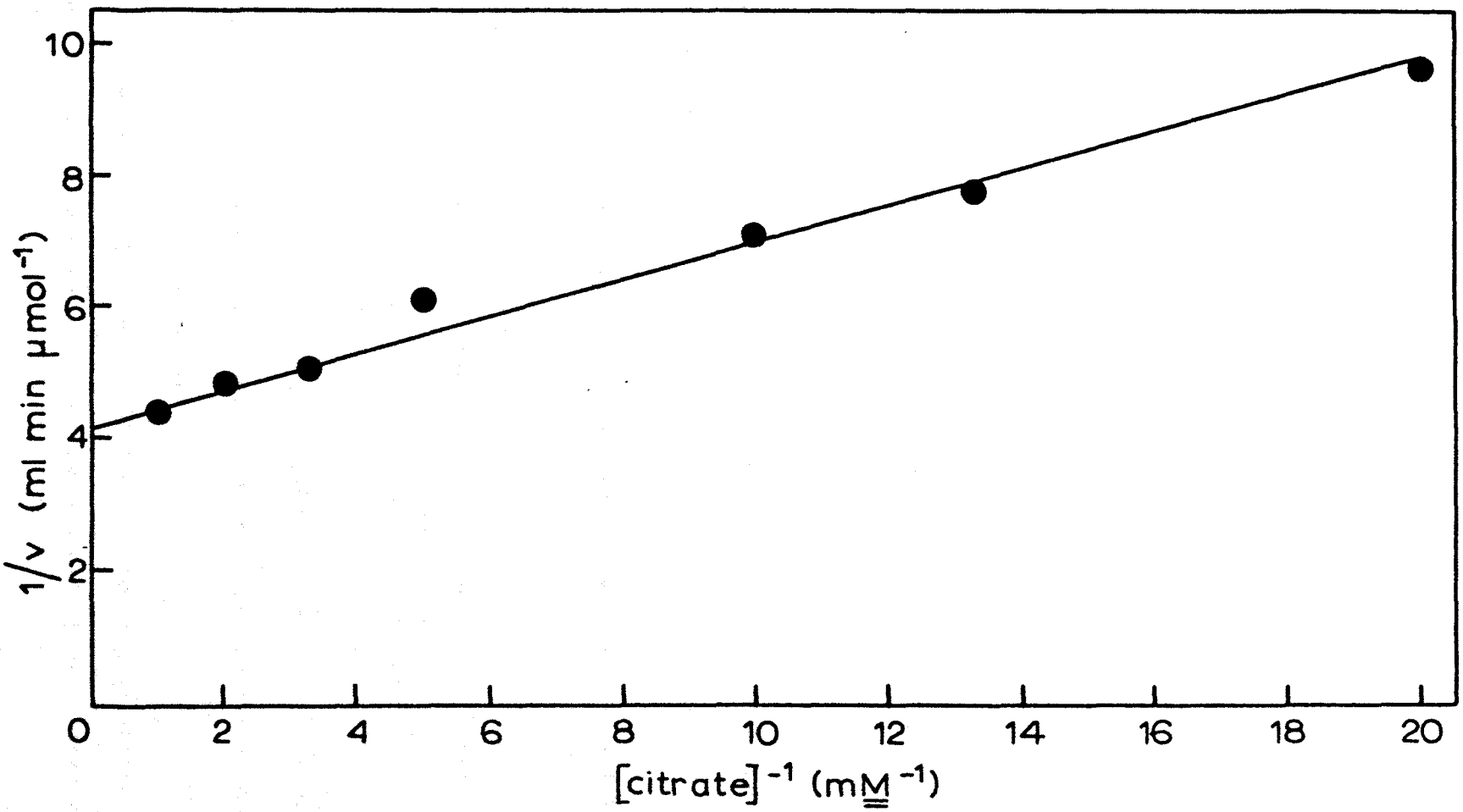


FIGURE 34

Lineweaver-Burk plot showing variations in initial velocity of ATP: citrate lyase from *Lipomyces starkeyi* CBS 1809 with variation in citrate concentration. ATP and coenzyme A concentrations were 1 m$\underline{\underline{M}}$ and 0.2 m$\underline{\underline{M}}$, respectively.

Fig. 34


10
8
6
4
2
1/v (ml min µmol^{-1})
0
2
4
6
8
10
12
14
16
18
20
[citrate]$^{-1}$ (m$\underline{\underline{M}}^{-1}$)

6.8 The effect of nucleotides on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

Various nucleotides were tested in the standard assay mixture at a final concentration of 1 mM (Table 16). Srere (1975) reported that ITP, GTP and UTP could partially substitute for ATP as a substrate for ATP:citrate lyase from rat liver. However, these nucleotides were not substrates for ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809, nor did they have any effect on the activity of the enzyme when ATP was used as substrate.

None of the other nucleotides tested modulated the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 with the exception of ADP, which was inhibitory (Figure 35). In a control experiment, 1 mM ADP was tested against malate dehydrogenase (a constituent of the ATP: citrate lyase assay mixture) using the assay procedure of Englard and Siegal (1969) but no inhibition was observed. Thus, the observed inhibition of ATP:citrate lyase by ADP was a genuine effect and not an artifact of the assay procedure.

6.9 The response of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 to mixtures of adenine nucleotides simulating various energy charge values

Atkinson and Walton (1967) reported that ATP:citrate lyase from rat liver was inhibited by ADP. Since ADP is a product of this reaction and ATP a substrate, they considered that ATP:citrate lyase might be regulated by changes in the prevailing adenylate energy charge. They pointed out that lipid may be regarded as stored ATP. Consequently, under high energy charge conditions ATP is utilized in the production of lipid, via the action of ATP:citrate lyase (and presumably other lipogenic enzymes). This results in an increase in ADP concentration which ultimately reduces the rate of lipogenesis by product-inhibition of ATP:citrate lyase.

ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 responded to

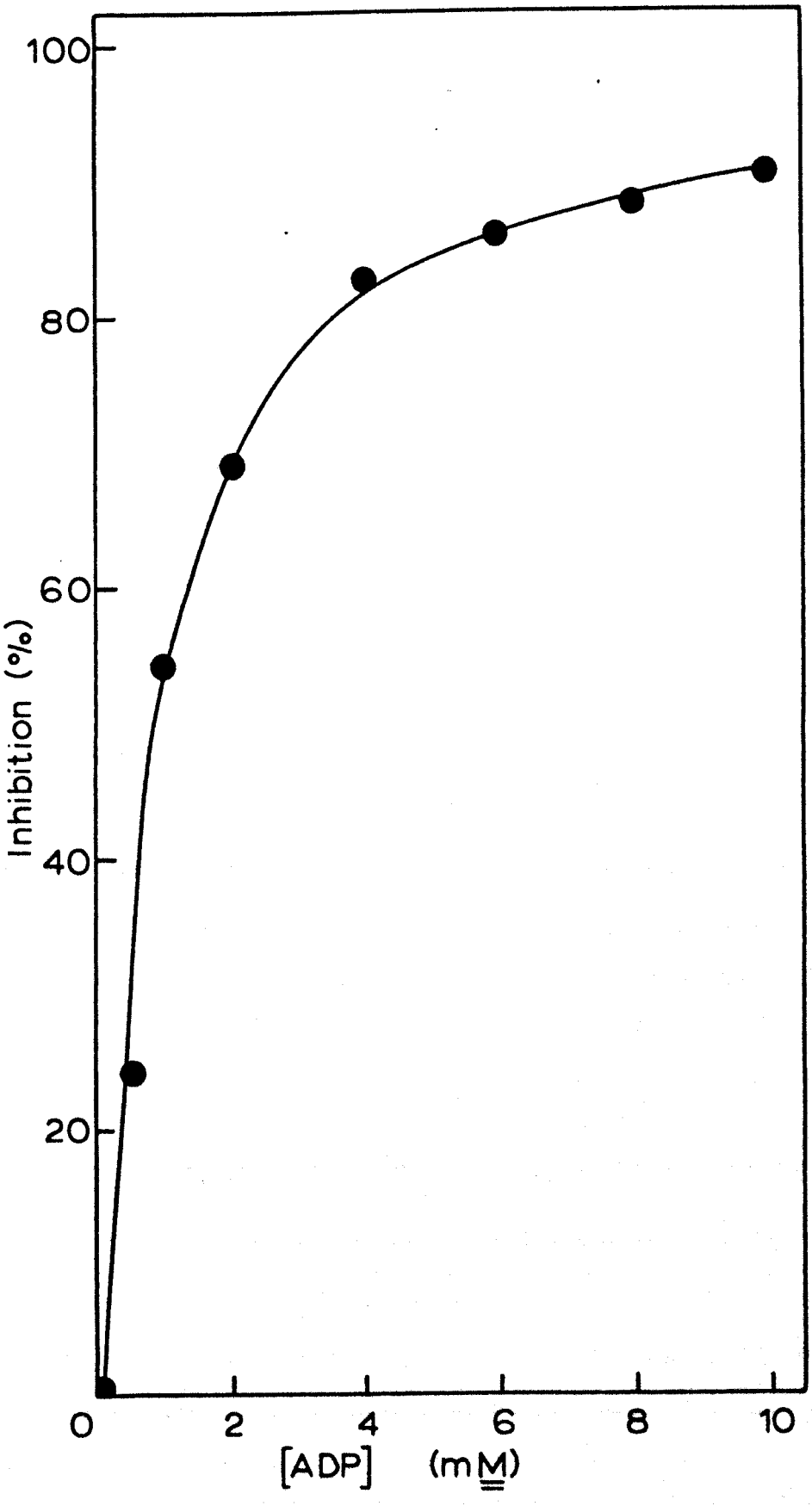
TABLE 16: The effect of nucleotides on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 when tested in the standard assay mixture at a final concentration of 1 mM. All reactions were initiated by the addition of ATP. Activities shown relative to that observed in the absence of added nucleotide, but in the presence of ATP [0.86 μmol min^{-1}(mg protein)$^{-1}$].

Nucleotide	Activity (%)
ATP only	100
ADP	45.3
AMP	90.6
Adenosine 3':5'-cyclic monophosphate	102.5
GMP	108.6
GDP	105.5
GTP	106.8
CMP	102.1
CDP	100.1
CTP	102.4
UMP	103.8
UDP	102.1
UTP	104.3
IMP	103.7
ITP	94.5

FIGURE 35

Inhibition of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 by ADP when tested in the standard assay mixture at the concentrations indicated. Reactions were initiated by the addition of ATP. The uninhibited activity was 0.61 μmol min^{-1}(mg protein)$^{-1}$.

Fig. 35



mixtures of adenine nucleotides simulating various energy charge values as predicted by Atkinson and Walton (1967) [Figure 36]. The *in vivo* significance of this phenomenon, however, was difficult to evaluate. When intracellular adenine nucleotide concentrations of *Lipomyces starkeyi* CBS 1809 were monitored during transitions from carbon- to nitrogen-limitation (weakly lipogenic to strongly lipogenic) a dramatic increase in adenylate energy charge was observed (Results, Section 1.3.3). However, this was almost entirely due to a decrease in AMP concentration, very little variation in the intracellular concentrations of ADP and ATP being observed. Thus, although energy charge did fluctuate as predicted, it was not accompanied by changes in the concentrations of those adenine nucleotides that would theoretically modulate the activity of ATP:citrate lyase.

On the other hand the determination of intracellular adenine nucleotide concentrations took no account of the undoubted intracellular compartmentalization of these metabolites. Thus, the possibility that variations in the cytosolic pool-size of ATP and ADP might be sufficient to regulate the activity of ATP:citrate lyase cannot be disregarded. The role of divalent metal ions in interactions between ATP:citrate lyase and adenine nucleotides cannot be questioned as this enzyme had an absolute requirement for Mg^{2+} (Results, Section 6.6) consequently, saturating concentrations of this ion were included in all assays.

6.10 The effect of other metabolites on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

Various metabolites were tested in the standard assay to see if any were capable of modulating the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 (Table 17). No effects were observed with the exception of glucose-6-phosphate, which was mildly inhibitory. In a control experiment the same metabolites were tested against malate dehydrogenase, a constituent of the ATP:citrate lyase assay. No effects

FIGURE 36

The response of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 to mixtures of adenine nucleotides simulating various energy charge values when tested in the standard assay mixture. Reactions were initiated by the addition of coenzyme A. Total adenine nucleotide concentrations were: 0.1 mM, ○; 1 mM, ● and 10 mM, ▲.

