The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms

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I. Introduction

The concept of using microorganisms as sources of oils and fats has a long history. The commercial opportunities of such processes have been continuously examined for nearly a 100 years, though today such opportunities are confined to the production of the very highest valued

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oils—those containing nutritionally important polyunsaturated fatty acids. It has also long been known that some microorganisms have a greater propensity to accumulate substantial amounts of oil, sometimes up to and even in excess of 70% of their biomass weight, while other microorganisms remain stubbornly slim even when given the greatest encouragement to become obese. However, the reasons for microbial obesity, to adapt the common parlance for excessive lipid accumulation within an organism, have remained obscure until relatively recently. This review has therefore been undertaken to describe the work that has been carried out, mainly in the authors' laboratory, to elucidate this phenomenon: Why can some organisms accumulate lipids to a considerable extent and others cannot? There has to be a biochemical explanation for this difference. Knowing this reason should then lead to an understanding of the genetic constitution of the lipid-accumulating organisms and a determination of which genes are important for the process.

There is, though, a subsidiary problem that, perhaps, is even more intriguing than understanding the basic mechanism for lipid accumulation itself. Even among lipid-accumulating microorganisms there is a considerable difference between the extent to which lipid might accumulate to a maximal level. What determines these differences? Why should some microorganisms have an apparent limit to the accumulation of oil of, say, 30% of their biomass, whereas other organisms, even closely related ones, accumulate 50% oil and still others go even higher. Uncovering a biochemical explanation for these differences should then lead to elucidation of the genetical basis of microbial obesity.

We hope, therefore, in this review to present evidence that allows us to establish working hypotheses to explain the process of lipid accumulation, as well as the factors governing the extent to which this can take place, in both biochemical and molecular biological terms. We believe these explanations will also be applicable outside the microorganisms and may provide useful insights into the possible mechanisms of lipid accumulation in plant oilseeds and maybe even in animal cells as well. The questions that we addressed over many years of research are central to lipid accumulation processes in all cells even though the vast majority of our work has been confined to microbial systems. Ultimately, by being able to identify, first, the biochemical reasons for lipid accumulation and then the genes coding for the key enzymes, whatever they may turn out to be, we hope that it will become possible to modulate lipid accumulation processes. It should be possible to increase the amount of lipid that a cell might accumulate, but equally, it should also be possible to reverse this and curtail lipid accumulation. The first would be advantageous if the lipid itself were the product; the second would be

desirable if lipid represented a wasteful drain of carbon away from some other more valuable product as might happen during the production of secondary metabolites (see, for example, Jacklin *et al.*, 2000).

Because the study of lipid accumulation has a long historical record, we begin this review by a brief synopsis of the background to this subject. We then go on to describe, again fairly briefly, some of the more recent attempts to produce commercially useful microbial oils, now euphemistically known as *Single Cell Oils*. This then provides the background to the biochemical explanation of how it all happens. Finally, we indicate how the biochemistry of the process has helped show how the whole process of lipid biosynthesis is a closely integrated series of reactions.

II. The Development of Single Cell Oils

The study of microbial lipids has a long history going back to the mid-1870s (Ficinus, 1873; Nageli and Loew, 1878; see also Ratledge, 1984, 1992). Considerations for using microbial oils as sources of commodity oils and fats were made throughout most of the last century, with serious efforts being made in Germany during both world wars to develop processes that would provide useful amounts of oils and fats for a country denied access to major supplies of such commodities. Not surprisingly, major advances in identifying appropriate lipid-producing organisms took place in Germany from about 1920 to 1945 (Bernhauer, 1943; Bernhauer and Rauch, 1948; Hesse, 1949). Interest in other countries, including the United States and the United Kingdom, in the possible commercial aspects of developing microbial oils was though evident right up to the end of the 1950s (see Woodbine, 1959, for an authoritative review of the work that was done in the first half of the twentieth century). However, the considerable developments in agriculture that took place after 1945 meant that very cheap supplies not only of oils and fats could be assured but also for all other food sources as well. Consequently, it was realized that oils derived from microorganisms would never be able to compete in terms of price with the bulk commodity oils, such as soybean oil, sunflower oil, and more recently, rapeseed (or canola) oil. Interest in developing biotechnological processes for microbial oil production then virtually ceased, being considered a complete waste of time.

In the early 1960s, however, considerable interest was awakened by the prospects of producing protein by growing selected yeasts on cheap alkane feedstocks derived from petroleum refineries. The era of Single Cell Protein (SCP) arrived (see, for example, Rose, 1979). The concept

was taken up by many of the major oil companies as a simple means of producing a cheap animal feed, and its use for human consumption was not ruled out. SCP production was not only an innovative idea but it also led to major developments in bioprocess technology, with fermentation units up to $5 \times 500\ m^3$ being developed, and for the conversion of natural gas (methane) or methanol derived from it into SCP, single fermenters of up to $1500\ m^3$ were eventually built.

It was then suggested (Ratledge, 1976, 1978) that, if SCP was an economic proposition with a value of no more than $300/tonne, then oils from microorganisms, which became known as Single Cell Oils (SCO) (Ratledge, 1976), could be an equally attractive commercial proposition. Unfortunately, historical events overtook both SCP and SCO processes, which led to major reconsiderations of their value. What had not been appreciated at the time of developing SCP processes was that agricultural developments would be enormous during the second half of the twentieth century. Greater yields of crops, with improved varieties of plants together with better agricultural technologies, drove down the price of major sources of animal feed materials, such as soybean meal, so that the prices in real terms hardly rose at all between 1960 and 1990. SCP processes became even more uneconomic when Organization of Petroleum Exporting Countries (OPEC) countries increased the world price of oil in the 1970s, thereby escalating the price of the very feedstock to be used in the these processes. Little has changed in the intervening years, and consequently there are no currently used SCP processes based on using alkanes as a feedstock.

With microbial oils there was, however, an alternative strategy. Although it was always evident that microbial oils could never compete commercially with the major commodity plant oils, there were commercially opportunities for the production of some of the higher valued oils. Although protein is always just "protein," with only minor variations in its nutritional qualities from different sources, oils are not just "oils." The price range of oils can vary enormously and the price of individual fatty acids that go to make up the oils can vary from as little as $0.30/kg to over $100/kg (Gunstone, 1997, 2001). If microorganisms could be identified that could produce some of the highest valued oils, then commercial development of them would still be a reality. And this is exactly what has happened to SCOs which, since the early 1980s, have moved increasingly toward the very highest valued materials, materials that are expensive simply because no abundant source of them currently exists.

Table I illustrates the wide range of fatty acids found in microorganisms, mostly eukaryotic species that produce triacylglycerol oils (see Fig. 1) and thus can be directly compared, in terms of their chemical

TABLE I

LIPID CONTENTS AND FATTY ACID PROFILES OF SOME OLEAGINOUS, HETEROPTROPHIC MICROORGANISMS USED, OR CONSIDERED FOR USE, AS SOURCES OF SCOs[a]

	Lipid (% w/w)	Major fatty acid residues (rel. % w/w)											
		14:0	16:0	16:1	18:0	18:1	18:2	18:3 (n-3)	18:3 (n-6)	20:4 (n-6)	20:5 (n-3)	22:6 (n-3)	Others
Yeasts													
Cryptococcus curvatus	58	—	32	—	15	44	8	—	—	—	—	—	
Lipomyces starkeyi	63	—	34	6	5	51	3	—	—	—	—	—	
Rhodosporidium toruloides	66	—	18	3	3	66	—	—	—	—	—	—	23:0 (3%) 24:0 (6%)
Rhodotorula glutinis	72	—	37	1	3	47	8	—	—	—	—	—	
Rhodotorula graminis	36	—	30	2	12	36	15	4	—	—	—	—	
Yarrowia lipolytica	36	—	11	6	1	28	51	1	—	—	—	—	
Molds													
Entomophthora coronata	43	31	9	—	2	14	2	—	4	4	—	—	20:1 (13%) 22:1 (8%)
Cunninghamella japonica	60	—	16	—	14	48	14	—	8	—	—	—	
Mortierella alpina	50	—	19	—	8	28	9	—	8	21	—	—	20:3 (7%)
Mucor circinelloides	25	—	22	1	5	38	10	—	15	—	—	—	
Pythium ultimum	48	7	15	—	2	20	16	1	—	11	14	—	20:1 (5%)
Algae(grown heterotrophically)													
Crypthecodinium cohnii	40	16	16	1	—	21	1	—	—	—	—	40	
Schizochytrium limacinum[b]	50	4	56	—	1	—	—	—	—	—	—	30	15:0 (2%) 22:5 (n-6) (6%)
Thraustochytrium aureum	15	3	8	—	—	16	—	—	—	3	—	52	

[a] Data mainly from Ratledge (1997, 2001).
[b] From Yokochi *et al.* (1998).