Fig. 36

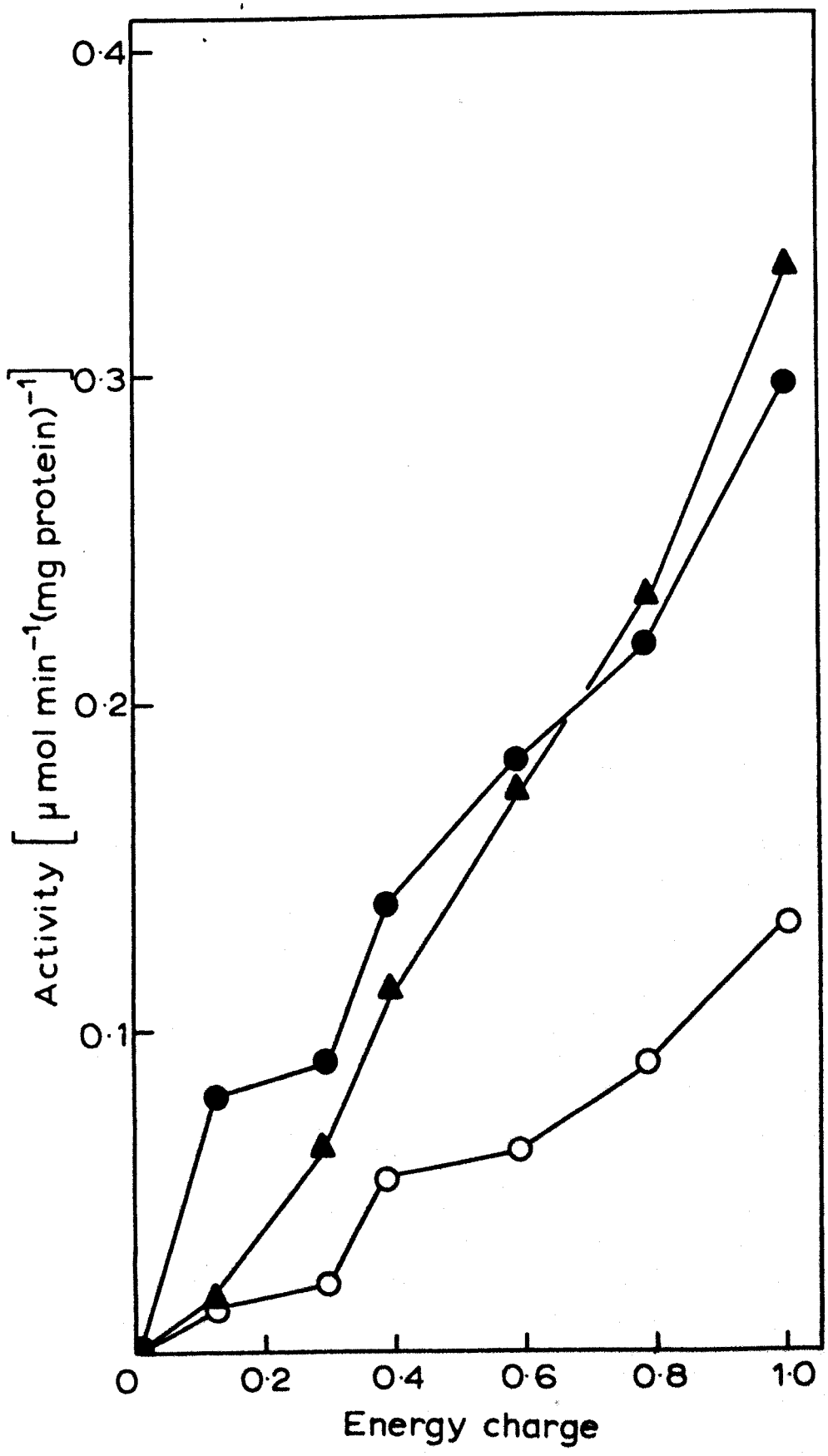

0·4
0·3
0·2
0·1
Activity [μ mol min⁻¹(mg protein)⁻¹]
0
0·2
0·4
0·6
0·8
1·0
Energy charge

TABLE 17: The effect of various metabolites on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 when tested in the standard assay at the concentrations indicated. Activities expressed relative to that observed in the absence of added metabolite [0.59 μmol min^{-1} mg $protein)^{-1}$].

Metabolite	Test concentration (mM)	Activity (%)
Phosphocreatine	1	109.3
Pyruvate	1	102.1
Glucose 6-phosphate	1	88.2
Glucose 6-phosphate	5	69.3
Fructose 1,6-bisphosphate	1	100
6-phosphogluconate	1	100.1
L-malate	3	107.1
Acetyl DL-carnitine	1	105.3
L-glutamine	1	101.8
Acetyl phosphate	1	101.7
DL-isocitrate	2	94.1
Diphosphoglycerate	1	103.5
Phospho(enol)pyruvate	1	108.7

were observed when the metabolites were tested at the concentrations indicated (Table 17).

6.11 The effect of fluorocitrate and fluoroacetate on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

Fluoroacetyl-CoA is a substrate for citrate synthase and the product, fluorocitrate inhibits aconitase. Consequently, both fluorocitrate and fluoroacetate inhibit the TCA cycle.

Neither of these compounds had any effect on the activity of ATP: citrate lyase from *Lipomyces starkeyi* CBS 1809 when tested in the standard assay at a final concentration of 1 mM.

6.12 The effect of fatty acyl-CoA esters on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

In common with citrate synthase from *Candida* 107 (Results, Section 4.9) fatty acyl-CoA esters were found to be potent inhibitors of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 (Table 18). No effects were observed when fatty acyl-CoA esters were tested, at a final concentration of 100 μM, against malate dehydrogenase, a constituent of the ATP:citrate lyase assay mixture.

6.12.1 Time-dependency of oleoyl-CoA inhibition of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

The inhibition of citrate synthase from *Candida* 107 was time-dependent (Results, Section 4.9.1). A similar effect was noted when oleoyl-CoA was incubated with ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 for various periods of time (Figure 37). In this latter case, however, a much longer incubation period was required (15 min as compared with 30 sec) before the maximum inhibition was observed.

6.12.2 The pattern of oleoyl-CoA inhibition of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

The pattern of oleoyl-CoA inhibition of ATP:citrate lyase differed from that observed with citrate synthase in that in the former case the response was not sigmoidal (Figures 20, 21 and 38). Thus, oleoyl-CoA was

TABLE 18; The effect of fatty acyl-CoA esters on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809. The enzyme was preincubated for 5 min at 30 °C with the test compound prior to initiating the reaction by the addition of coenzyme A. Activities expressed relative to that observed in the absence of test compound [0.81 μmol min^{-1} $(mg\ protein)^{-1}$].

Compound		Concentration (μM)	Activity (%)
Acetyl-CoA		10	100
Acetyl-CoA		100	100
Malonyl-CoA		10	101
Malonyl-CoA		100	99.8
Lauroyl-CoA	($C_{12:0}$)	10	51.8
Myristoyl-CoA	($C_{14:0}$)	10	4.8
Palmitoyl-CoA	($C_{16:0}$)	10	5.4
Stearoyl-CoA	($C_{18:0}$)	10	44.9
Oleoyl-CoA	($C_{18:1}$)	10	20.5

FIGURE 37

Time-dependency of the inhibition of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 by oleoyl-CoA. Enzyme [specific activity: 0.81 μmol min^{-1}(mg protein)$^{-1}$] was incubated with 10 μM oleoyl-CoA at 30 °C, for the time indicated, in the standard assay mixture prior to initiating reactions by the addition of coenzyme A.

Fig.37

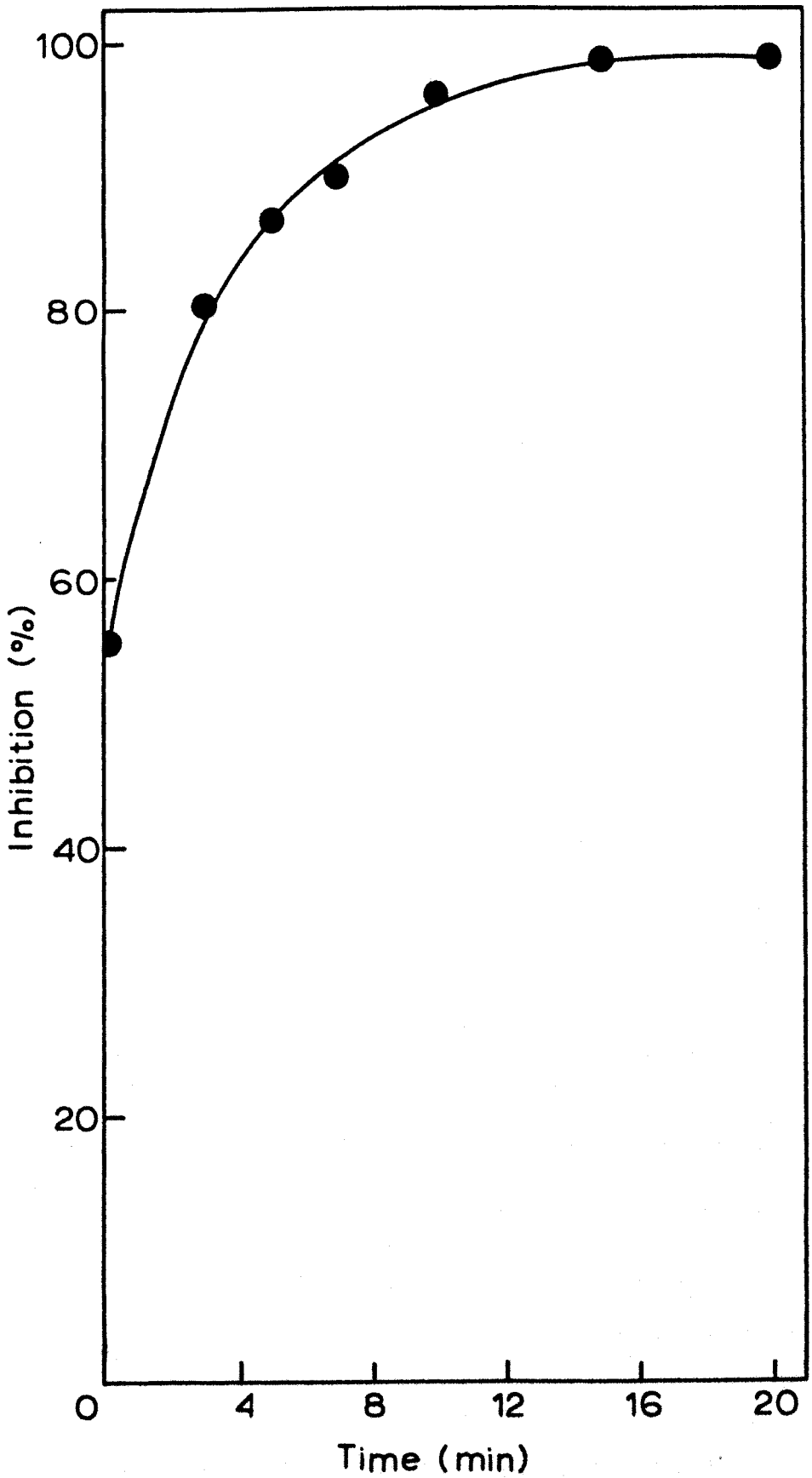
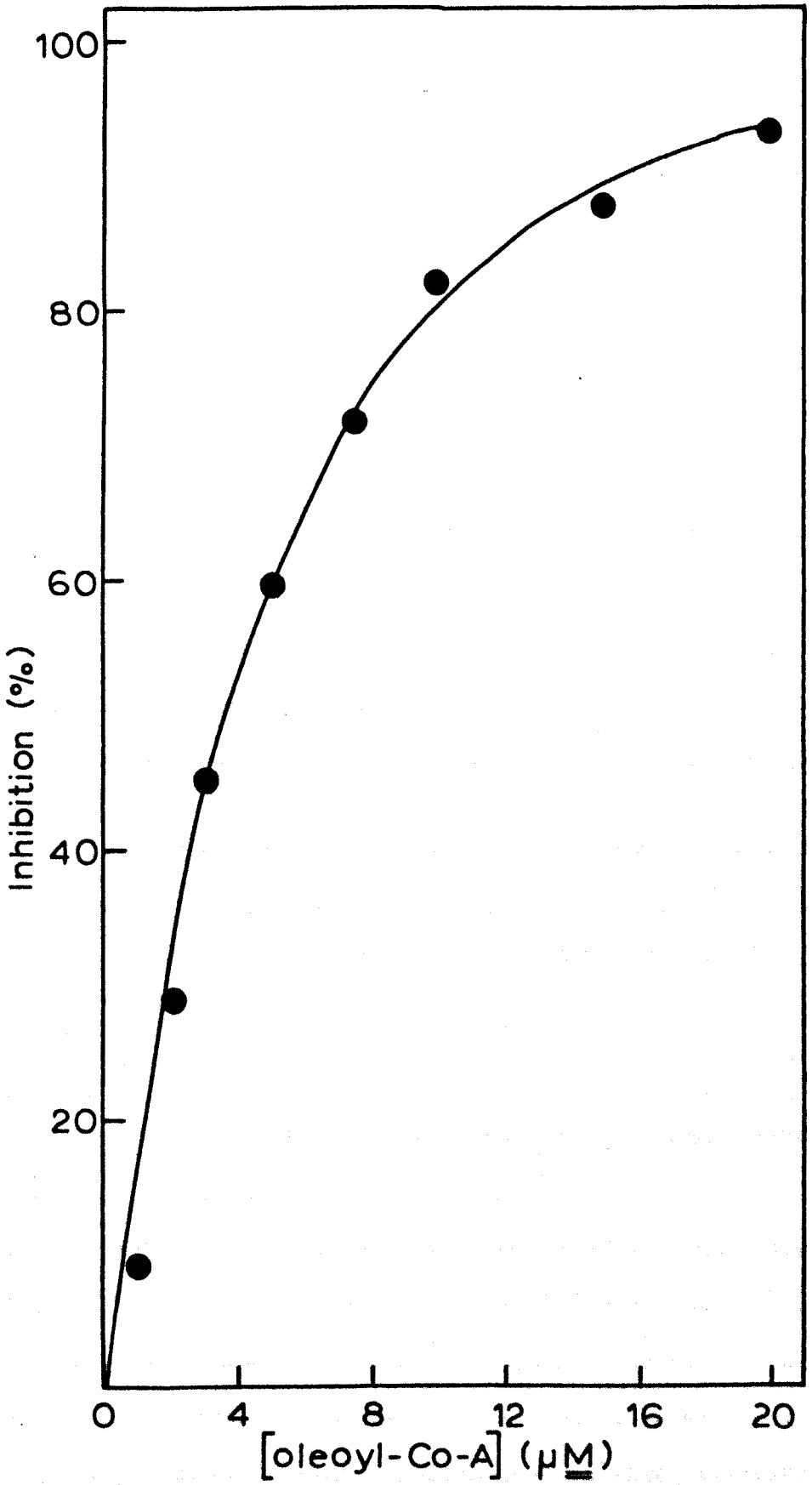