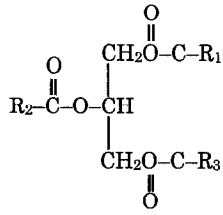

FIG. 1. Structure of a triacylglycerol and the nomenclature used to note the various fatty acids, where R_1CO—, R_2CO—, and R_3CO— are long acyl chains that may be either saturated, mono-unsaturated, diunsaturated, or polyunsaturated. *Nomenclature* of fatty acids is usually given in the from of *x*:*y*, where *x* denotes the number of C atoms and *y* the number of double bonds. As the double bonds in polyunsaturated fatty acids ($y = 3$ or more) are usually methylene interrupted (i.e., —CH:CH—CH_2—CH:CH—), it is only necessary to specify the position of the final bond in a chain. This is normally using the n-a (or ω-a) system where "a" denotes the number of C atoms from the methyl end of the chain to the position of the last double bond. Thus 18:3 (n-3) denotes a C_{18} chain with three double bonds at positions 9, 12, and 15 (counting the carboxyl group as no. 1) so that the final bond (between C15 and C16) is n-3 from the end. The alternative is to specify the position of each double bonds individually, i.e., 18:3(9, 12, 15). Unless stated otherwise, all double bonds may be arranged to be in the *cis* (or *Z*) configuration. (For further details and information, see Ratledge and Wilkinson, 1988.)

composition, to the oils and fats obtained from plant oilseeds. Another major consideration in evaluating the potential of a microorganism for oil production is the amount of oil it can produce. Obviously the more oil a microbial cell can accumulate, the more attractive it will be from a commercial viewpoint. Both the quality and quantity of the oil varied from organism to organism. Reviews on microbial lipids, and particularly on yeast lipids, divided species into high oil producers and low oil producers (Rattray *et al.*, 1975; Rattray, 1989; Ratledge and Evans, 1989). Some yeasts, such as *Saccharomyces cerevisiae* or *Candida utilis,* never accumulated much above 10% of their cell mass as lipid, but other yeasts, such as species of *Rhodotorula* and *Lipomyces,* could accumulate 70% and even more of their biomass as lipid. Moreover, the majority of this lipid was in the triacylglycerol form (Rattray, 1989), and therefore equivalent in chemical composition to the commercial oils and fats. Those microorganisms that could accumulate lipid to more than about 20% of their biomass (this was an arbitary, though useful, cutoff point dividing the accumulators from the nonaccumulators) were termed the *oleaginous* species (Thorpe and Ratledge, 1972), an epithet that seems to have stuck in just the same way that SCOs appears to have stayed in common use. [It should be pointed out, though, that the word *oleaginous* was first used in the 17th century (Oxford English Dictionary) and is not a neologism.]

III. Microorganisms as Sources of High-Valued Oils

A. Yeast Oils as Possible Cocoa Butter Equivalent Material

In the quest for microbial oils that could be produced economically, attention has increasingly focused on the highest valued materials. In the 1980s, selected yeasts were used to produce a cocoa butter equivalent (CBE) (see Table II)—that is, a triacylglycerol with equal amounts of stearate, oleate, and palmitate esterified to glycerol (see Smith, 2001). The research was undertaken principally in the Netherlands and New Zealand, but also in the United Kingdom and Canada (Moreton, 1988; Beaven *et al.*, 1992; Davies, 1992; Smit *et al.*, 1992), and used a variety of strategies to increase the amount of stearate in the yeast lipid as this fatty acid was normally less than 10% of the total fatty acids (see Table II). The most successful strategy used a mutant in which the Δ9 desaturase for the conversion of stearate to oleate (see also Fig. 2) was partially blocked so that stearate accumulated at the expense of oleate. The ensuing lipid then had the correct properties for its use as a CBE (Davies, 1992; Davies and Holdsworth, 1992). Unfortunately, during the time that it took to carry out this research the world price of cocoa butter, and to which of course the price of a CBE is related, fell from over $8000/tonne to less than $2500. The margin for profit from the yeast process then vanished.

TABLE II

Profile of Fatty Acid Composition of Yeast SCOs Used as Sources of CBE Fats and Compared to Cocoa Butter Itself[a]

	Relative fatty acyl composition (% w/w)				
	16:0	18:0	18:1	18:2	18:3
Yeast isolate K7-4[b]	20	24	40	7	2
Rhodosporidium toruloides[c]	28	7	40	18	5
Rhodosporidium toruloides[c,d]	20	47	22	5	2
Cryptococcus curvatus F33.10[e]	24	31	30	6	—
Cocoa butter	28	35	35	2	—

[a] (For further information, see Ratledge, 1994, 1997).

[b] From Davies and Holdsworth (1992).

[c] From Moreton (1988).

[d] With Δ9 and Δ12 cyclopropene $C_{18:1}$ fatty acids added each at 0.3 mg/liter to inhibit the Δ9 and Δ12 desaturases.

[e] From Verwoert *et al.* (1989): this is a hybrid yeast derived from an auxotrophic mutant with a diminished activity of the Δ9 desaturase (see Fig. 2).

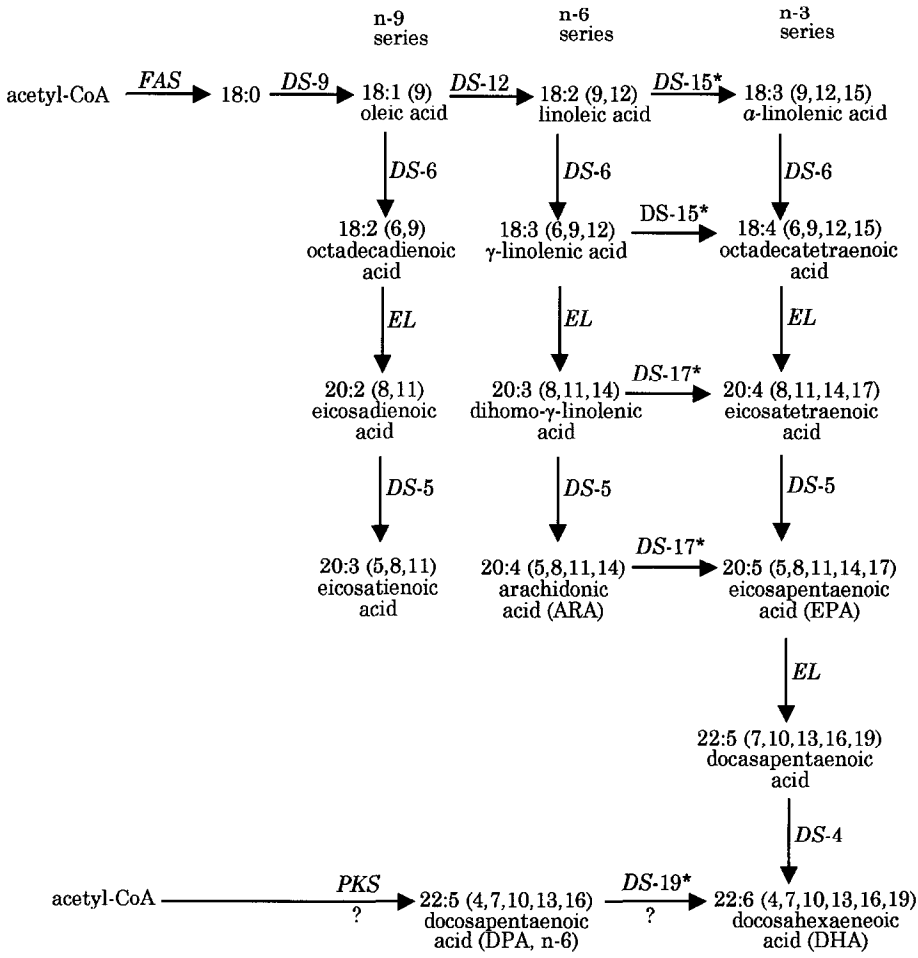

FIG. 2. Routes of polyunsaturated fatty acid biosynthesis in fungi, microalgae, and thraustochytrids (Updated from Ratledge, 2001). FAS: Fatty acid synthase; PKS: polyketide synthase. DS: A desaturase operating the carbon atom indicated (thus DS-15 is the Δ15 desaturase introducing a double bond between carbons 15 and 16 in the fatty acyl group). EL: An elongase; this is a four-component system involving a condensing enzyme, a reductase, followed by a dehydratase and a further reductase. Asterisks: These could all be regarded as n-3 desaturases—that is, introducing a double bond between the n-3 and n-2 carbon atoms. Question mark: Uncertain sequence in thraustocytrids leading to formation of DPA (n-6) and DHA. The synthesis of these fatty acids may not occur via the usual FAS route but by a complete separate PKS (see Metz *et al.*, 2001).

Nevertheless, the process know-how for the production of a CBE–SCO (i.e., a cocoa butter equivalent–single cell oil) as developed in New Zealand and the Netherlands (Davies, 1992; Smit *et al.,* 1992) is still valid today. The process is currently not considered to be economic even though the feedstock used for the growth of the yeast is whey, which is essentially zero cost. The fermentable substrate within the whey was lactose and the whey itself was derived from cheese creameries that, in New Zealand, posed environmental problems for its disposal. Even under these conditions, the operating costs of the fermentation plant coupled with the costs of oil extraction and refinement (which are still needed with microbial SCOs) were higher than could be borne by the final selling price that was dictated by the world price of cocoa butter itself (see Davies, 1992). Interestingly, it is predicted that by 2004, there is likely to be a shortfall in cocoa beans (and therefore of cocoa butter itself) of some 250,000 tonnes (Smith, 2001). The prospects therefore of the price of a CBE rising considerably in the next three years cannot be ruled out. Opportunities for a profitable CBE–SCO process may once more arise.

B. Possibilities for Producing Polyunsaturated Fatty Acids

Since the demise of the CBE–SCO process, interests in the commercial development of SCOs have concentrated almost exclusively on the newly developing market for polyunsaturated fatty acids. This area has been recently extensively reviewed (Certik and Shimizu, 1999; Ratledge, 2001), and readers who require details of the current approaches being taken to produce these materials are referred to one or both of these reviews for details that may not be provided here.

Polyunsaturated fatty acids (PUFAs), whose pathways of synthesis are given in Figure 2, are currently in increasing demand as dietary supplements, loosely termed *nutraceuticals,* for both adults as well as infants. Nutritional recommendations from a number of authoritative sources advise for the inclusion of PUFAs, especially the longer chained and more unsaturated fatty acids, in the diet for the prevention of coronary heart problems and also for the improvement of retinal and brain functions (Huang and Sinclair, 1998). At present, the nonmicrobial sources of these materials are from marine sources including many endangered species of fish. Thus, there is now a major activity in producing some of these PUFAs from alternate microbial sources.

1. γ-Linolenic Acid (18:3n-6)

The first PUFA–SCO that was produced commercially was γ-linolenic acid, 18:3n-6 (for the notation used to describe fatty acids, see Fig. 1).