FIGURE 38

Inhibition of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 by oleoyl-CoA. Enzyme [specific activity: 0.81 μmol min^{-1} (mg protein)$^{-1}$] was incubated for 5 min at 30 °C with oleoyl-CoA, at the concentration indicated in the standard assay mixture, prior to initiating reactions by the addition of coenzyme A.

Fig 38



inhibitory to ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 at concentrations below the critical micellar concentration of 4.7 μM (Hsu and Powell, 1975). A similar pattern of inhibition was observed when reactions were initiated by the addition of either ATP or citrate (results not shown).

6.12.3 The effect of protein concentration on the inhibition of ATP: citrate lyase from *Lipomyces starkeyi* CBS 1809 by oleoyl-CoA

When ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 was assayed in the simultaneous presence of oleoyl-CoA and bovine serum albumin, no inhibition was observed (Table 19 - 2). Therefore, in common with oleoyl-CoA inhibition of citrate synthase from *Candida* 107 (Results, Section 4.9.4) the inhibition of ATP:citrate lyase by oleoyl-CoA was apparently dependent on protein concentration. However, contrary to the previous observations made with citrate synthase (Table 10), when bovine serum albumin was added to inhibited ATP:citrate lyase the inhibition was reversed (Table 18.3).

6.12.4 Determination of the molecular weight of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

Srere (1975) reported that rat liver ATP:citrate lyase had a molecular weight of 520,000 and was composed of 4 identical subunits. The molecular weight of ATP:citrate lyase from *Lypomyces starkeyi* CBS 1809 was determined by passing a sample of enzyme down a column of Sephacryl-S300 (Pharmacia, Uppsala, Sweden) and comparing the elution volume with the elution volumes of proteins of known molecular weight (Methods, Section 7.3). The elution profile of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 indicated a molecular weight of 501,000 (Figure 39).

Since a homogeneous preparation of ATP:citrate lyase was not available it was not possible to investigate whether or not the enzyme was composed of subunits. However, the molecular weight of the yeast enzyme was very similar to that reported for rat liver ATP:citrate lyase.

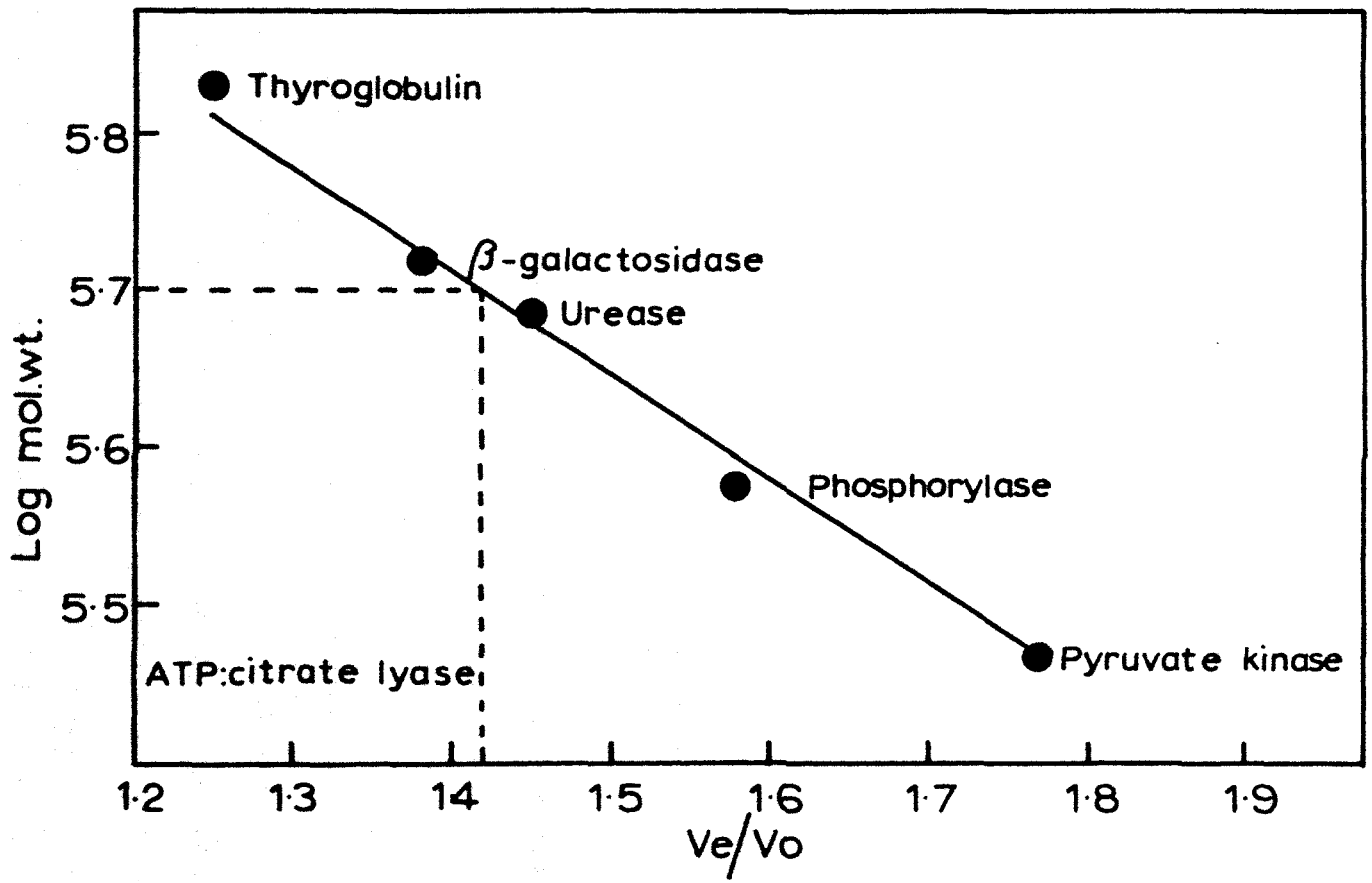
TABLE 19: The effect of bovine serum albumin on the inhibition of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 by oleoyl-CoA. Initial rates were measured using the standard assay mixture under the conditions described. Activities expressed relative to that observed in the absence of oleoyl-CoA [0.81 μmol min^{-1}(mg protein)$^{-1}$].

	Conditions	Relative activity (%)
1.	Enzyme preincubated for 5 min at 30 °C with 10 μM oleoyl-CoA prior to initiating the reaction by the addition of coenzyme A.	19.7
2.	Enzyme preincubated for 5 min at 30 °C with 10 μM oleoyl-CoA and 1 mg BSA prior to initiating the reaction by the addition of coenzyme A.	101.6
3.	As 1.	18.0
	1 mg BSA added to inhibited enzyme.	95.1

FIGURE 39

Determination of the molecular weight of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 by column chromatography on Sephacryl-S300. The column was calibrated, using the marker proteins indicated, as described in Methods (Section 7.3). Purified ATP: citrate lyase [2 ml, specific activity: 0.71 μmol min^{-1}(mg protein)$^{-1}$] was loaded onto the calibrated column, which had been previously equilibrated in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM ATP and 20 mM tripotassium citrate, and eluted in similar buffer. Elution volume of peak activity of ATP: citrate lyase and marker enzymes = Ve and void volume of column = Vo.

Fig. 39


Thyroglobulin
β-galactosidase
Urease
Phosphorylase
Pyruvate kinase
ATP:citrate lyase
Log mol.wt.
5·8
5·7
5·6
5·5
1·2
1·3
1·4
1·5
1·6
1·7
1·8
1·9
Ve/Vo

In both cases the molecular weights were high and the subunit structure of the mammalian enzyme has been well-documented (Srere, 1975) consequently, it was considered likely that the yeast enzyme also consisted of subunits.

6.12.5 Gel permeation column chromatography of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 in the presence and absence of oleoyl-CoA

If the inhibition of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 by oleoyl-CoA was due to non-specific detergent effects then it would be more than likely that it would result in the dissociation of the enzyme into inactive subunits. Furthermore, the reversal of the inhibition by the addition of bovine serum albumin (Results, Section 6.12.3) probably occurred because the oleoyl-CoA was bound in a non-specific manner by the inert protein, thereby allowing the subunits to reaggregate into active native enzyme.

To test this possibility, ATP:citrate lyase was subjected to gel permeation column chromatography, on Sephacryl-S300, in the presence and absence of oleoyl-CoA (Figure 40). The results showed that the elution profile of uninhibited ATP:citrate lyase was identical to that observed when the enzyme was developed in the presence of oleoyl-CoA. This indicated that the inhibition and its reversal by the addition of bovine serum albumin were not associated with disaggregation and reaggregation of the ATP:citrate lyase molecule, because if this had been the case the prediction would have been that the inhibited enzyme would have behaved as though it had an apparently smaller molecular weight.

6.13 The effect of lipid intermediates and detergents on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809

Various lipid intermediates, other than fatty acyl-CoA esters, and general detergents did not inhibit ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 when tested at a final concentration of 100 μM in the standard assay (Table 20).

FIGURE 40

Gel permeation column chromatography of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 in the presence (B) and absence (A) of oleoyl-CoA. Samples of a crude cell-free extract (2 ml) were developed on Sephacryl-S300 (2.5 x 30 cm columns) equilibrated in 50 mM Tris/HCl buffer, pH 7.5, containing 1 mM $MgCl_2$, 1 mM ATP, 20 mM tripotassium citrate and 10 mM 2-mercaptoethanol. In B the buffer was supplemented with 5 μM oleoyl-CoA. Prior to loading extract onto column B the sample was equilibrated with 200 μM oleoyl-CoA for 30 min at 25 °C. Key: ●, A_{280}; ○, ATP:citrate lyase activity; ■ , ATP:citrate lyase activity, 1 mg BSA added to assay mixture.