γ-Linolenic acid (GLA) is found in a relatively small number of plant seed oils, principal among which is evening primrose oil, where it constitutes only about 8–10% of the total fatty acids. Evening primrose oil commands a price of about $15/kg. Borage oil, which contains 20–23% GLA, sells at about $35/kg (Clough, 2001). Both these oils sell as over-the-counter nutraceuticals in the United Kingdom and Europe for the relief or treatment of a number of minor complaints and problems, of which the relief of premenstrual tension is a major claim. It is also prescribed for the treatment of eczema, especially in children where it appears to be particularly effective (Huang and Ziboh, 2001). Development of a biotechnological route to produce an equivalent SCO rich in GLA has been described in detail elsewhere (Ratledge, 1992). This process, which was in commercial production in the United Kingdom from 1985 to 1990, and therefore was the world's first SCO to be offered for sale, used the filamentous fungus, *Mucor circinelloides*. The fatty acyl composition of the oil from this organism is given in Table I. The oil was given a clean bill of health and approved for human consumption as the fungus itself has long been associated with oriental food materials and therefore has a record of safe ingestion over several millennia.

Although the fungal oil contained about twice the concentration of GLA as did evening primrose oil, it experienced, perhaps not surprisingly, some marketing problems. Moreover, the price of evening primrose was deliberately decreased so as to become more competitive and, simultaneously, borage oil (also known as starflower oil) was developed as an improved plant source of GLA. "Oil of Javanicus," as the GLA–SCO was known, was thus forced out of the market by price reductions in the material it sought to replace and by the arrival of a cheaper product on the market. Should there come a demand for GLA at a high purity, then the fungal oil probably represents the best source of starting material as purification of GLA is easiest starting with an oil with a low content of other PUFAs that will then not interfere with the isolation of GLA. Both borage oil and evening primrose oil contain relatively high contents of linoleic acid (18:2)—40 and 70%, respectively—but this fatty acid is much less in the *Mucor* oil (see Table I). However, a commercial demand for a high-purity GLA material has not yet arisen.

2. Arachidonic Acid (20:4n-6)

After GLA, arachidonic acid (ARA) (20:4n-6) was the next PUFA–SCO to be developed and processes for its production continue today. ARA is incorporated into infant feed formula where it is considered a desirable component to be added along with docosahexaenoic acid (DHA)—see below. Traditional sources of ARA are egg yolks and animal livers. The former source yields various phospholipids rich in ARA that

are then used as such while the latter source, although containing ARA as a triacylglycerol, is not acceptable vegetarians. Microbial sources of ARA being actively developed have mainly used *Mortierella alpina* as the best producing organism. Currently, large scale processes exist in both Japan and Europe for its production. Although *Mortierella alpina* is related to *Mucor,* it has not been recorded as having a long term association with any oriential foodstuff as had *Mucor;* so a lot of work has been done to demonstrate its safety (summarized by Streekstra, 1997; see also Kyle, 1997a, 1997b). Approval for the use of this ARA–SCO has recently been given by the Food and Drug Authority (FDA) of the United States (Anonymous, 2001; see also FDA net link: www.cfsan.fda.gov/~rdb/opa-g041.html).

3. Eicosapentaenoic Acid (20:5n-3) and Docosahexaenoic Acid (22:6n-3)

Other PUFAs that are being produced by, or are capable of production by, microorganisms are eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3). These two PUFAs occur together in the oils of many fish, and recommendations for their use as dietary supplements for the prevention of cardiac problems in older people have long been advocated. Stocks of many fish species are now dwindling rapidly and the increasing presence of pollutants in the marine environment (many of which are concentrated in the livers of fish that are the major sources of these PUFAs) cause concern for the future and safe supplies of these fatty acids.

Additionally, and in favor of an SCO route to production, is the requirement for DHA to be produced in an oil without the presence of EPA, which is thought to compete against DHA for incorporation into key retinal and neural brain lipids (Gibson *et al.,* 1998). As it is very difficult to remove EPA from the mixture of DHA and EPA in fish oils, this has provided further impetus to develop microorganisms as sources of DHA as species are known that produce this PUFA without the presence of EPA (see Table II).

There are two principal organisms currently used commercially for production of an oil rich in DHA: *Crypthecodinium cohnii* and the thraustochytrid group of marine microorganisms. The former is a marine dinoflagellate that has been known for some time as a DHA producer (Harrington and Holz, 1968), but it is only within the last 10 years or so that it has been developed into a commercial process (Kyle, 1992). The organism is nonphotosynthetic and is therefore grown heterotrophically. The process is operated in the United States by Martek Biosciences Corp. and uses stirred tank fermenters up to 110 m^3 with glucose as the principal feedstock (Kyle, 1996, 2001). A refined triacylglycerol oil is

produced that contains 40% of the fatty acids as DHA. Its approval for incorporation into infant food formula has been given by the FDA (Anonymous, 2001) provided that it is given along with arachidonic acid (see above). The oil is also available in many countries as an over-the-counter nutritional supplement for adults (Becker and Kyle, 1998; Haumann, 1997, 1998).

The thraustochytrid group of marine organisms, originally classified as marine fungi, are now placed into a unique phyllum—*Heterokonta,* within the class of Labyrinthista (Dick, 2001). *Thraustochytrium* spp. and *Schizochytrium* spp. are the principal organisms that have been investigated for DHA production (Bajpai *et al.,* 1991a, 1991b; Barclay, 1991; Kendrick and Ratledge, 1992a; Barclay *et al.,* 1994; Nakahara *et al.,* 1996; Yaguchi *et al.,* 1997; Bowles *et al.,* 1999). In all cases, the oil not only contains DHA (see Table I) but also docosapentaenoic acid, DPA (22:5n-6). Although this particular PUFA is somewhat rare in oils from any source, and it is uncertain by which route it may be synthesized (see Fig. 2), it does not seem to be deletrious to the efficacy of DHA in its incorporation into key membrane lipids of the human body. While thraustochytrid oils are not yet incorporated into infant formula, the whole organism is currently used as a supplement for poultry feeding, which then produce eggs rich in DHA. The presence of DPA in the egg is very much lower than in the original oil (Abril and Barclay, 1998).

Both thrautochytrid and *Crypthecodium* biomass and oils can also be used in fish feeding, particularly to increase the rate of growth of young fish larvae and fry within hatcheries.

Opportunities to develop microorganisms for the production of oils rich in EPA have also been considered (Yongmanitchai and Ward, 1989; Vazhappilly and Chen, 1998; see also Ratledge, 2001) but the market for such oils is uncertain though various claims have recently been made for the efficacy of such materials in the treatment of certain mental disorders including schizophrenia (Fenton *et al.,* 2000; Peet *et al.,* 2000). At the moment, no process for the commercial production of such an oil is in operation, but this may have to be quickly rectified if current reports on the effectiveness of EPA to act against "wasting," which is symptomatic of cancer patients with a poor prognosis, prove to be substantiated in further clinical trials of this PUFA (Tisdale, 1999).

While emphasis for the production of desirable PUFAs has been placed on heterotrophic organisms (*Crypthecodium* and thrautochytrids are heterotrophs), considerable research is underway in many places throughout the world to develop processes to produce DHA and the other desirable fatty acids using photosynthetic algae. However, costs of both building and operating photobioreactors are prohibitively expensive (Borowitzka, 1999) and the prospects of using open lagoons for algae growth are unlikely to meet the stringent safety requirements for

subsequent use of the oils in baby foods or even for consumption by adults. Nevertheless, algae are considered by many proponents to be worthy of serious consideration as sources of PUFA-rich oils (Cohen, 1999). They also can be used for fish feeding and may represent the best sources of a range of nutrients for larvae and fish fry that might not be available from other sources.

IV. Biochemistry of Oleaginicity

For the details of fatty acid biosynthesis in cell systems, almost any standard college biochemistry textbook can be consulted by the erudite reader. In this review we are only concerned with using this information to help elucidate the key questions surrounding the causes of oleaginicity in microorganisms. We do not discuss in any detail the activity and organization of enzymes such as fatty acid synthetase or of acetyl-coenzyme A carboxylase except where these impinge upon other activities that we consider can explain the reasons of oleaginicity in microorganisms.

A. Patterns of Lipid Accumulation

Lipid accumulation in oleaginous microorganisms has long been known to be triggered by a nutrient imbalance in the culture medium. When cells run out of a key nutrient, usually nitrogen, excess carbon substrate continues to be assimilated by the cells and converted into storage fat. This is shown diagrammatically in Figure 3A. This pattern is observed in the lipid-accumulating yeasts and filamentous fungi, though it might not apply in photosynthetic algae, or the heterotrophic algae, *Crypthecodinium cohnii,* nor in thraustochytrids, that are of current interest for PUFA production (see above). In these organisms the growth rate is probably lower than the intrinsic rate of lipid biosynthesis. Cells assimilate carbon quicker than they can convert it into new cells so mechanism for storage the excess carbon is then found by converting it into lipid. A possible scenario for lipid accumulation in these organisms is shown in Figure 3B. This pattern of growth-associated lipid accumulation has also been found with a single strain of an oleaginous yeast, *Cryptococcus terricolus* (Boulton and Ratledge, 1984), but this seems to be an exception among yeasts.

With the "normal" oleaginous yeast or mold, the process of lipid accumulation can also be achieved in continuous culture (see Fig. 3C), where is it necessary to grow the cells at a sufficiently low dilution rate (= growth rate) to allow the cells to assimilate the glucose. The results from continuous cultivation studies clearly indicate that the rate of lipid synthesis is slower than the maximum growth rate.