Fig. 40

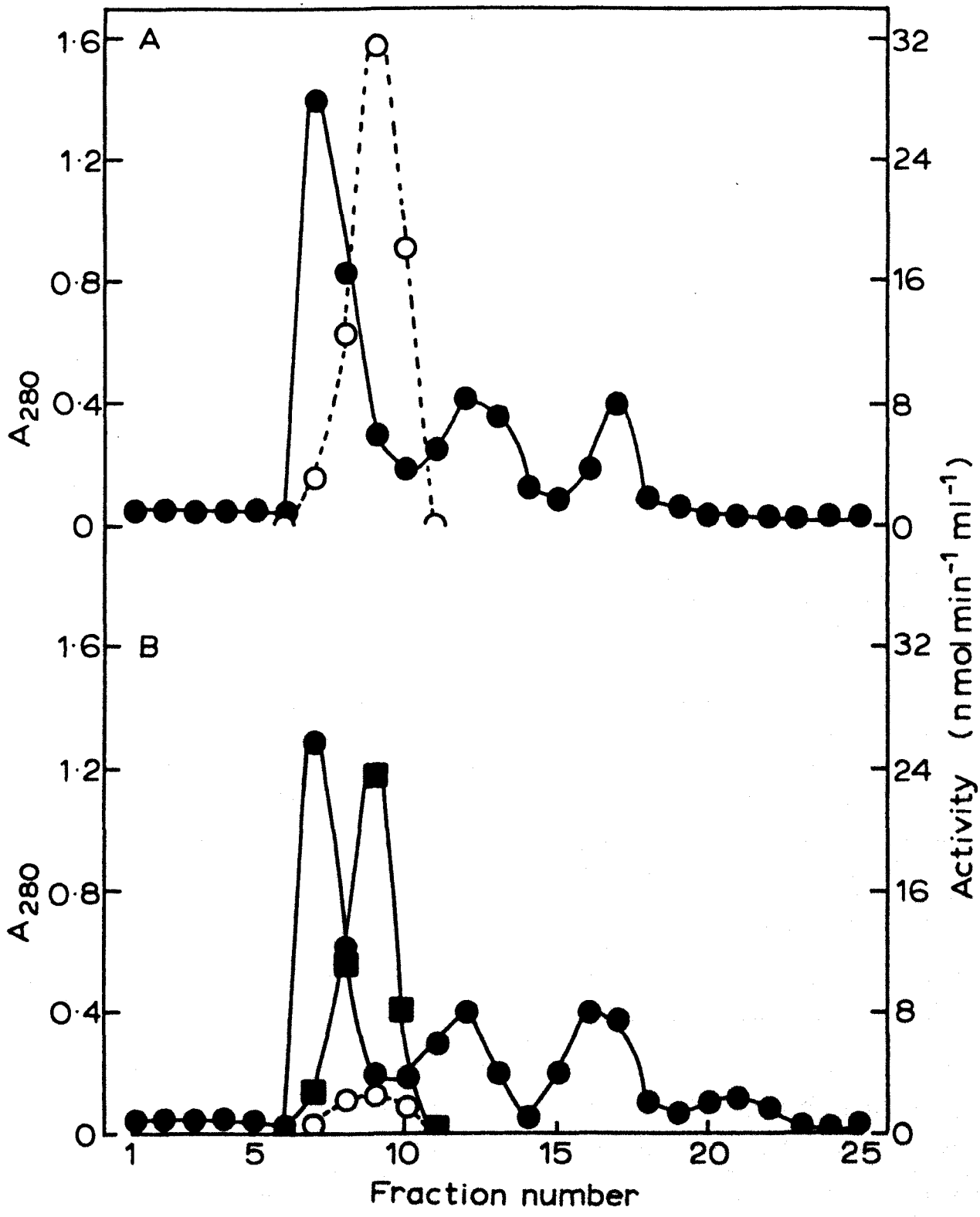

A
B
1·6
1·2
0·8
0·4
0
32
24
16
8
A280
Activity (n mol min-1 ml-1)
1
5
10
15
20
25
Fraction number

TABLE 20: Effect of lipid intermediates and detergents on the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809. Enzyme [specific activity: 0.91 μmol min^{-1} $(mg\ protein)^{-1}$] incubated for 5 min at 30 °C with test compound at a concentration of 100 μM prior to initiating reactions by the addition of coenzyme A.

Compound	Relative activity (%)
Oleic acid methyl ester	97.2
Oleoyl alcohol	100
Oleoyl laurate	89.6
Trioleoyl glycerol	98.6
Oleoyl acetate	92.0
Palmitic acid methyl ester	98.6
Palmitic acid ethyl ester	94.4
Palmitic acid myristyl ester	91.3
Palmityl acetate	100
Palmityl carnitine chloride	89.0
Myristoyl chloride	99.5
Myristic acid palmityl ester	85.3
Sodium dodecyl sulphate	95.8
Triton X-100 (0.1% w/v)	94.4

7. Occurrence of malic enzyme in oleaginous yeasts

Frenkel (1975), in a recent review, reported that malic enzyme is implicated in lipogenesis in many mammalian systems. The evidence for this was that dietary and hormonal manipulation of rates of lipogenesis could be positively correlated with the activity of the enzyme. The presumed role for malic enzyme was that it provided reducing equivalents, in the form of NADPH, for lipogenesis.

Whitworth and Ratledge (1975a) detected malic enzyme in the oleaginous yeast, *Candida* 107, and this lead Botham and Ratledge (1979) to propose a similar function in these organisms. They reasoned that the cytosolic oxaloacetate, produced by ATP:citrate lyase, was reduced to malate (via malate dehydrogenase) which was then converted to pyruvate by malic enzyme with the concomitant evolution of NADPH. The pyruvate could then enter the mitochondrion and be utilized in the production of further acetyl units for fatty acid biosynthesis.

The presence of malic enzyme in *Candida* 107 was confirmed and in common with the observations of Whitworth and Ratledge (1975a) there was no correlation with the prevailing lipid content (Table 21). The enzyme was not detected, however, in two strains of *Lipomyces starkeyi* cultivated under carbon- or nitrogen-limiting conditions. Clearly, therefore, malic enzyme was not essential for lipid accumulation to occur in these organisms. Presumably in those yeasts not possessing malic enzyme the reducing power required for lipogenesis must be provided by some other pathway such as the pentose phosphate cycle.

8.1 Attempts to assay acetyl-CoA carboxylase from *Lipomyces starkeyi* CBS 1809

Acetyl-CoA carboxylase is probably the most widely studied lipogenic enzyme. It is often regarded as catalyzing the first committed step in the biosynthesis of fatty acids and as such it may catalyse the rate-limiting step for the sequence (Volpe and Vagelos, 1976). Attention has focussed on two aspects of its possible regulation. Firstly, activation

TABLE 21: **Specific activities of malic enzyme and lipid content of *Candida* 107 and *Lipomyces starkeyi* CBS 1807 and 1809 cultivated under conditions of carbon- and nitrogen-limitation, in vortex-aerated batch cultures**

	Candida 107		*L. starkeyi* CBS 1807		*L. starkeyi* CBS 1809	
	CLC	NLC	CLC	NLC	CLC	NLC
Activity of malic enzyme [nmoles min^{-1}(mg protein)$^{-1}$]	21.2	17.9	0	0	0	0
Lipid content (% w/w)	12.2	26.2	16.4	32.4	20.9	35.0

by citrate and secondly, feed-back inhibition by long chain fatty acyl-CoA esters (Volpe and Vagelos, 1976).

With the exception of our own laboratory, the study of acetyl-CoA carboxylases from oleaginous microorganisms is a much neglected topic. Furthermore, the early reports from our laboratory have not been entirely confirmed by subsequent workers.

Gill and Ratledge (1973) reported that acetyl-CoA carboxylase from *Candida* 107 was activated by citrate, a concentration of 50 mM being required for maximum stimulation. Fructose 1,6-bisphosphate also stimulated activity, whereas fatty acyl-CoA esters were inhibitory. In this latter case the effects were complex and dependent on the chain-length of the acyl moiety of the ester. For example, lauroyl-CoA ($C_{12:0}$) and myristoyl-CoA ($C_{14:0}$) at concentrations of 200 µM were not inhibitory. Palmitoyl-CoA ($C_{16:0}$) at a concentration of 100 µM was not itself inhibitory but it prevented the activation of the enzyme by citrate. Stearoyl-CoA ($C_{18:0}$) at a concentration of 100 µM was inhibitory irrespective of the presence or absence of citrate.

In a later report, Botham and Ratledge (1979), working with the same yeast confirmed that citrate activated acetyl-CoA carboxylase, however, much smaller concentrations (1-5 mM) were required for maximum stimulation. In contrast to the results of Gill and Ratledge (1973) fructose 1,6-bisphosphate was found to be inhibitory and moreover no effect was observed when 200 µM stearoyl-CoA was included in enzyme assays. These apparent inconsistencies were probably due to the fact that crude preparations were being used and also the lack of a reliable assay procedure.

It was decided, therefore, to study acetyl-CoA carboxylase from *Lipomyces starkeyi* CBS 1809 paying special regard to its possible regulation by fatty acyl-CoA esters and in particular comparing the effects,

if any, of these metabolites with those observations already made with citrate synthase (Results, Section 4.9) and ATP:citrate lyase (Results, Section 6.12).

Of the many assay methods available for acetyl-CoA carboxylase the method of choice was that of Inoue and Lowenstein (1975) in which the incorporation of label from [^{14}C] $NaHCO_3$ into acid-stable malonyl-CoA was measured. This method has the advantage that it is the only one in which the activity of the enzyme is measured directly. In previous studies in this laboratory (Gill and Ratledge, 1973; Botham and Ratledge, 1979) the procedure of Matsuhashi (1969) was used in which incorporation of label from [^{14}C] acetyl-CoA or [1-^{14}C] acetate, into saponifiable lipid was measured. This method has the disadvantage that it relies on the remainder of the lipid-synthesizing enzymes being in excess, consequently, interpretation of inhibitor studies is difficult.

At least 20 separate attempts were made to assay acetyl-CoA carboxylase from crude extracts of *Lipomyces starkeyi* CBS 1809 using the procedure of Inoue and Lowenstein (1975) [Methods, Section 3.6] but no activity was detected. Negative results were obtained irrespective of the condition of the yeast, i.e. the stage in the growth cycle or whether cultivated under carbon- or nitrogen-limited conditions. Preparation of crude cell-free extracts by various methods, for example, ultrasonic disruption, French pressure cell or homogenization with glass beads also failed to elicit activity, as did the addition of glycerol or bovine serum albumin to extraction buffers (Methods, Section 8.1). The manipulation of assay pH, incubation temperature and substrate concentrations also failed to produce demonstrable activity. Botham and Ratledge (1979) reported that the activity of acetyl-CoA carboxylase from *Candida* 107 apparently increased if prior to preparing ultrasonic extracts the cells were first lyzed with toluene. This procedure was

followed using *Lipomyces starkeyi* CBS 1809 (Methods, Section 8.2) but no activity was observed.

It was concluded, therefore, that for unknown reasons it was not possible to assay acetyl-CoA carboxylase from *Lipomyces starkeyi* CBS 1809 using the direct assay procedure of Inoue and Lowenstein (1975).

8.2 Investigation of the pathway of carbon into lipid in *Lipomyces starkeyi* CBS 1809 and *Saccharomyces cerevisiae*

Since it was not possible to assay acetyl-CoA carboxylase from *Lipomyces starkeyi* CBS 1809 it was decided to attempt to demonstrate that the pathway of carbon into lipid, in this organism, was that predicted for oleaginous yeasts. As a control, the pathway of carbon into lipid in a non-oleaginous yeast, *Saccharomyces cerevisiae* was also investigated.

The incorporation of label from various radioactive substrates into lipid, in washed toluenized cell suspensions of yeast, harvested in the exponential phase of growth, was monitored (Methods, Section 8.3). The toluenization step was included in order to render the cells permeable for the uptake of substrates from the suspending medium.

The results (Table 22) indicated that label was incorporated into lipid, in both yeasts from [1-^{14}C] acetate and [1-^{14}C] acetyl-CoA. There was little incorporation from these substrates in the absence of added ATP and $NaHCO_3$, and in the case of [1-^{14}C] acetate, added coenzyme A. Incorporation was not inhibited by added fluorocitrate, indeed the addition of this inhibitor tended to increase the amount of label appearing in the lipid fraction. This indicated that the labelled substrates were incorporated directly as the fluorocitrate would have inhibited the aconitase of the TCA cycle. Thus, these results were consistent with the presence of an active acetyl-CoA carboxylase in both yeasts.

The most striking feature of the results, however, was that significant incorporation of label into lipid from [1,5^{14}C] citrate only

TABLE 22: Incorporation of label into lipid fraction of washed cell suspensions of *Lipomyces starkeyi* CBS 1809 and *Saccharomyces cerevisiae*. Yeasts were harvested in the mid-exponential phase of growth after cultivation under nitrogen-limiting conditions, rendered permeable with toluene, then suspended in the incubation mixtures indicated for 1 h at 30 °C. The lipid fraction was then isolated and radioactivity measured using a toluene-based scintillation fluid.

Incubation mixture	*L. starkeyi* CBS 1809		*S. cerevisiae*	
	Activity (cpm)	Relative activity (%)	Activity (cpm)	Relative activity (%)
1. *a.* [1,5-^{14}C] citrate (5mM,0.1μCiμmol^{-1})+1mM CoA, 5mM ATP, 5mM $NaHCO_3$, 1mM NADPH	3546.4	100	1122.2	100
b. as *a.* stopped at zero time	640.1	18.0	701.8	62.5
c. as *a.* - CoA	670.1	18.9	725.3	64.6
d. as *a.* - ATP	685.0	19.3	801.2	71.4
e. as *a.* - $NaHCO_3$	652.1	18.4	711.7	63.4
f. as *a.* + 0.5mM fluorocitrate	6832.0	192.7	880.2	76.2
2. *a.* [1-^{14}C] acetate (5mM,0.1μCiμmol^{-1})+1mM CoA, 5mM ATP, 5mM $NaHCO_3$, 1mM NADPH	2082.7	100	2229.3	100
b. as *a.* stopped at zero time	612.4	29.4	437.8	19.7
c. as *a.* - CoA	792.4	38.1	502.1	22.5
d. as *a.* - ATP	854.5	41.3	539.2	24.2

e. as *a.* - $NaHCO_3$	650.3	31.2	510.0	22.9
f. as *a.* + 0.5mM fluorocitrate	2328.8	111.8	3098.4	138.9
3. *a.* [1-^{14}C] acetyl-CoA (5mM, 0.1μCiμmol^{-1})+5mM ATP, 5mM $NaHCO_3$, 1mM NADPH	3445.4	100	2010.0	100
b. as *a.* stopped at zero time	592.4	17.2	420.2	20.9
c. as *a.* - ATP	611.3	17.7	479.8	23.9
d. as *a.* - $NaHCO_3$	601.7	17.5	443.7	22.1
e. as *f.* + 0.5mM fluorocitrate	4222.2	122.6	1999.9	99.5

occurred in the oleaginous yeast and that the incorporation was dependent on the presence of coenzyme A, ATP and $NaHCO_3$. This provided clear evidence that the ATP:citrate lyase pathway of lipogenesis was predominant in *Lipomyces starkeyi* CBS 1809, the oleaginous yeast, whereas, this pathway was inoperative in *Saccharomyces cerevisiae*, the non-oleaginous yeast. The increase in incorporation of label from [1,5^{14}C] citrate, in *Lipomyces starkeyi*, in the presence of fluorocitrate was probably due to the decrease in that portion of the citrate that would have been oxidized via the TCA cycle in the absence of inhibitor. Similarly, the relatively small incorporation of label from citrate into lipid in *Saccharomyces cerevisiae* was probably due to the production of acetyl-CoA from citrate via TCA cycle oxidation. Thus, when the TCA cycle was inhibited by fluorocitrate, the incorporation of label from citrate into lipid in *Saccharomyces cerevisiae* was virtually eliminated.

In conclusion, it was not possible to assay for acetyl-CoA carboxylase from *Lipomyces starkeyi* CBS 1809 and consequently, the effect of citrate and fatty acyl-CoA esters upon the enzyme could not be tested. However, this experiment did confirm that the pathway of carbon into lipid, in this organism, was as predicted, i.e. via ATP:citrate lyase.

DISCUSSION

1. Physiological changes accompanying lipid accumulation in oleaginous yeasts

Initial batch culture experiments confirmed that *Lipomyces starkeyi* CBS 1809 and *Candida* 107 were capable of accumulating substantial amounts of lipid in the stationary phase when growth was limited by nitrogen depletion. However, closer examination of the parameters monitored during the batch growth of *Lipomyces starkeyi* CBS 1809 (Fig. 4) reveals that the changes that occurred during the growth of the yeast were more complex than is superficially apparent. Thus, growth in terms of protein synthesis must have ceased some time after 30 h cultivation because of nitrogen exhaustion from the medium. At 30 h the biomass was approx. 7 $g l^{-1}$ and the lipid content approx. 6%, therefore, the lipid-free biomass was 6.6 $g l^{-1}$. At 70 h the biomass had increased to 30 $g l^{-1}$ of which 33% was lipid, i.e. the lipid-free biomass had increased to 20 $g l^{-1}$. Since there can have been no further growth following nitrogen exhaustion from the medium, after 30 h cultivation, it is apparent that the 13.4 $g l^{-1}$ increase in lipid-free biomass observed after 70 h cultivation must be accounted for. Recent work in this laboratory had indicated that the increase in the lipid-free biomass is due to the accumulation of intracellular carbohydrate (C. T. Evans, Personal Communication) and investigations are currently in progress in order to determine the nature of the carbohydrate and how the flow of carbon between carbohydrate- and lipid-synthesizing pathways is regulated. In fact, although the carbohydrate content of *Lipomyces starkeyi* CBS 1809 was not determined in the batch culture experiment illustrated in Figure 4 it is likely that it would be less than the calculation, described above, suggests. This may be inferred in that although nitrogen disappeared from the medium after approx. 30 h cultivation there would have presumably been

further protein synthesis after this time during which stored nitrogen in the form of the intracellular amino acid pool would have been utilized. Thus, if the same calculation is performed, assuming that protein synthesis ceased after 40 h cultivation, then this would indicate that the lipid-free biomass (carbohydrate) increased by 6.1 gl^{-1} between this time and 70 h.

The lipid content of *Lipomyces starkeyi* CBS 1809 cultivated in a chemostat under conditions of nitrogen-limitation was of a similar order to that observed in batch cultures and decreased with increasing growth rate. However, the specific rate of lipid synthesis (expressed as g lipid synthesized per g fat-free yeast per h) showed an opposite trend. Thus, when the dilution rate was increased from 0.03 h^{-1} to 0.2 h^{-1} the specific rate of lipid synthesis increased from 0.023 to 0.036 (g lipid synthesized per g fat-free yeast per h) yet the lipid content of the yeast decreased from 42.8% (w/w) to 15.3% (w/w). When the yeast was cultivated in a chemostat under conditions of carbon-limitation the lipid content of the cells was low and did not vary greatly over the range of dilution rates tested. Therefore, as expected the specific rate of lipid synthesis in carbon-limited cells was also low and showed little variation with growth rate.

These results supported the view of Gill *et al.* (1977) who observed when working with *Candida* 107 that rates of lipid synthesis did not necessarily correlate with lipid content. Thus, they inferred that lipid does not accumulate when growth is limited by nitrogen-depletion because of increased rates of lipid synthesis but rather other biosynthetic processes dependent on a supply of nitrogen decline such that lipogenic pathways become a dominant part of the metabolism of the yeast.

The lack of correlation between the observed lipid contents and calculated rates of lipid synthesis in both *Candida* 107 and *Lipomyces*

starkeyi CBS 1809 is illustrated in Table 23.

In order to test the hypothesis of Gill *et al.* (1977) a technique was introduced that has not hitherto been applied to the study of oleaginous yeasts growing in continuous culture. This was to study the physiological changes that occurred when chemostat cultures of oleaginous yeasts underwent a transition from carbon- to nitrogen-limitation. It was considered that if nitrogen-limitation altered the flux of metabolism in favour of lipogenesis, at the expense of other nitrogen-requiring pathways, then this was most likely to be reflected in changes in the intracellular concentrations of amino acids and proteins.

Initial experiments with *Candida* 107 confirmed that the biomass and lipid content of the yeast did increase during transitions from carbon-limitation to carbon-excess. The increase in biomass began as soon as excess glucose appeared in the growth vessel and this was mirrored by a rapid decline in the NH_4^+ content of the medium. However, there was a considerable delay between the onset of nitrogen-limitation (as measured by its disappearance from the medium) and any marked increase in lipid content. This presumably indicated the intervention of other physiological changes.

In subsequent transition experiments with *Candida* 107 it was demonstrated that the onset of nitrogen-limitation was followed by a decline in the intracellular amino acid pool and the total protein concentration of the yeast. In a typical experiment with the dilution rate set at 0.06 h^{-1}, at the start of the transition the biomass of 2.7 $g l^{-1}$ contained 48% (w/w) protein and 15% (w/w) lipid. When the transition was completed the biomass increased to 6.5 $g l^{-1}$ of which 28% (w/w) was protein and 27% (w/w) lipid. Thus, although in absolute terms the amount of protein synthesized ($g l^{-1} h^{-1}$) increased during the transition

TABLE 23: Comparison of the growth, lipid content and specific rates of lipid synthesis of *Lipomyces starkeyi* CBS 1809 and *Candida* 107 cultivated in a chemostat under nitrogen-limited conditions. The data for *Candida* 107 was taken from Gill *et al.* (1977)

		Dilution rate (h^{-1})				
		0.03	0.06	0.10	0.15	0.20
Biomass (gl^{-1}):	*Candida* 107	16.4	18.1	14.0	9.7	7.2
	L. starkeyi CBS 1809	26.3	18.1	15.3	9.5	1.3
Lipid content (% w/w):	*Candida* 107	21.8	37.1	27.9	21.5	19.5
	L. starkeyi CBS 1809	42.8	30.0	22.7	19.4	15.3
Specific rate of lipid synthesis: (g lipid.g fat-free yeast^{-1}.h^{-1})	*Candida* 107	0.008	0.035	0.039	0.041	0.048
	L. starkeyi CBS 1809	0.023	0.026	0.029	0.036	0.036

from 0.078 to 0.108, this was a much smaller increase than that observed for lipid (0.025 to 0.107 $g l^{-1} h^{-1}$).

The amino acid pool was also observed to decline during a transition from carbon- to nitrogen-limitation. This in itself was probably not surprising in that in addition to providing precursors for macromolecular biosynthesis the amino acid pool has been ascribed the function of acting as a nitrogen store (Brown and Stanley, 1972; Johnson and Ellwood, 1975). Thus, it would be expected that when growth was limited by nitrogen-depletion from the medium, a portion of the amino acid pool would be utilized.

In conclusion, the results of early transition experiments were consistent with the view of Gill *et al.* (1977) that nitrogen-limitation diverted biosynthetic processes to the production of lipid at the expense of other biosynthetic processes dependent upon nitrogen.

Botham and Ratledge (1979) postulated that the flux of metabolism between lipogenic and other biosynthetic processes was regulated by changes in intracellular energy charge (Atkinson, 1968). In particular they regarded the intramitochondrial AMP concentration as being of key importance. They observed that the NAD^+-dependent isocitrate dehydrogenase from *Candida* 107 and other oleaginous yeasts had an absolute requirement for AMP. This phenomenon was not observed with the NAD^+-dependent isocitrate dehydrogenases from non-oleaginous yeasts. Botham and Ratledge (1979) demonstrated that the *in vivo* energy charge of *Candida* 107 grown under conditions of nitrogen-limitation was considerably higher than that observed for carbon-limited cultures. This led them to postulate that lipid accumulated under conditions of nitrogen-limitation because AMP depletion prevented the NAD^+-dependent isocitrate dehydrogenase from functioning, thereby, resulting in an over-production of citrate which was then utilized to synthesize lipid via the ATP:citrate lyase pathway.

I confirmed the variations in energy charge but extended the observations to encompass the changes occurring during transitions from carbon- to nitrogen-limitation. During a typical transition experiment with *Lipomyces starkeyi* CBS 1809 the energy charge increased from 0.13 to 0.57, the increase being almost entirely due to a decrease in the intracellular AMP concentration. These results were consistent with those of Botham and Ratledge (1979) and supported their hypothesis.

The results presented here did differ, however, in that only a small increase in the intracellular concentrations of ADP and ATP was observed during the transition, indicating that the overall increase in energy charge was not simply due to the conversion of AMP to ADP or ATP. Yoshino and Murakami (1981) reported that AMP deaminase may be important in stabilizing adenylate energy charge such that the enzyme increases in activity with decreasing energy charge. AMP deaminase has been detected in non-oleaginous yeasts (Yoshino *et al.*, 1979) and it would be interesting to see if its regulation in *Lipomyces starkeyi* CBS 1809 is abnormal. This however, has not been undertaken in this study.

In general, the adenylate energy charge values determined for *Lipomyces starkeyi* CBS 1809 and the degree of variation observed were clearly atypical with respect to the majority of other systems so far examined. Atkinson (1977) reported that the adenylate energy charge values of nearly all cells under all or nearly all conditions lie within the range 0.87 to 0.94. However, standard procedures were used in this study and due care was paid to the choice of quenching agent and to the time of quenching. Furthermore, the results were in agreement with those obtained by Botham and Ratledge (1979), therefore, unless future studies provide evidence to the contrary the results must be accepted at face value.

It should be stated, however, that the adenylate energy charge values

reported in this study were average cellular values at it was not possible to differentiate between mitochondrial and cytosolic pools of adenine nucleotides. Indeed it is not easy to conceive of a method by which this might be accomplished bearing in mind the twin problems of the need for rapid quenching and then being able to separate the cytosolic and mitochondrial adenine nucleotide pools. Possibly a method may be developed in the future in which adenine nucleotides could be determined *in situ* although no such method was available at the time of this study. However, if the assumption is made that the constitution of the adenine nucleotide pool in the mitochondria is similar to that of the cytosol then the results of this study provided clear evidence that transitions from carbon- to nitrogen-limitation were accompanied by a decrease in the intracellular AMP concentration. *In vitro* studies with NAD^+-dependent isocitrate dehydrogenase supported the hypothesis that the decrease in intracellular AMP concentration would be sufficient to inactivate this enzyme and divert the flow of carbon into lipid production.

Transition experiments carried out with *Lipomyces starkeyi* CBS 1809 indicated that there was still considerable delay between the decrease in intracellular AMP concentration to the lowest observed values and the onset of significant lipid accumulation. This suggested that further physiological changes had occurred.