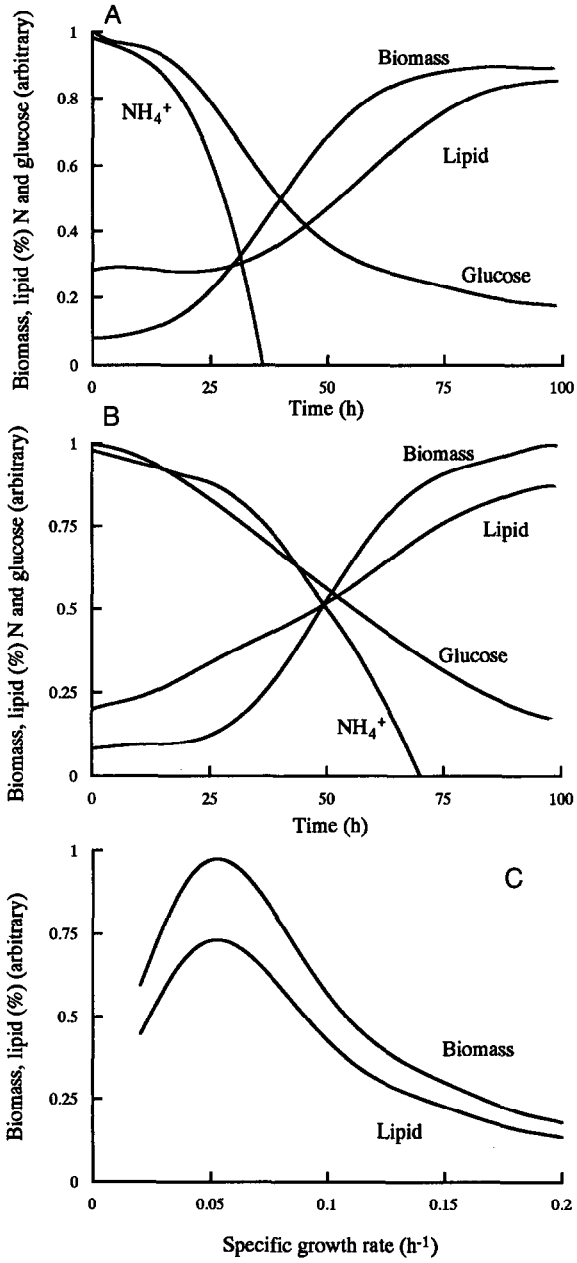

FIG. 3. Schematic representation of the course of lipid accumulation in oleaginous microorganisms. (A) Lipid accumulation in a batch culture system that is typical of oleaginous yeasts and filamentous fungi showing that lipid accumulation does not commence until nitrogen is exhausted from the medium. (B) Pattern of lipid accumulation in a

B. Possible Biochemical Reasons for Oleaginicity

With respect to lipid accumulation in yeasts and fungi, where a nutrient imbalance is need to engender the process, and where the pathway of fatty acid biosynthesis is the same in both oleaginous organism and nonoleaginous organisms (as indeed it is), the obvious question to ask is: What is the biochemical difference between these two groups of very distinct microorganisms?

Our laboratories starting studying this question over 20 years ago. We wanted to understand how two yeasts, placed in exactly the same growth medium, with the same nitrogen limitation after 24 h growth, would result in one accumulating in excess of 40% of its biomass as lipid, while the other would not. The yeasts used in this initial comparison were the nonoleaginous *C. utilis,* otherwise known as the food yeast, and an oleaginous yeast known as *Candida* sp. no. 107. We could have used *S. cerevisiae,* or indeed, any one of about 570 other species of yeast as controls. Only about 25–30 species are known to be capable of lipid accumulation—i.e., are "oleaginous" species (Ratledge and Evans, 1989; Rattray, 1989).

We considered four possible reasons why some yeasts might accumulate lipid (Botham and Ratledge, 1979):

- That upon nitrogen exhaustion from the medium, the nonoleaginous species would cease to assimilate glucose and thus no acetate units would be generated to act as the starting point for fatty acid biosynthesis.
- That acetyl-CoA carboxylase, the first committed reaction of fatty acid biosynthesis [considered by many at that time (Volpe and Vagelos, 1976), and even still today (Ivessa *et al.,* 1997; Davis *et al.,* 2000) to be the rate-limiting step of fatty acid biosynthesis] may be hyperactive in the oleaginous yeast. Alternatively, in the nonoleaginous yeast, this enzyme could be repressed or subject to feedback inhibition by a fatty acyl-CoA ester as the end product of fatty acid synthetase.
- That in the nonoleaginous yeast, there may be a futile cycle of lipid biosynthesis simultaneously accompanied by lipid oxidation so that there would be no net lipid accumulation.

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heterotrophically grown algae such as *Crypthecodium cohnii* or a thraustochytrid. Lipid accumulates during the growth phase and does not depend upon exhaustion of the nitrogen supply. (C) Pattern of lipid accumulation in continuous culture of a yeast or filamentous fungus growing in N-limited medium. Lipid accumulation requires a slow growth rate of the cells to allow the excess carbon to be assimilated faster than it can be converted into biomass so that the surplus carbon is channeled into lipid.

- Intermediary metabolism may be differently regulated in the two types of yeast so that in the oleaginous species there would be an increased flux of carbon into acetyl-CoA, or alternatively in the nonoleaginous species, this flux would be diminished by cellular regulatory processes.

We were able, by appropriate experimentation, to eliminate the first three possibilities: the two yeasts, the oleaginous and the nonoleaginous ones, were more or less the same with respect to glucose assimilation both before and after nitrogen exhaustion from the growth medium; both had equal activities of acetyl-CoA carboxylase, there was no discernable difference in their regulation, and there was no lipid turnover in either of them (Botham and Ratledge, 1979). This left the fourth possibility, which was not as clearly defined as the other options, and therefore a certain amount of guesswork had to take place as to what might be an appropriate experiment to carry out to determine if there were differences in cellular regulation between the two types of yeast.

C. The Role of AMP

Fortunately, at the time of the mid-1970s, the concept of the "energy charge," in which the prevailing cellular concentrations of ATP, ADP, and AMP were computed (Atkinson, 1977) to give a numerical value for the metabolic standing of the cell, was in current vogue. In order to calculate this energy charge, it was necessary to measure the intracellular concentration of the adenine nucleotides in the cell. This was done, and while the calculated energy charge values were different between the two yeasts, the most obvious difference between the yeasts was that in the oleaginous strain the content of AMP fell under N-limited growth conditions to less than 5% of its value under C-limited conditions. In the nonoleaginous yeast, *C. utilis,* the AMP concentration fell by very little (Botham and Ratledge, 1979).

In summary, we were able to see a massive change in the intracellular concentration of AMP during the lipid accumulation stage in the oleaginous yeast. These changes were subsequently confirmed by Boulton and Ratledge (1983b) using chemostat cultures of oleaginous yeasts undergoing a transition from C-limited growth (with N in excess) to N-limited growth (with C now in excess) (see Fig. 4). The AMP concentration in the cells fell abruptly as soon as the cells exhausted the nitrogen supply. This change, in fact, preceded the actual onset of lipid accumulation. N-limitation clearly started a cascade of biochemical events in the oleaginous yeast.

The sharp decrease in AMP concentration was not accompanied by an increase in either ADP or ATP (see Fig. 4B) and AMP deaminase

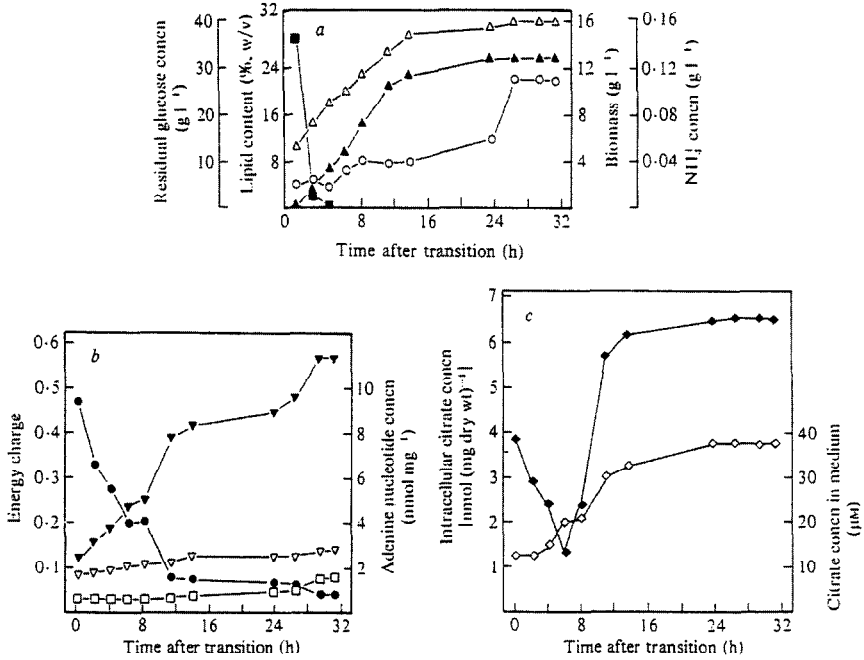

FIG. 4. Pattern of lipid accumulation in the oleaginous yeast, *Lipomyces starkeyi*, during transition from carbon-limited growth to nitrogen-limited growth. The yeast was in steady-state continuous culture growing at a constant rate of 0.06 h^{-1}; at zero time the medium was switched and effectively all residual NH_4^+ was consumed in about 3–4 h; the biomass began to increase immediately from time zero but lipid accumulation did not commence until after 8 h (A) during which time the AMP concentration had dropped by 80% (B) and citrate had begun to accumulate (C). (A) Biomass (△), lipid content of cells (○), concentration of NH_4^+ (■) and glucose (▲) in medium; (B) intracellular concentration of AMP (●); ADP (▽), ATP (□) and energy change (▼); c): intracellular (◆) and extracellular (◇) concentrations of citrate. (From Boulton and Ratledge, 1983a.)

was identified as the enzyme causing this change (Evans and Ratledge, 1985c):

$$\text{AMP} \longrightarrow \text{IMP} + \text{NH}_4 \qquad (1)$$

where IMP = inosine monophosphate. *AMP deaminase,* which had been characterized in *S. cerevisiae* (Yoshino *et al.*, 1979; Yoshino and Murakami, 1985), showed a sharp increase in activity in the oleaginous yeast as soon as the cells ran out of available nitrogen in the medium. (How this increase in activity is brought about is still not understood. It could well involve posttranscriptional modification of the enzyme as a result of a change in the intracellular concentration of NH_4^+ or of some key amino acid at the onset of nitrogen exhaustion from the medium. The enzyme can be regarded as an NH_4^+-scavenging enzyme, and therefore its increased activity when cells enter N-limitation could

be viewed as a means of garnering further nitrogen for protein and nucleic acid biosynthesis.) A similar sharp increase in AMP deaminase activity has been noted in *Mucor circinelloides* at the point of nitrogen exhaustion from the medium and the onset of lipid accumulation (Wynn *et al.*, 2001). This may therefore be a common event in oleaginous microorganisms whose growth is limited by nitrogen availability in the medium (Solodovnikova *et al.*, 1998).

D. Events Leading to the Biosynthesis of Acetyl-CoA

The rapid drop in AMP concentration at the onset of nitrogen limitation profoundly affected the activity of *isocitrate dehydrogenase (ICDH) (NAD$^+$-dependent)* within the oleaginous yeast (Botham and Ratledge, 1979). The reaction catalyzed by this enzyme,

$$\text{Isocitrate} + \text{NAD}^+ \longrightarrow \text{2-Oxoglutarate} + \text{NADH}. \quad (2)$$

is within the mitochondria, and in the oleaginous yeasts and molds, its activity is absolutely dependent upon AMP (Botham and Ratledge, 1979; Evans and Ratledge, 1985c; Wynn *et al.*, 2001). However in the non-oleaginous yeast, there was no discernable requirement of ICDH for AMP to be active (Botham and Ratledge, 1979). Related work with a citric acid-accumulating strain of *Candida lipolytica* (Mitsushima *et al.*, 1978) also found that nitrogen-limited growth of this yeast led to low AMP concentrations, a consequent shift-down in ICDH activity with the concomitant accumulation of citric acid in the cells and in the medium.

Nitrogen limitation would lead to an increase in AMP deaminase activity, which would then decrease the prevailing AMP concentration in the cells and the mitochondria (Mitsushima *et al.*, 1978; Bartels and Jensen, 1979), with the consequential drop in ICDH activity. The isocitrate, no longer effectively metabolised via the tricarboxylic acid cycle, would then equilibrate to citrate via the action of aconitase:

$$\text{Isocitrate} \longleftrightarrow \text{Aconitate} \longleftrightarrow \text{Citrate}. \quad (3)$$

Aconitase was found, as expected, to be equally active under both carbon-limited and nitrogen-limited growth conditions (Evans and Ratledge, 1983a; Evans *et al.*, 1983a, 1983b).

What we could not explain was the involvement of citrate in lipid biosynthesis. The obvious enzyme activity to investigate at this stage was the ATP:citrate lyase:

$$\text{Citrate} + \text{ATP} + \text{CoA} \longrightarrow \text{Acetyl-CoA} + \text{Oxaloacetate} + \text{ADP} + \text{P}_\text{i}. \quad (4)$$

A review on this enzyme (Srere, 1972) had stated that the enzyme had widespread distribution in most animal cell systems but "was absent

in yeast"! What was meant was that the enzyme was absent in *Saccharomyces cerevisiae*. However, ATP:citrate lyase was duly found in the oleaginous yeast but not in the nonoleaginous species, and was the first major biochemical difference to be identified between the two types of yeast. ATP:citrate lyase is discussed in further detail below as it has proved to be one of the key enzymes that must be present in a eukaryotic microbial cell for it to be able to accumulate substantial amounts of triacylglycerol lipids.

We could now provide a rational explanation as to how there could be metabolic channeling of carbon from glucose directly into fatty acid biosynthesis. Figure 5 shows how this information was used to describe the likely sequence of events in oleaginous yeasts; this metabolic channeling is, though, slightly different in oleaginous fungi (Wynn *et al.*, 2001).

As with yeasts, the process of lipid accumulation in the fungal species (*Mucor circinelloides* and *Mort. alpina*) begins when they run out of assimilatable N in the culture medium, which immediately causes a rapid increase in AMP deaminase activity. This, in turn, affects ICDH activity within the mitochondrion, causing a downturn in the TCA cycle

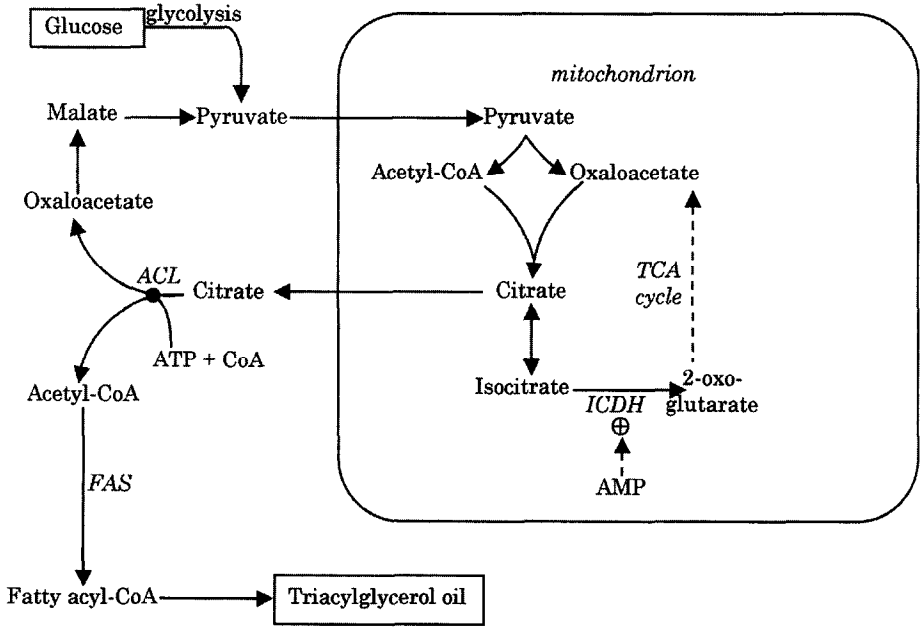

FIG. 5. Outline of the main sequence of events leading to lipid accumulation in oleaginous yeasts and molds. Lipid accumulation is triggered by a sequence of events described in the text. ICDH: isocitrate dehydrogenase (AMP dependent); TCA cycle: tricarboxylic acid cycle; ACL: ATP:citrate lyase; FAS: fatty acid synthase (see also Fig. 7).

activity, which can be detected by the falling output of CO_2 from the cultures. The time from N depletion to detecting a fall in levels of CO_2 was about 15–20 min. However, unlike the metabolic situation in yeasts, in the filamentous fungi the decline in AMP was matched by simultaneous downturns in concentrations of ADP and so that the overall energy charge with the cells remained largely unaltered during this transition into N-limited growth conditions. The key, though, to the initiation of lipid accumulation was considered to be the severe limitation to ICDH activity caused by the decrease in AMP concentration (Wynn *et al.*, 2001).

The following sections discuss the principal enzymes that are considered to be involved in the process of lipogenesis starting from glucose as the carbon source.

E. Glucose Uptake and Glycolysis

Very little is known about the regulation of glucose uptake in oleaginous microorganisms. It is likely to be a tightly regulated process. Work from Kubicek and his colleagues (Arisan-Atac *et al.*, 1996), examining the role of hexokinase in the regulation of glucose uptake into the citric acid-producing *Aspergillus niger,* showed that trehalose-6-phosphate was a major controlling metabolite for the activity of hexokinase. Genetic deletion of trehalose-6-phosphate synthetase led to a 20% increase in the productivity of citric acid accumulation by ensuring that hexokinase operated at its maximum possible activity. With respect to oleaginous yeasts, Botham and Ratledge, (1979) examined the rate of ^{14}C-labeled glucose into *Candida* sp. no. 107 and *Candida utilis,* and showed there was no evidence that glucose transport was a rate-limiting process for the growth of either yeast.

Of the various glycolytic enzymes, most attention has been paid to the possible regulation of *phosphofructokinase* (PFK) (see Fig. 6). Citrate acts as a strong inhibitor of this enzyme in most cells, but NH_4^+ can relieve this inhibition in both yeasts and fungi (Evans and Ratledge, 1984c; Wynn *et al.*, 2001). At physiological concentrations of fructose-6-phosphate and ATP (i.e., 1 m*M* in each case), the activity of PFK in *Rhodosporidium toruloides* was decreased to zero in presence of 5 m*M* citrate. However, in the presence of 10 m*M* NH_4^+ the K_i value for citrate was raised from 0.9 to 7.2 m*M*. What happens to the glycolytic flux once the nitrogen supply has been exhausted from the medium and the intracellular concentration of NH_4^+ is low? Glucose continues to be taken up by the cells and converted into lipid (see Fig. 3). In accordance with earlier work on this enzyme by Mavis and Stellwagen (1970), it was hypothesized that PFK could form a stable complex with ammonium