Mitsushima *et al.* (1978) reported that citrate accumulation in the yeast, *Candida lipolytica*, was regulated by the availability of AMP by an identical mechanism to that already discussed. In oleaginous yeasts citrate may have two roles in lipogenesis. Firstly, to provide precursor acetyl-CoA units via the action of ATP:citrate lyase and secondly, as an activator of acetyl-CoA carboxylase. Citrate activation of acetyl-CoA carboxylases from many sources has been well-documented (Volpe and Vagelos, 1973) including an oleaginous yeast, *Candida* 107 (Gill and Ratledge, 1973).

The intracellular citrate concentration of *Lipomyces starkeyi* CBS 1809 was observed to fluctuate during transitions from carbon- to nitrogen-limitation. Initially, when growth was limited by neither carbon nor nitrogen the intracellular citrate concentration fell, as compared with the steady state carbon-limited value. However, once the culture became limited by nitrogen-depletion from the medium the intracellular citrate concentration increased rapidly. Thus, in a typical experiment the citrate concentration was 0.95 to 1.9 m$\underline{\underline{M}}$ under steady state conditions of carbon-limitation and this increased to 1.6 to 3.2 m$\underline{\underline{M}}$ when the culture became nitrogen-limited.

The interpretation of these results remains unclear. Gill and Ratledge (1973) reported that 50 m$\underline{\underline{M}}$ citrate was required to fully activate acetyl-CoA carboxylase from *Candida* 107, whereas, the observed intracellular citrate concentration of *Lipomyces starkeyi* CBS 1809 was an order of magnitude lower than this. However, in a more recent study Botham and Ratledge (1979) also working with *Candida* 107 reported that acetyl-CoA carboxylase was maximally stimulated by 1 m$\underline{\underline{M}}$ citrate. As no methods were available to distinguish mitochondrial and cytosolic metabolite pools in yeasts it was not possible to determine the actual effective intracellular citrate concentration.

However, it should be noted that Capuzzi *et al.* (1974) investigated the compartmentalization of citrate in mammalian liver and concluded that 70% was associated with mitochondria and thus, would not be available to activate the cytosolic acetyl-CoA carboxylase. Similarly, Greenbaum *et al.* (1971) reported that the cytosolic citrate concentration in mammalian liver cells was 0.1-0.2 m$\underline{\underline{M}}$ yet citrate activated hepatic acetyl-CoA carboxylase with an apparent K_m of 2.6 m$\underline{\underline{M}}$. Consequently, it is not possible to conclude with any certainty that the observed increase in intracellular citrate concentration would be sufficient to cause any

significant activation of acetyl-CoA carboxylase.

It may be stated, however, that the observation that the intracellular citrate concentration did increase during the transition, following the establishment of a basal concentration of AMP, can be regarded as circumstantial confirmatory evidence for the premise that the inactivation of NAD^+-dependent isocitrate dehydrogenase by AMP-depletion is responsible for the channelling of carbon flow into lipogenic pathways. In addition, the observed increase in the intracellular citrate concentration following the establishmemt of steady-state nitrogen-limiting conditions may indicate that either the rate of citrate efflux from the mitochondria or the rate of citrate cleavage might be the rate-limiting step for lipogenesis under these conditions. It is not possible to identify which of these two steps might be rate-limiting because it was not possible to differentiate between the cytosolic and mitochondrial citrate pools. Clearly if the increase in intracellular citrate concentration occurred principally within the mitochondria this would indicate that the transport of this metabolite to the cytosol might be rate-limiting. Similarly, if the increase principally occurred within the cytosol this would indicate that ATP:citrate lyase catalyzed the rate-limiting step. However, it is worthy of note that when the transition was initiated the extracellular citrate concentration was immediately increased. Since this must have been derived from the cytosolic citrate pool this is perhaps evidence that citrate did accumulate in the cytosol during the transition and, therefore, indicative of ATP: citrate lyase catalyzing the rate-limiting step.

In conclusion, the sequence of events observed to occur during transitions from carbon- to nitrogen-limitation may be summarized as follows:

1. There was a brief initial period (0 to 4 h) in which growth was neither limited by carbon nor nitrogen. During this time the

biomass increased but intracellular citrate and AMP fell. The lipid content was low and unchanged.

2. An intermediate phase (4 to 10 h) commencing when the culture became nitrogen-limited, during which the intracellular AMP concentration fell to a basal value but the intracellular citrate concentration increased. The lipid content remained low and unchanged during this period.

3. A final phase (10 to 24 h) during which the lipid content of the yeast increased rapidly following the establishment of constant AMP and citrate concentrations.

These results were consistent with the hypothesis of Botham and Ratledge (1979) that the primary event responsible for the diversion of carbon flow into lipid production is the inactivation of the mitochondrial NAD^+-dependent isocitrate dehydrogenase by AMP depeltion. This results in an increase in the intracellular citrate concentration which may then be utilized to furnish acetyl-CoA units for lipid synthesis, via the action of ATP:citrate lyase, and possibly to activate acetyl-CoA carboxylase.

2. Experiments with isolated mitochondria from oleaginous yeasts

Although considerable efforts were made to isolate mitochondria from oleaginous yeasts it was not possible to develop a satisfactory procedure for this. The reasons for this failure are discussed below.

The preferred methods of isolating mitochondria from yeasts is one in which sphaeroplasts are initially prepared. These may then be disrupted by gentle methods, thus, minimising damage. However, the cell walls of the oleaginous yeasts tested - namely, *Candida* 107, *Lipomyces starkeyi* CBS 6047, *Lipomyces lipofer* CBS 5842 and *Rhodosporidium toruloides* CBS14 - were resistant to enzymatic hydrolysis. This resistance was independent of the stage in the growth cycle. Pre-treatment with thiol-

containing reagents had no effect.

The reasons for the failure to produce sphaeroplasts from oleaginous yeasts are obscure. Phaff (1971) reported that the susceptibility of yeast cell walls to enzymic degradation showed great inter-species variation. It seems likely, therefore, that the resistance must be a consequence of a novel cell wall structure such that the glucan moiety does not provide a suitable substrate for the lytic enzymes, or possibly the glycosidic linkages are not accessible to the lytic enzymes.

Since sphaeroplasts could not be prepared from oleaginous yeasts it was necessary to resort to mechanical methods to effect the initial cell disruption step in mitochondrial isolation. These methods have the disadvantage that the force which must be used to rupture the cell wall is sufficient to render inevitable damage to mitochondria. Evidence for this damage was provided by the fact that when mitochondria were isolated from oleaginous yeasts it was not possible to demonstrate respiratory control and the cytosolic fraction was always contaminated with enzymes of the mitochondrial matrix. It proved impossible to reach a compromise where there was sufficient yeast disruption and only limited mitochondrial damage.

Of all the methods tested the most satisfactory proved to be that in which yeast was suspended in isotonic medium and then disrupted by passage through a French pressure cell. Mitochondria were then recovered from the crude cell-free extract by differential centrifugation. However, these preparations were not considered suitable, for the reasons discussed, to pursue the initial aims of the investigation, namely, to study the mechanism of citrate efflux and if possible to quantify relevant metabolite pool sizes. Consequently, these preparations were only used to investigate the intracellular location of key lipogenic enzymes.

It was confirmed that the proposed key lipogenic enzymes did occupy

the locations suggested by the hypothesis of Botham and Ratledge (1979). Thus, the possibility that cytosolic acetyl-CoA might be generated in oleaginous yeasts by an unusual enzyme distribution, such as the provision of a cytosolic pyruvate dehydrogenase, was eliminated.

In subsequent work in this laboratory (C. T. Evans, Personal Communication) good quality mitochondrial preparations have been isolated from several oleaginous yeasts (*Lipomyces starkeyi* spp and *Lipomyces lipofer* spp). Mitochondria were isolated from sphaeroplasts which were prepared using a commercially-available cell wall-hydrolyzing enzyme, Zymolyase 5000 (Miles Laboratories Ltd., Stoke Poges, U.K.). This enzyme preparation, derived from the bacterium, *Arthrobacter luteus*, was not available at the time of these investigations.

Using these mitochondrial preparations, malate has been identified as the counter-ion for citrate efflux and the process found to be inhibited by long chain fatty acyl-CoA esters when tested in the range 10-100 μM. In addition, preliminary evidence has been presented indicating that citrate efflux from mitochondria of oleaginous yeasts is inhibited by cytosolic AMP, whereas, this has not been observed with mitochondrial preparations isolated from non-oleaginous yeasts. Thus, this has tended to confirm the hypothesis of Botham and Ratledge (1979) that AMP-depletion restricts the activity of NAD^+-dependent isocitrate dehydrogenase thereby diverting the flow of carbon into the production of cytosolic citrate. Furthermore, the possibility that citrate efflux from mitochondria of oleaginous yeasts is a regulated step and indeed the rate-limiting step in lipogenesis cannot be discounted. This work is still in progress and it is hoped that further information regarding the importance of citrate transport and its regulation may be forthcoming.

3. Phosphoketolase activity in oleaginous yeasts

In early studies on the biochemistry of oleaginicity in micro-organisms

the failure to detect phosphofructokinase led to the proposal that the principal pathway of glucose catabolism was the pentose phosphate cycle (Brady and Chambliss, 1967; Höfer, 1968; Whitworth and Ratledge, 1975 a, b). However, since this pathway gives only 1 mol acetyl-CoA for each mol glucose utilized it was realized that there must be other mechanisms to supplement the supply of C_2 units in order to account for the high molar growth and lipid yields in these organisms (Gill *et al.*, 1977; Höfer *et al.*, 1971; Brady and Chambliss, 1967). Thus, it was proposed that the supply of C_2 units for lipid synthesis, in these organism, might be supplemented by phosphoketolases (Höfer *et al.*, 1969, 1971; Höfer, Betz and Becker, 1970).

Whitworth and Ratledge (1977) detected hexose and pentose phosphoketolases in the oleaginous yeasts, *Rhodotorula graminis*, *R. glutinis* and *Candida* 107 but not in the non-oleaginous yeast *Saccharomyces cerevisiae*. However, the significance of these results was later cast in some doubt when it was subsequently discovered that oleaginous yeasts such as *Candida* 107 did possess an active but labile phosphofructokinase, thereby indicating that the glycolytic pathway of glucose catabolism was operative (Ratledge and Botham, 1977).

It is important to evaluate the importance of phosphoketolases as the presence of these activities in oleaginous yeasts would result in the direct production of cytosolic acetyl-CoA from glucose and thus, cast into some doubt the significance of the ATP:citrate lyase pathway of lipogenesis in these organisms.

The presence of fructose 6-phosphate phosphoketolase was confirmed in crude cell-free extracts of *Candida* 107 and *Rhodotorula graminis*, however, no activity was detected in extracts of *Hansenula saturnus* CBS 5761 and *Lipomyces starkeyi* CBS 1807 and 1809 with either xylulose 5-phosphate or fructose 6-phosphate as substrate.

Phosphoketolases were not essential, therefore, for lipid accumulation to occur in oleaginous yeasts. Furthermore, the presence of phosphoketolases did not confer an increased ability to accumulate lipid in those yeasts possessing the activities. Thus, the physiological role of phosphoketolases in oleaginous yeasts remains unclear, however, since they are not ubiquitous in these organisms it seems likely that they are of only minor importance in supplying cytosolic acetyl-CoA for lipogenesis.

4. Activities of TCA cycle enzymes during lipid accumulation

4.1 Citrate synthase

The observed kinetic properties of citrate synthase from *Candida* 107 were similar to those reported for the enzyme from *Saccharomyces cerevisiae* (Hathaway and Atkinson, 1965). The K_m values for acetyl-CoA and oxaloacetate were similar and in each case ATP was a potent inhibitor. The K_i was 0.14 mM with citrate synthase from *Candida* 107 and 0.11 mM with citrate synthase from *Saccharomyces cerevisiae* and in each case the inhibition was competetive with respect to acetyl-Coa. ATP inhibition of citrate synthase from *Saccharomyces cerevisiae* was non-competitive with respect to oxaloacetate (Hathaway and Atkinson, 1965) whereas the enzyme from *Candida* 107 exhibited kinetics that were mixed competitive/non-competetive with respect to this substrate. This difference, however, is probably not of great significance.

Mixtures of adenine nucleotides giving simulated energy charge values were inhibitory towards citrate synthase from *Candida* 107 the degree of inhibition increasing with increasing energy charge. This observation was paradoxical in that if the ATP:citrate lyase pathway of lipogenesis is operative in oleaginous yeasts there should be no restriction in the supply of cytosolic citrate. However, Botham and Ratledge (1979) reported that both the intracellular ATP concentration and the *in vivo*

energy charge of *Candida* 107 cultivated under nitrogen-limiting conditions were considerably higher than those observed for carbon-limited cultures. Thus, the *in vitro* evidence that citrate synthase might be inhibited when cultural conditions favoured lipid accumulation must be reconciled.