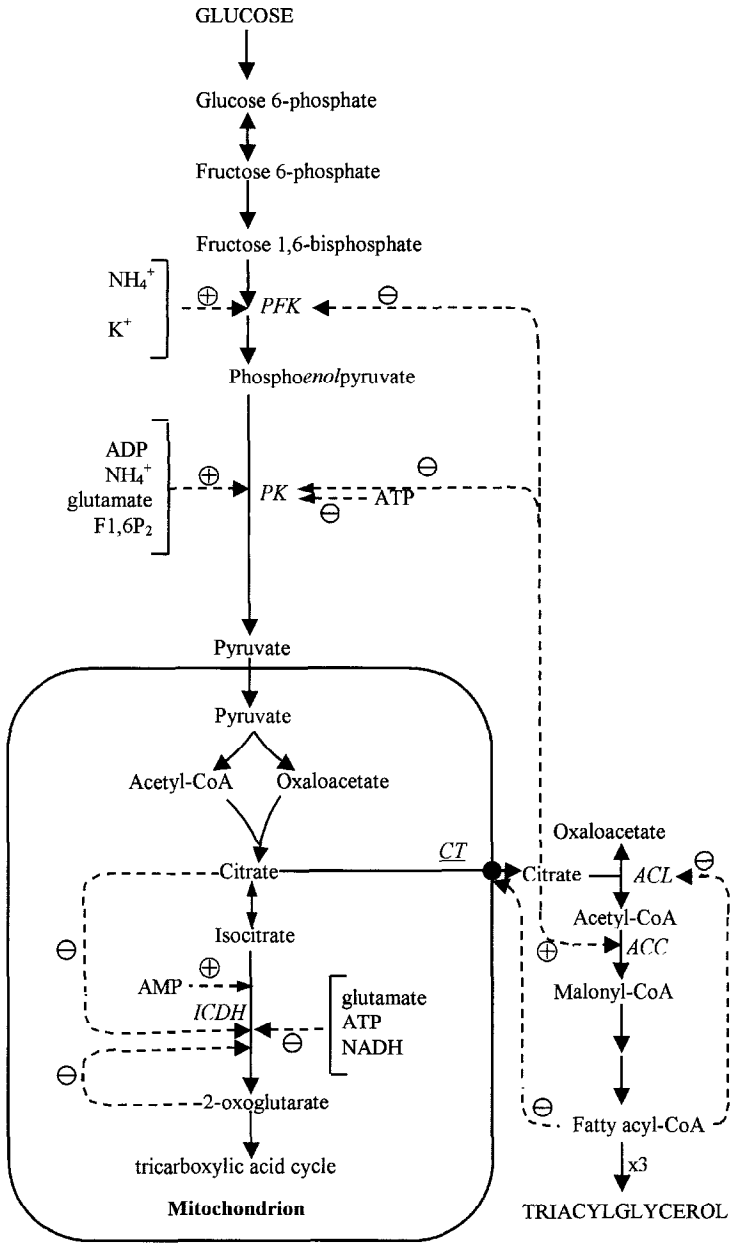

FIG. 6. Regulatory controls exerted by citrate and other metabolites on the flux of carbon to lipid. The key regulatory points appear to be at phosphofructokinase (PFK) pyruvate kinase (PK) and isocitrate dehydrogenase (ICDH). Acetyl-CoA carboxylase (ACC) may also be regulated by citrate. (Adapted from Evans and Ratledge, 1986.)

ions before the onset of nitrogen limitation. This complex was surmised not be susceptible to feedback inhibition by citrate (Evans and Ratledge, 1984c). However, the work of Evans and Ratledge (1984a, 1984b) was able to account for the increased concentration of lipid (up to 50% of the biomass) that accumulated in *Rh. toruloides* when it was grown with glutamate as a nitrogen source rather than ammonium salts (where the lipid content was less than 20% of the cell dry weight) by finding that the intracellular concentration of NH_4 was highest with the glutamate-grown cells and lowest with those grown on ammonium salts. Thus, in the glutamate-grown cells, the activity of PFK was not being controlled by feedback inhibition by citrate to the same extent that it was in the ammonium-grown cells.

Similar results have been recently reported for the feedback inhibition of PFK by citrate in the oleaginous mold, *Mucor circinelloides* (Wynn *et al.*, 2001), and these observations probably parallel those found in other fungi, including the citric acid producing *A. niger* (Roehr *et al.*, 1996). In both cases, it has to be argued that PFK is able to remain active in spite of the obvious increases in the concentration of intracellular citrate both in the oleaginous species and in the citrate producing one. It therefore seems reasonable to argue that PFK can indeed form a stable complex with NH_4, as was suggested above, which then resists the inhibitory effects of citrate.

Although PFK and pyruvate kinase (see below) are considered as the major regulatory enzymes for the control of the glycolytic flux, the overexpression of these enzymes by genetic manipulation (Ruijter *et al.*, 1997) has not led to any significant increase in the enhancement of the flux of carbon, at least in *A. niger,* suggesting that their regulation is more complex than previously thought.

F. The Role of Citrate

The key role of citrate was revealed through the work of Boulton and Ratledge (1983a) during an investigation into the changes in metabolite concentrations during the transition of oleaginous yeasts, *Candida* sp. no. 107 and *Lipomyces starkeyi,* from carbon limitation to nitrogen limitation (see Fig. 4). This work was done in continuous culture where the culture medium was changed abruptly from one that was low (limiting) in glucose, but with NH_4 in excess, to one that was high in glucose but with nitrogen now being the growth-limiting nutrient. One of the key events that occurred during the transition, and that preceded the onset of lipid accumulation, was in the large increase in intracellular citrate concentration (Fig. 4c). Biochemical events must have preceded

the accumulation of citrate itself; moreover, citrate was probably exerting metabolic control over the reactions described in Figure 6.

Besides regulating the activity of PFK (see above), citrate also regulates the activity of *pyruvate kinase* (Evans and Ratledge, 1985a). Like PFK, pyruvate kinase can be activated and inhibited by several metabolites (see Fig. 6). During lipogenesis it is crucial that the overall activity of the enzyme remains high so that a flux of carbon through pyruvate is maintained. Citrate also regulates the activity of several other key events (see Fig. 6 and also Evans and Ratledge, 1985d, where the role of citrate in the control of metabolism in the oleaginous yeast is discussed in detail). The intracellular concentration of citrate is a key determinant in both stimulating enzymes for fatty acid biosynthesis, e.g., acetyl-CoA carboxylase (see Fig. 6), and for down-regulating the activity of the tricarboxylic acid cycle, particularly at the level of isocitrate dehydrogenase (see above), as well as in regulating glycolysis (see Fig. 6).

Citrate must efflux from the mitochondrion, where it is formed, as a necessary prerequisite for its cleavage into acetyl-CoA by ATP:citrate lyase, which is a cytosolic enzyme. This efflux is a carefully controlled system. The *citrate translocase* (Evans *et al.*, 1983a, 1983b, 1983c) sometimes known as the *citrate/malate translocase,* is the translocating protein within the mitochondrial membrane, and involves the participation of malate moving into the mitochondrion as part of a concerted sequence of events. Malate is first synthesized in the cytosol by the action of malate dehydrogenase on oxaloacetate. The initial oxaloacetate to prime the cycle of events comes from the carboxylation of pyruvate. Once the citrate translocase has been primed with malate, then the effluxing citrate provides all the necessary oxaloacetate for the cycle to continue. Figure 7 shows how this process might operate in fungi (Wynn *et al.*, 2001).

In yeasts, the system may be slightly different as it is considered, though not universally accepted, that pyruvate carboxylase (PC) is within the mitochondrion (Evans and Ratledge, 1983a, 1983b) and a variation of Figure 7 therefore has been described (Evans and Ratledge, 1985c, 1985d) that takes account of this different location. The net reaction remains the same and the stoichiometry is unaltered.

PC has been described as a mitochondrial enzyme in certain oleaginous yeasts (Evans and Ratledge, 1983b), as well as be in animal cells (Bottger *et al.*, 1969; Taylor *et al.*, 1978). However, a cytosolic location for it has been reported in both oleaginous and nonoleaginous yeasts (van Urk *et al.*, 1989; Rohde *et al.*, 1991; Sokolov *et al.*, 1995). In filamentous fungi, in spite of an earlier report to the contrary (Purohit and Ratledge, 1988), the prevailing view is that in PC is a cytosolic enzyme

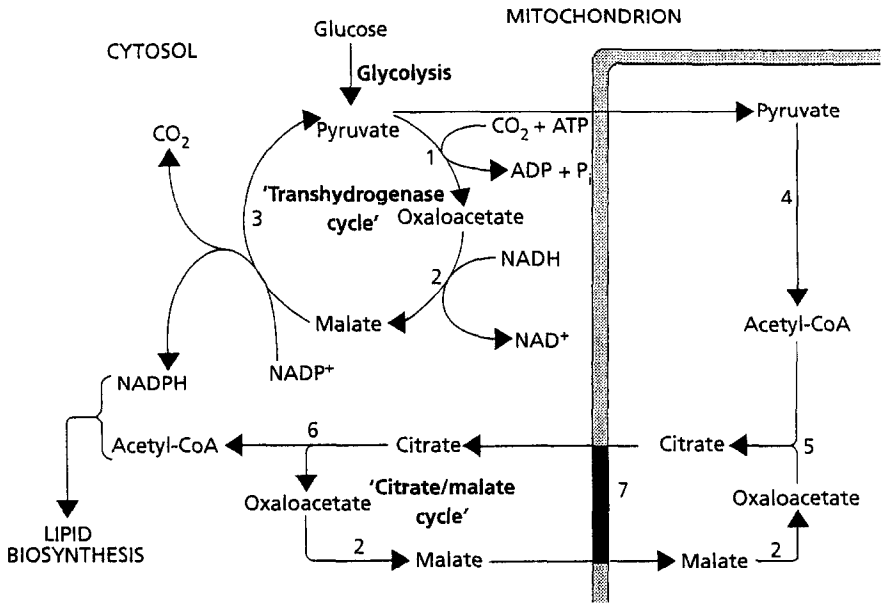

FIG. 7. Scheme showing how the proposed citrate/malate cycle and the cytosolic transhydrogenase cycle could provide sufficient precursors (acetyl-CoA and (NADPH) for lipogenesis in filamentous fungi. Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase; 3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase; 6, ATP:citrate synthase; 7, citrate/malate translocase Net carbon balance: pyruvate $\longrightarrow$ acetyl-CoA + CO_2. Net reaction for NADPH production (the transhydrogenase cycle): NADH + $NADP^+$ + ATP $\longrightarrow$ NAD^+ + NADPH + ADP + P_i. The transhydrogenase cycle can operate independently of the citrate/malate cycle and provide all the NADPH required both for fatty acid synthesis and for fatty acid desaturation.

(Osmani and Scrutton, 1985; Jaklitsch *et al.*, 1991), and has recently been confirmed for two oleaginous molds, *Mucor circinelloides* and *Mortierella alpina* (Wynn *et al.*, 2001). The scheme shown in Figure 7 is proposed with of PC being considered a cytosolic enzyme.

On the basis of the combined details given in Figures 6 and 7, some stochiometry of the total carbon flux from glucose to triacylglycerol can also be suggested (see also Ratledge, 1997). One mole glucose, when metabolized exclusively via glycolyis, generates two moles pyruvate; thus, it can be calculated that approximately 15 mole glucose are needed to synthesize 1 mole triacylglycerol; i.e., 100 g glucose will provide maximally 32 g lipid, assuming that glucose is not used for the synthesis of any other product—which, of course, it is. Under the best growth condition (i.e., in a chemostat) the highest yields of lipid that have been obtained are 22 g/100 g glucose used (Ratledge, 1988; Ykema *et al.*, 1988; Davies and Holdsworth, 1992).

V. The Key Enzymes of Lipid Accumulation

A. General Considerations

All enzymes within a cell could be regarded as essential for some function or other. But some are more essential than others. Considerable cellular organization must be in place for lipid biosynthesis and storage to occur. However, a simple examination of the various reactions leading from glucose, with its initial uptake into the cell via linkage to hexokinase thereby generating glucose 6-phosphate, right through to triacylglycerol biosynthesis does not immediately indicate which enzymes may be "more essential" than others. If we compare which enzyme activities are present in oleaginous microorganisms and which appear to be absent or different in the nonoleaginous organism, we would consider that two activities stand out as possible candidates for fulfilling important roles in microbial obesity. These are *ATP:citrate lyase* and *malic enzyme*. In addition, it is highly likely that *acetyl-CoA carboxylase* (ACC), the truly first committed enzyme of lipid biosynthesis, is also vitally important for lipid accumulation. But ACC, a ubiquitous enzyme, is found in all cells that generate their lipids from acetyl-CoA. Detailed studies have been carried out on the enzyme from *S. cerevisiae* (see, for example, Ivessa *et al.*, 1997), showing an integration of the enzyme with the endoplasmic reticulum and thereby suggesting a possible route for the product from ACC, malonyl-CoA, to be channeled directly into the fatty acid synthetase itself. If this is the case, then the commitment of acetyl-CoA for its subsequent conversion into fatty acids is absolute; the regulation of acetyl-CoA carboxylase therefore need not be "exceeding complicated" as has been suggested for the enzyme in animals (Allred and Reilly, 1997), as there is no flexibility in the use of the product if the conclusions of Ivessa *et al.* (1997) are correct. Overproduction of ACC activity in *Escherichia coli* led to an increase in the rate of fatty acid biosynthesis (Davis *et al.*, 2000), but even with a 100-fold increase in the content of intracellular malonyl-CoA, there was only a 6-fold increase in the rate of fatty acid biosynthesis. It was therefore concluded that the limitation (bottleneck) to lipid biosynthesis must be later in the pathway. No information was given, though, concerning whether the total lipid content of the genetically modified *E. coli* cells had been increased by increasing the activity of ACC—which is, of course, what this present review is seeking to clarify.

The gene coding for ACC in *Aspergillus nidulans* has been isolated, sequenced, and characterized (Morrice *et al.*, 1998). The enzyme itself is allosterically regulated with citrate being a positive effector. When the activity of the enzyme was inhibited *in vivo* by the fungicide, soraphen A, growth of the fungus was not restored by adding C_{16-18} fatty acids into

the medium, thereby suggesting that ACC maybe fulfilling an additional and essential function besides its involvement in fatty acid biosynthesis.

Other enzymes besides ACC have also been suggested as possible rate-limiting steps in fatty acid biosynthesis. (Indeed, it might be said that almost every single enzyme associated with lipid biosynthesis has been suggested by someone to be the rate-limiting step!) Thus, as an example, Heath and Rock (1996) have suggested that in *E. coli* it is the *condensation reaction* between acetyl-CoA and malonyl-CoA that controls the rate of fatty acid initiation, and therefore the total amount of fatty acid produced. However, up to now, no one has succeeded in substantially increasing the storage lipid content of a cell through genetic modification. This, though, remains a prime goal for many research teams dealing with both microbial and plant systems.

The following two sections give detailed information on two possible candidate enzymes that are, in the opinion of the authors of this review, critical for lipid accumulation to occur in oleaginous microorganisms.

B. ATP:Citrate Lyase

As indicated above, the first major biochemical difference delineated between an oleaginous and a nonoleaginous microorganism was the presence in the former of the citrate cleaving enzyme, ATP:citrate lyase. This enzyme had been known for some time to be of major importance in animal metabolism (Srere, 1972, 1975). There were two earlier reports (Attwood, 1973; Mahlen, 1973) that had shown the presence of this enzyme in, respectively, *Mortierella* spp. and in *Penicillium spiculisporum,* but without drawing the conclusion that the enzyme was essential for oleaginicity. While formation of citrate and its subsequent efflux from the mitochondria of the oleaginous yeast were clearly key events during lipogenesis (Evans *et al.,* 1983a, 1983b, 1983c), the detectable activity of ATP:citrate lyase (ACL) did not vary much between the balanced phase of growth and the lipid accumulation phase (Boulton and Ratledge, 1981a, 1981b, 1983b). Nevertheless, the ability of a yeast to accumulate lipid closely correlated with the possession of ACL (see Table III). In those yeasts without ACL, lipid contents of the cell were invariably low. However, some yeasts had ACL activity but did not accumulate lipid (Ratledge and Gilbert, 1985), thus indicating that other enzyme activities were needed to ensure lipid accumulation (see Table III). Interestingly, there was no correlation between the specific activity of ACL and the amount of lipid that a cell could accumulate (see Table III). In summary, while the possession of ACL activity will not automatically engender lipid accumulation in a microorganism, if the enzyme is absent the cells will be unable to accumulate lipid and

TABLE III

POSSIBLE CORRELATION OF ATP: CITRATE LYASE ACTIVITY WITH HIGH LIPID CONTENTS IN OLEAGINOUS YEASTS[a]

Yeasts	ACL activity (nmol min^{-1} mg $protein^{-1}$)	Lipid content (% dry wt)
Cryptococcus curvatus	7	34
Candida tropicalis	0	4
Candida utilis	0	4
Hansenula saturnus	11	25
Lipomyces lipofer NCYC 944	50	36
Lipomyces lipofer NCYC 692	0	2
Rhodosporidium toruloides CBS 6016	0	3
Rhodosporidium toruloides CBS 5490	42	26
Rhodosporidium toruloides ML 2590	45	4
Rhodosporidium toruloides ML 2921	52	5
Rhodotorula graminis	42	24
Saccharomyces cerevisiae	0	6

[a] From Boulton and Ratledge, 1981, and Ratledge and Gilbert, 1985. All filamentous fungi appear to possess ATP:citrate lyase activity irrespective of their lipid contents (Wynn *et al.*, 1998).

will therefore be nonoleaginous. Clearly, other enzyme activities must be in place to determine the extent to which lipids may accumulate in individual organisms.

In filamentous fungi, ACL appears to be universally distributed (Wynn *et al.*, 1998) irrespective of the lipid contents of these microorganisms. It is only in yeasts that ACL is of variable occurrence.

ACL, surprisingly, has even been found in the citric acid producing fungus, *A. niger* (Pfitzner *et al.*, 1987), but because ACL has a rather low affinity for citrate (2.5 m*M*) (in yeast the K_m value for citrate is 0.07 m*M*—Boulton and Ratledge, 1983a), it was considered that it did not significantly affect citrate accumulation but at the same time did function to supply acetyl-CoA units for lipid biosynthesis. ACL has been purified to homogeneity from the yeast, *Rhodotorula gracilis* (Shashi *et al.*, 1990). It consists of four identical subunits, each about 120 kDa in size, giving a total molecular weight of 520,000. Activity was stimulated by NH_4^+ and inhibited by long-chain acyl-CoA esters, as had been noted earlier by Boulton and Ratledge (1983a) for the enzyme from *Lipomyces starkeyi* where an M_r value of 510 kDa had been calculated for the molecular size of ACL. Evans and Ratledge (1985c) had calculated the molecular size of ACL from *Rh. toruloides* as being 480 kDa; this enzyme was stimulated by NH_4^+ ions at nonsaturating concentrations of citrate (0.1 m*M*) and was inhibited by fatty acyl-CoA esters. These features would then seem to be common among ACL from oleaginous yeasts. The inhibition by

fatty acyl-CoA esters was considered to be a rapid response mechanism to ensure, when the storage lipid of an oleaginous cell began to be broken down, as would occur under starvation conditions, that lipid biosynthesis would be instantly inhibited (Holdsworth *et al.*, 1988; Holdsworth and Ratledge, 1987; Naganuma *et al.*, 1987), thereby preventing both lipid biosynthesis and lipid degradation occurring simultaneously. [As well as inhibiting ACL activity, fatty acyl-CoA esters also inhibit citrate translocase—see Fig. 7 (Evans *et al.*, 1983c)—thereby reinforcing their stringent control over the initial steps leading to acetyl-CoA formation.]

In yeasts ACL consists of four homomeric subunits, each with 120 kDa polypeptides (Shashi *et al.*, 1990), and is similar to the structure of ACL in humans and rats (Elshourbagy *et al.*, 1990, 1992). The inference is, then, that the enzyme in yeasts will be coded for by a single gene, as has been shown to be the case for both human and rats (Elshourbagy *et al.*, 1990, 1992). This does not, though, seem to be the case for the enzyme from filamentous fungi. Both Adams *et al.* (1997, 2002) and Nowrousian *et al.* (2000), working, respectively, with *Aspergillus nidulans* and *Sordaria macrospora,* have found that ACL in these filamentous fungi consists of two different polypeptides. The gene (*acl1*) has been sequenced from *Sor. macrospora* and codes for a 73 kDa subunit of ACL. With *A. nidulans,* ACL has been isolated and purified to homogeneity, and shown to be a hexamer of 371 kDa comprised of three polypeptides, each 55 kDa, and three further ones of 70 kDa (Adams *et al.*, 2002). The gene that codes for the smaller subunit polypeptide remains to be identified and sequenced. In *Sor. macrospora,* ACL may be involved in fruiting body formation, possibly by providing additional fatty acyl-CoA esters which become the trigger for sexual reproduction (Nowrousian *et al.*, 2000). In *Asp. nidulans,* the role of ACL was considered to be for "normal" lipid accumulation (Adams *et al.*, 2002). In summary, in fungi each subunit of ACL is being coded for by separate genes instead of the single one found in yeasts and animals. (Plants are as yet unknown in this respect.)