When considering the regulation of citrate synthase by changes in the prevailing energy charge two additional factors must also be taken into account. Firstly, the size of the adenine nucleotide pool and secondly, possible interactions between adenine nucleotides and divalent metal ions, especially Mg^{2+}.

Any discussion of the regulatory significance of energy charge must consider not only the prevailing energy charge but also the total adenine nucleotide concentration in relation to the concentrations of enzyme and substrates. There is no information regarding the precise micro-environment of the enzyme. However, the total adenine concentration within the mitochondrion is constant as passage of ATP into the cytosol occurs only via a 1:1 exchange with ADP (Klingenberg, 1970). Botham and Ratledge (1979) reported that the total adenine nucleotide concentration of *Candida* 107 lies within the range 0.2 to 4 mM and the average energy charge varies between 0.1 and 0.6 according to cultural conditions. It is known that the prevailing energy charge within the mitochondrion may differ from that in the cytosol (Heldt *et al.*, 1972) however, the total adenine nucleotide concentration may not be significantly different in each compartment. Thus, it is important that when simulated energy charge mixtures are tested against citrate synthase the total adenine nucleotide concentration should be in the correct range. This is illustrated by the fact that an increase from 2.5 to 5 mM in the total adenine nucleotide concentration resulted in a doubling of the inhibition of citrate synthase from *Candida* 107 at all energy charge values. Hence,

it is not possible to determine what the *in vivo* activity of citrate synthase would be if neither the prevailing energy charge nor the total adenine nucleotide concentration are known.

Any consideration of the effect of simulated energy charge mixtures on the activity of citrate synthase must take into account the effect of divalent metal ions, especially Mg^{2+}. It has been reported that ATP exists predominantly, *in vivo*, as the magnesium-ATP complex (Burt *et al.*, 1976) and Knowles (1977) considers that the available concentration of Mg^{2+} is of great importance in calculating the effect of energy charge as a regulatory parameter. Adenylate kinase is essential to the energy charge hypothesis of enzyme regulation in that it must be present to catalyze the return to equilibrium following alterations in the concentration of any adenine nucleotide. This enzyme, which is present in all cells, has an absolute requirement for Mg^{2+}, thus, it is likely that Mg^{2+} will be involved in all interactions between adenine nucleotides and other potentially regulated enzymes.

There is no information regarding the available intracellular Mg^{2+} concentration in *Candida* 107. However, when Mg^{2+} was included in citrate synthase assay mixtures inhibition due to ATP was considerably lessened as was the inhibition caused by mixtures of adenine nucleotides simulating high energy charge values. In fact, it was difficult to demonstrate any inhibition due solely to adenine nucleotides if comparison was made to the activity of the enzyme in the presence of excess Mg^{2+}. Thus, it is likely that citrate synthase from *Candida* 107 and other oleaginous yeasts is not subject to rigorous control by adenine nucleotides.

This view is supported by other reports of the effect of adenine nucleotides on citrate synthases from various other micro-organisms. Weitzman and Hewson (1973) confirmed the inhibition of citrate synthase from *Saccharomyces cerevisiae* by ATP, *in vitro*. However, no effect was

observed when the enzyme was assayed *in situ*, in cells made permeable by treatment with toluene. Similarly, Kubicek and Röhr (1980) concluded that citrate synthase of the citric acid-accumulating fungus, *Aspergillus niger* was only weakly regulated by changes in the prevailing energy charge since they also observed that ATP inhibition was entirely relieved in the presence of an excess of Mg^{2+}.

No other metabolites tested had any marked effect on the activity of citrate synthase from *Candida* 107 with the exception of long chain fatty acyl-CoA esters. These compounds were found to be potent inhibitors of the enzyme although the degree of inhibition was markedly reduced with reduction in the number of carbon atoms in the fatty acid moiety of the ester.

This phenomenon has been reported for various mammalian citrate synthases and certain authors consider it might be of regulatory significance (Hsu and Powell, 1975; Caggiano and Powell, 1979). However, these compounds are powerful detergents, consequently, caution must be exercised when interpreting results. Whether or not a regulatory function can be ascribed to fatty acyl-CoA ester inhibition of citrate synthase from *Candida* 107 is far from certain.

Hsu and Powell (1975) reported that oleoyl-CoA was a potent inhibitor of pig heart citrate synthase, whereas, its structural analogue, oleoyl-(1,N^6-etheno)-CoA, was less effective by an order of magnitude. However, this latter compound was a more powerful detergent as measured by its critical micellar concentration (3.2 $\mu\underline{M}$ as compared with 4.7 $\mu\underline{M}$ for oleoyl-CoA). Thus, these authors concluded that the inhibition of pig heart citrate synthase by oleoyl-CoA was unlikely to be due simply to the detergent properties of the molecule and consequently, could be of regulatory significance.

Citrate synthase from *Candida* 107 was also potently inhibited by

oleoyl-CoA but the results differed from those of Hsu and Powell (1975) in that no effect was observed when oleoyl-CoA was tested at a concentration below its critical micellar concentration. This would tend to suggest that the inhibition was a non-specific detergent effect, a conclusion further reinforced by the observation that the addition of bovine serum albumin failed to reverse the inhibition.

Kosicki and Lee (1966) reported that the inhibition of pig heart citrate synthase by palmitoyl-CoA was relieved by the presence of excess Mg^{2+}. The ameliorating effect of Mg^{2+} on the inhibition of *Candida* 107 by oleoyl-CoA was observed in this study. The arguments which have already been advanced concerning the physiological role of Mg^{2+} on the inhibition of citrate synthase by adenine nucleotides must also apply here. Thus, if the intracellular Mg^{2+} concentration is sufficient to saturate the adenine nucleotide pool then this would presumably also apply to free fatty acyl-CoA esters. If this were the case then further doubt must be cast on the *in vivo* significance of the inhibition of citrate synthase from *Candida* 107 by fatty acyl-CoA esters.

In conclusion, there was little evidence that citrate synthase from *Candida* 107, and by extrapolation other oleaginous yeasts, is subject to rigorous control. Therefore, the contention that citrate synthase must remain active during lipid accumulation in oleaginous micro-organisms was supported.

4.2 NAD^+-dependent isocitrate dehydrogenase

Botham and Ratledge (1979) observed that NAD^+-dependent isocitrate dehydrogenase from two oleaginous yeasts, *Candida* 107 and *Rhodotorula glutinis* and an oleaginous mould, *Mucor circinelloides*, had an absolute requirement for AMP, whereas, this adenine nucleotide had no effect on the activity of the enzyme from the non-oleaginous yeast, *Candida utilis*. Since these authors had previously observed that when cultural conditions favoured lipid accumulation the intracellular AMP concentration of

Candida 107 was low, they postulated that this would restrict the activity of NAD^+-dependent isocitrate dehydrogenase and thereby, divert carbon flow into lipogenic pathways.

These studies, however, were performed using crude cell-free extracts and no attempts were made to test the effects of other adenine nucleotides either singly or in combination. Since this phenomenon is of prime importance in controlling the flux of carbon flow between lipogenic and other biosynthetic pathways in oleaginous micro-organisms and constitutes one of the few biochemical characteristics that distinguish oleaginous micro-organisms from non-oleaginous types it was worthy of detailed investigation.

Botham and Ratledge (1979) reported that enzyme activity from *Candida* 107 was negligible in the absence of added AMP and that the addition of 0.5 mM AMP resulted in a 30-fold stimulation in activity. However, I observed no activation in the activity of the enzyme purified from *Lipomyces starkeyi* CBS 1809 when AMP was added to assay mixtures at concentrations up to 10 mM. Furthermore, this lack of response was not due to endogenous AMP as dialysis of extracts failed to elicit AMP-sensitivity. AMP did stimulate activity, however, when the D-isocitrate concentration was made non-saturating, although only 50 μM AMP was required for the maximum observed stimulation of 3.5 fold, and the enzyme was still active in the absence of added AMP. Thus, it appeared that NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 was not regulated simply by the availability of AMP.

In subsequent experiments it was demonstrated that ATP was a potent inhibitor of enzyme activity in the absence of added AMP and that this effect was independent of the concentration of D-isocitrate. Thus, the addition of 50 μM ATP reduced activity by 83%. However, when AMP was included in assays considerably more ATP was required to achieve

the same degree of inhibition. Thus, in the presence of 100 $\mu\underline{M}$ AMP, the ATP concentration had to be increased to 6.8 $m\underline{M}$ (138-fold increase) to produce 83% inhibition. The evidence was, therefore, that the relative concentrations of AMP, ATP and possibly D-isocitrate are of importance in regulating the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809.

Mixtures of adenine nucleotides simulating various energy charge values modulated the activity of the enzyme such that activity decreased with increasing energy charge. The total adenine nucleotide concentration was of little importance in determining the activity of the enzyme in that a 10-fold increase from 0.1 to 1 $m\underline{M}$ caused little or no alteration in enzyme activity. The role of divalent metal ions in interactions between adenine nucleotides and enzymes has been discussed previously. However, NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 had an absolute requirement for Mg^{2+}, therefore, saturating concentrations of this ion were included in all assays.

The evidence from *in vitro* studies was, therefore, that the activity of NAD^+-dependent isocitrate dehydrogenase from *Lipomyces starkeyi* CBS 1809 is regulated by variations in adenylate energy charge. It has been discussed previously that transition experiments with *Lipomyces starkeyi* CBS 1809 showed that the onset of nitrogen-limitation resulted in a dramatic increase in the observed intracellular energy charge. *In vitro* experiments indicated that this increase would be sufficient to restrict the activity of NAD^+-dependent isocitrate dehydrogenase and thereby, divert carbon flow into the production of lipid.

5. The role of ATP:citrate lyase in lipogenesis

Although the occurrence of ATP:citrate lyase in mammalian tissues has been well-documented, until comparatively recently it was not considered to be present in microorganisms (Srere, 1972). However,

ATP:citrate lyase has been detected in the oleaginous yeast, *Rhodotorula gracilis* (Guerritore and Hanozet, 1970) and several species of the oleaginous fungus, *Mortierella* (Attwood, 1973), although I was not aware of these reports until part way through this study.

In our own laboratory Botham and Ratledge (1979) reported the presence of ATP:citrate lyase in the oleaginous yeasts, *Candida* 107 and *Rhodotorula glutinis* and also the oleaginous mould, *Mucor circinelloides*. However, these authors also detected the enzyme in non-oleaginous strains of *M. circinelloides*, although the activities were 100-fold lower than those observed for oleaginous strains.

These initial reports have been considerably extended by an examination of some 23 yeasts for the occurrence of ATP:citrate lyase in comparison with their lipid content. The results have clearly indicated that the enzyme was only detectable in those yeasts capable of accumulating substantial amounts of lipid, i.e. above 25% w/w. However, there was no correlation between the activity of ATP:citrate lyase and the actual lipid content of the yeast. Thus, when the yeasts were cultivated under carbon-limiting conditions the lipid content was lower than that observed for nitrogen-limiting cultures but there was no reduction in ATP:citrate lyase activity. It would seem, therefore, that the possession of ATP:citrate lyase by any particular yeast indicates a potentiality to accumulate lipid but does not give any information regarding the prevailing lipid content. In this context a rapid screening procedure might be developed for the identification of as yet unknown oleaginous organisms based on the detection of ATP:citrate lyase in cultures grown on agar plates.

Further investigations with ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 confirmed that the enzyme is constitutive in this organism, as no alteration in its activity was detected during

transitions from carbon- to nitrogen-limitation. Therefore, the regulation of microbial ATP:citrate lyase apparently differs from its regulation in many mammalian tissues where induction by high carbohydrate diet has been reported, for example, rat hepatic enzyme (Yen and Mack, 1980).

Although ATP:citrate lyase from oleaginous yeasts may not be inducible this does not, *of* course, preclude the possibility that its activity might be rigorously regulated. In fact, when *Lipomyces starkeyi* CBS 1809 was cultivated in a chemostat under conditions of nitrogen-limitation, at various dilution rates, the specific activity of ATP:citrate lyase positively correlated with the specific rate of lipid synthesis. This observation is consistent with the possibility that ATP:citrate lyase catalyzes the rate-limiting step, in lipogenesis, in this organism.

Conversely, there was no reduction in ATP:citrate lyase activity when the previously discussed experiment was repeated under conditions of carbon-limitation, although observed specific rates of lipid synthesis were considerably reduced. These findings can possibly be explained in that under conditions of carbon-limitation the regulation of ATP:citrate lyase may not be a significant determinant in the control of lipogenesis. In this case the intramitochondrial NAD^+-dependent isocitrate dehydrogenase would be active, for reasons previously discussed, and thus, the *in vivo* activity of ATP:citrate lyase would probably be limited by the availability of citrate.

Two possible mechanisms were identified whereby the activity of ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 could be modulated. The first of these was the regulation of activity by changes in the prevailing adenylate energy charge. The second involved the control of ATP:citrate lyase activity by feed-back inhibition by long chain fatty acyl-CoA esters.

ATP:citrate lyase could be controlled by changes in adenylate energy charge because ATP is a substrate and ADP, a product, was discovered to be a potent inhibitor. Mixtures of adenine nucleotides simulating various energy charge values modulated the activity of ATP:citrate lyase as predicted by Atkinson (1968) for those enzymes involved in sequences where ATP is utilized. In this case the role of metal ions cannot be questioned as the enzyme was shown to have an obligate requirement for Mg^{2+}, consequently, this was included in all assays.

Whether the control of ATP:citrate lyase by changes in the adenylate energy charge is of importance in regulating rates of lipogenesis or in limiting the absolute amount of lipid synthesized is debatable. Previous studies reported here have shown that the *in vivo* energy charge of *Lipomyces starkeyi* CBS 1809 was high when the yeast was cultivated under conditions which favoured lipid accumulation (nitrogen-limitation), whilst energy charge was low when conditions did not favour lipid accumulation (carbon-limitation). This would support the view that ATP: citrate lyase would be fully active under conditions of nitrogen-limitation, whereas, under conditions of carbon-limitation the low energy charge would restrict the activity of the enzyme and consequently limit lipogenesis. However, as discussed previously, the observed variations in energy charge due to changes in nutrient limitation were mainly a result of changes in the intracellular AMP concentration and this would not be expected to influence the activity of ATP:citrate lyase.

The evidence for the control of ATP:citrate lyase activity by long chain fatty acyl-CoA esters was more substantial. In this context it is illuminating to compare the effects of these compounds on ATP: citrate lyase and citrate synthase. Long chain fatty acyl-CoA esters were potent inhibitors of both enzymes and in both cases the inhibition was time-dependent. However, oleoyl-CoA was inhibitory to ATP:citrate

lyase at concentrations below the critical micellar concentration of the molecule, whereas citrate synthase was not inhibited under such conditions. Furthermore, Mg^{2+} alleviated the inhibition of citrate synthase by oleoyl-CoA, whereas, since ATP:citrate lyase had an absolute requirement for Mg^{2+} this ion was included in all assays. More importantly oleoyl-CoA inhibition of ATP:citrate lyase was reversible whilst the inhibition of citrate synthase by oleoyl-CoA was not so.

There was considerable evidence, therefore, that the inhibition of ATP:citrate lyase by long chain fatty acyl-CoA esters was not merely a non-specific detergent effect. Further confirmation was provided by the fact that fatty acyl-CoA esters did not cause dissociation of the ATP:citrate lyase molecule, thus, opening up the possibility that there might be a specific binding site. Citrate synthase, on the other hand, appeared to be inhibited as a result of the detergent properties of these compounds presumably causing a breakdown in the quaternary or tertiary structure of the enzyme.

There was considerable evidence, therefore, that ATP:citrate lyase from *Lipomyces starkeyi* CBS 1809 is regulated by changes in the intracellular concentration of fatty acyl-CoA esters. No information is available regarding the intracellular concentration of fatty acyl-CoA esters in oleaginous yeasts. However, it is pertinent to note that these molecules are the ultimate end-products of fatty acid biosynthesis in yeasts, whereas, in mammalian tissues free fatty acids are produced (Bloch and Vance, 1977). This may explain why mammalian ATP:citrate lyases have generally been found to be relatively insensitive to fatty acyl-CoA ester inhibition (Bloch and Vance, 1977).

Gill and Ratledge (1973) reported that the inhibition of acetyl-CoA carboxylase, from oleaginous yeasts, by fatty acyl-CoA esters might be important in controlling rates of lipogenesis. However, in this case

comparatively high concentrations (100-200 μM) were required to effect significant inhibition. Thus, ATP:citrate lyase was more sensitive, by almost two orders of magnitude, to this type of inhibition. This is in keeping with the proposal that ATP:citrate lyase should now be considered as catalyzing the first step in lipid biosynthesis in oleaginous yeasts.

The mechanism, if any, by which fatty acyl-CoA esters might be released to act upon ATP:citrate lyase is at present unknown, although elucidation of this process could possibly shed light on what limits the amount of lipid any particular yeast can store. It is known that lipid accumulates in droplets bound by a membrane structure (Uzuka *et al.*, 1974). This suggests that the lipid-synthesizing and membrane-synthesizing enzymes might be organised in close physical and functional proximity. Indeed, Christiansen (1979) has reported that the enzymes for triacylglycerol biosynthesis, which utilize fatty acyl-CoA esters as substrates, are associated with the 'lipid particles' of bakers' yeast and are thus likely to be on the surface phospholipid which encompasses the droplet. If the same applies to oleaginous yeasts, then as these droplets expand with increasing lipid storage so might the enzymes become less securely attached to the droplet. This, in turn, would decrease triacylglycerol synthesis, lead to the appearance of free fatty acyl-CoA esters in the cytosol and thus lead to the inhibition of ATP:citrate lyase and consequently, lipid biosynthesis. Although the concentration of free fatty acyl-CoA esters remains to be determined values of between 15 and 140 μM have been recorded in mammalian and avian liver (Volpe and Vagelos, 1976).

ATP:citrate lyase occupies a unique position in the schemes of fatty acid biosynthesis. Although this enzyme is present in both animals and plants, the microbial enzyme appears distinct with its high sensitivity to feedback inhibition by fatty acyl-CoA esters as well as possibly being

the rate-limiting step in lipid biosynthesis.

6. The role of malic enzyme in lipogenesis

Malic enzyme is presumed to be involved in lipogenesis, in mammalian tissues, its role being to supply reducing equivalents in the form of NADPH. Evidence for this role is provided by the fact that dietary and hormonal manipulation of rates of lipogenesis can be positively correlated with the activity of the enzyme (Frenkel, 1975).

The enzyme has been detected in the oleaginous yeast, *Candida* 107 (Whitworth and Ratledge, 1975a). Thus, Botham and Ratledge (1979) proposed a similar function for malic enzyme from oleaginous yeasts. The presence of malic enzyme in *Candida* 107 was confirmed but no activity was detected in two oleaginous strains of *Lipomyces starkeyi* (CBS 1807 and CBS 1809), furthermore, the observed activities from *Candida* 107 did not correlate with the prevailing lipid content.

Malic enzyme, therefore, appears to be not essential for the process of lipid accumulation to occur in oleaginous yeasts. This raises the question as to what role the enzyme does perform in those organisms where it has been detected, and what other mechanisms exist to supply reducing equivalents for lipogenesis in those organisms where malic enzyme is apparently absent.

It seems probable that malic enzyme would contribute to the production of reducing equivalents for lipogenesis in those organisms where it has been detected. However, this contribution may well be quite small since the conversion of malate to pyruvate would tend to restrict citrate export from mitochondria as malate is required to act as the citrate antiporter. In those organisms where malic enzyme is absent reducing equivalents for lipogenesis are probably supplied by the pentose phosphate pathway.

7. Studies with acetyl-CoA carboxylase

In early studies in this laboratory it was reported that acetyl-CoA carboxylase from *Candida* 107 was activated by citrate, a concentration of 50 mM being required for maximum stimulation, and that this activation was prevented by the inclusion of 100 μM palmitoyl-CoA in assay mixtures (Gill and Ratledge, 1973). Subsequently, Botham and Ratledge (1979) confirmed that citrate activated the enzyme from *Candida* 107 but in this case only 5 mM citrate was required for maximum stimulation and fatty acyl-CoA esters had no effect on enzyme activity.

In this study it did not prove possible to assay acetyl-CoA carboxylase from *Lipomyces starkeyi* CBS 1809 using the direct procedure of Inoue and Lowenstein (1975) in which the incorporation of label from [^{14}C] $NaHCO_3$ into acid-stable malonyl-CoA is measured. Other assay methods for this enzyme (e.g. Matsuhashi, 1969) measure the incorporation of label from [1-^{14}C] acetyl-CoA or [1-^{14}C] acetate into saponifiable lipid and therefore, require the remainder of the lipid-synthesizing enzymes to be present in excess and are consequently unsuitable for inhibitor studies. It was not possible, therefore, to confirm citrate activation of acetyl-CoA carboxylase in this organism nor was it possible to compare the effects of long chain fatty acyl-CoA esters on this enzyme with those already observed for ATP:citrate lyase.

In subsequent experiments the uptake of label from various radioactive substrates, into lipid, in cell suspensions of *Lipomyces starkeyi* CBS 1809 and *Saccharomyces cerevisiae*, rendered permeable by treatment with toluene, was measured.

Label was incorporated into lipid in both yeasts from [1-^{14}C] acetate and [1-^{14}C] acetyl-CoA only in the presence of added ATP and $NaHCO_3$, and in the case of [1-^{14}C] acetate, added CoA. The labelled substrates were incorporated directly into lipid as the presence of

fluorocitrate, which would have inhibited the aconitase of the TCA cycle, had no effect on the degree of incorporation of label. Thus, in both yeasts it was confirmed that label was incorporated into lipid in a manner consistent with the presence of an active acetyl-CoA carboxylase.

Incorporation of label from [1,5-^{14}C] citrate into lipid was only significant in the oleaginous yeast. In this case incorporation was dependent on the presence of ATP, Coenzyme A and $NaHCO_3$ and was roughly doubled in the presence of fluorocitrate. This was consistent with the presence of an active ATP:citrate lyase in the oleaginous yeast. The increased incorporation in the presence of fluorocitrate, which had previously been shown to have no effect on ATP:citrate lyase from this organism, was probably due to the decrease in the portion that would have been oxidized via the TCA cycle in the absence of inhibitor. The relatively small incorporation of label from [1,5-^{14}C] citrate, into lipid, in *Saccharomyces cerevisiae* was probably due to the production of acetyl-CoA, from citrate, via TCA cycle oxidation. To a certain extent this was confirmed in that incorporation of label from [1,5-^{14}C] citrate in *Saccharomyces cerevisiae* was reduced in the presence of fluorocitrate.

In conclusion, the pathway of carbon into lipid was, as predicted, via ATP:citrate lyase, and this pathway was only operative in the oleaginous yeast.

SUMMARY

The most significant findings reported in this thesis may be summarized as follows.

Initial studies confirmed that batch cultures of two typical oleaginous yeasts, *Candida* 107 and *Lipomyces starkeyi* CBS 1809 only accumulated substantial lipid in the stationary phase when growth was limited by the depletion of nitrogen from the medium. Furthermore, similar lipid concentrations were achieved in chemostat cultures of these yeasts providing the medium had a high glucose to nitrogen ratio and a dilution rate of 0.02 h^{-1} to 0.2 h^{-1} was used. In nitrogen-limited chemostat cultures of *L. starkeyi* CBS 1809 the calculated specific rates of lipid synthesis (g lipid synthesized per g fat-free yeast per h) were higher than those observed for carbon-limited cultures, but this parameter showed no correlation with the prevailing lipid content when measured as a function of growth rate. Therefore, lipid did not accumulate simply because nitrogen-limitation induced increased lipogenic rates.

In subsequent experiments various parameters were monitored during transitions from steady state carbon-limited to steady state nitrogen-limited conditions. A sequence of events was identified initiated by the onset of nitrogen-limitation and culminating in the accumulation of lipid. The results were consistent with the premise that the primary event responsible for the diversion of carbon flow from oxidative pathways into lipogenesis was the decrease in the intracellular AMP concentration with a concomitant increase in adenylate energy charge.

Isotopic uptake studies indicated that the principal lipogenic pathway operating in oleaginous yeasts was that involving ATP:citrate lyase and that this pathway was non-operative in non-oleaginous yeasts. In certain oleaginous yeasts there was evidence that the supply of acetyl units for lipogenesis might be supplemented via a pathway involving

phosphoketolases but that this was not ubiquitous.

In studies with isolated enzymes citrate synthase purified from *Candida* 107 was shown to be inhibited by adenine nucleotides (ATP > ADP > AMP) and by long chain fatty acyl-CoA esters. In each case evidence was provided to show that the inhibition was unlikely to be of physiological significance. Therefore, there was no reason to think that citrate synthase activity would be restricted when cultural conditions favoured lipogenesis.

Studies with NAD^+-dependent isocitrate dehydrogenase indicated that enzyme activity was likely to be dependent on the relative concentrations of AMP, ATP and isocitrate and that variations in the concentrations of these metabolites would probably be sufficient to control the flux of carbon-flow between oxidative and lipogenic pathways.

ATP:citrate lyase was shown to be present only in oleaginous yeasts but its presence gave no indication of the prevailing lipid content. However, strong evidence was provided that the enzyme might be of regulatory importance in controlling the rate and possibly extent of lipid accumulation by a feed-back control mechanism involving interactions with long chain fatty acyl-CoA esters. In addition, there was some evidence that ATP:citrate lyase might catalyze the rate-determining step for lipogenesis in oleaginous yeasts and not acetyl-CoA carboxylase as has hitherto been supposed.

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