The complexity of the structure of ACL from all cells suggests that it fulfills a key role in the generation of acetyl-CoA units. One might reasonably expect that its activity should be carefully controlled so that the changing needs of the cell for acetyl-CoA units can be met at all stages of cell growth and development. Indeed, initial work with ACL had suggested that it may be the key regulatory enzyme for lipid biosynthesis in yeast (Boulton and Ratledge, 1981b, 1983a; Evans and Ratledge, 1985c), and may even be the rate-limiting step of lipid biosynthesis in some cases (Boulton and Ratledge, 1981a).

Although no work has been done to increase the activity of ACL in microorganisms, either by gene cloning or by placing the existing gene/s

under different regulatory controllers, the cloning of the gene coding for ACL from rat into plastids of tobacco plant has been carried out (Rangasamy and Ratledge, 2000). Although a functional protein was produced in these genetically modified plants, with a 4-fold increase in ACL activity, there was only a 16% increase in the amount of lipid accumulated in tissue cultures of the modified plants. This would therefore suggest that ACL activity is not a major bottleneck for the production of lipid in this plant, although earlier work (Ratledge *et al.*, 1997) had suggested a positive correlation between ACL activity with lipid accumulation in the developing seeds of *Brassica napus*. However, until someone clones an additional gene for ACL into a microbial cell, we shall not know whether it is the rate-limiting step to fatty acid biosynthesis in microbes. But, as the following section indicates, we would not consider this to be likely.

C. Malic Enzyme

Malic enzyme catalyzes the reaction:

$$\text{L-MALATE} + \text{NADP}^+ \longrightarrow \text{Pyruvate} + \text{CO}_2 + \text{NADPH}. \quad (5)$$

It occurs in a range of fungi and yeasts, though is not ubiquitous. It also occurs in animals and its association with lipogenesis has been suggested for many years (see, for example, Wise and Ball, 1964). The considered view is that malic enzyme is just one of several activities (the others being glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP-dependent isocitrate dehydrogenase) that generate NADPH, which is used by fatty acid synthetase, or indeed, other enzymes requiring this cofactor. Malic enzyme would then contribute to a general cytosolic pool of NADPH, but would assume prime importance only if a substrate, such as pyruvate or even acetate, were being used instead of glucose (Flatt and Ball, 1964; Wise and Ball, 1964).

In fungi, malic enzyme appears to be of widespread distribution. It has been purified to homogeneity from *Mucor circinelloides* (Song *et al.*, 2001), where it occurs in a number of isoforms (see below). The native enzyme of the principal form has a molecular size of about 160 kDa (Savitha *et al.*, 1997), and is composed of two identical monomers (Song *et al.*, 2001). The function of malic enzyme, however, has never been too clear. Some workers have suggested that its main role is in the metabolism of pyruvate (Zink, 1972; Zink and Katz, 1973; McCullough and Roberts, 1974).

While malic enzyme activity has always been regarded as an essential component for the *transhydrogenase cycle* (see Fig. 7), its absolute requirement for fatty acid biosynthesis and for fatty acid desaturations has only been clear following the initial observations of Kendrick

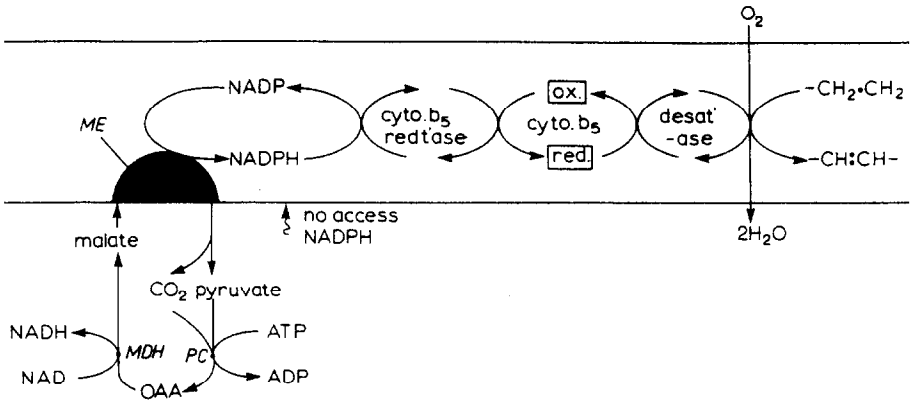

FIG. 8. Proposed structure of the election transfer chain involved in the microsomal membrane desaturation of fatty acids in *Mucor circinelloides*. Desaturation is driven by the provision of exogenous malic acid to the membranes. ME: malic enzyme (malic dehydrogenase decarboxylating-NADP linked); PC: pyruvate carboxylase (which is cytosolic enzyme in this fungus); and MDH: malate dehydrogenase (from Kendrick and Ratledge, 1992b).

and Ratledge (1992b) that the formation of polyunsaturated fatty acids using microsomal (endoplasmic reticulum) membranes from *Mucor circinelloides* could be achieved only when malate was included in the preparations and not NADPH. The added NADPH apparently was not able to access the desaturases within the membranes, but malate was. It appeared that there must be a second malic enzyme within the membrane beside the soluble one that occurred in the cytosol. This membrane-associated malic enzyme was indeed shown to be distinct from the cytosolic form (Kendrick and Ratledge, 1992b), and hypothesized to drive the desaturase reaction through the usual linkages of cytochrome b_5 and cytochrome b_5 reductase (see also Section V). This is shown schematically in Figure 8 (see also Kendrick and Ratledge, 1992c).

The suggestion that NADPH was not able to penetrate membrane preparations of *M. circinelloides,* and thus drive the desaturases directly, was surprising but should not have been in view of the pronounced lipophobic nature of this cofactor. On the other hand, a lipophilic enzyme, as this particular isoform of malic enzyme appeared to be, would certainly be able to penetrate, at least partially, into the membranes where fatty acyl desaturations were taking place. If the enzyme is indeed located as shown in Figure 8, then NADPH could easily be generated in close proximity to cytochrome b_5 reductase; malic enzyme could be even be physically associated with the reducatase, thereby ensuring direct channeling of NAPDH right through the

desaturases itself. Malic enzyme would function by the provision of malate on the "open" (cytoplasmic) side of the enzyme—a "transhydrogenase cycle" (see Fig. 7) would be in operation at this location.

While working on the membrane-bound malic enzyme, we attempted to block its activity by using selective inhibitors. Shimizu *et al.* (1989) had previously found that a nonoil component of sesame seed oil diminished the formation of arachidonic acid (20:4) in growing cultures of *Mortierella alpina*. This compound was identified as a bicyclic aromatic molecule, sesamin, which specifically inhibited the $\Delta 5$ desaturase of the fungus and caused the accumulation of the precursor of arachidonic acid, dihomo-γ-linolenic acid (20:3) (see Fig. 2). Kendrick and Ratledge (1996) and Wynn *et al.* (1997) then went on to test sesamol, which is 3,4-methylene-dioxyphenol, for its effect on fatty acid desaturases in several fungi. Wynn *et al.* (1997) showed that sesamol acted as a highly specific inhibitor of malic enzyme activity in *Mucor circinelloides*. When cultures were grown in its presence (at 3–5 m*M*), the content of lipid in the cells was decreased by almost 90%, from 24% of the cell biomass to 2% but—and this was of major significance—without causing any major effect on growth. Not only was the lipid content of the cells affected by sesamol, but the content of γ-linolenic acid (18:3n-6) dropped from 16% of the total fatty acids to 2%. Thus, as only malic enzyme activity appeared to have been affected by the inhibitor, it was concluded that sesamol was specifically inhibiting both the cytoplamic and membrane-bound malic enzymes, and that without malic enzyme the cell was unable to accumulate lipid or to carry out desaturations of it. The essentiality of malic enzyme for lipid biosynthesis appeared to be established.

Wynn and Ratledge (1997) went on to show that in a mutant of *Asp. nidulans* lacking malic enzyme activity, only half the lipid (12% of the cell dry weight) that had been produced by the competent strain under nitrogen-limited growth conditions was now produced. (Sesamol, for reasons of its impermeability into this fungus, had no effect on malic enzyme activity.) Interestingly, without the metabolic burden of having to synthesize the storage lipid in such large amounts, the mutant devoid of malic enzyme activity now grew slightly better than the parent cells. However, there was no diminution in the linoleic acid content of the oil from the mutant compared to the original culture having malic enzyme activity. Probably, therefore, a membrane-bound form of malic enzyme (see Fig. 8) is not universal among fungi though one has been detected in *Pythium ultimum* (Savitha *et al.*, 1997).

The hypothesis is advanced that only malic enzyme can provide the NADPH that is needed for fatty acid biosynthesis and, therefore, is vital to the process of lipid *accumulation*. If the actvity of the enzyme

is prevented, either by inhibition or by mutation, then lipid accumulaton ceases. Fatty acid biosynthesis per se is still functional and phospholipids can be produced. Thus, the cell can manage without malic enzyme—it is not absolutely vital—but the cell cannot produce storage triacylglycerols in any abundance. Without malic enzyme activity the flux of carbon, from glucose to lipid, is considerably diminished and only essential lipids are produced—presumably by using other sources of NADPH.

The activity of malic enzyme controls the extent of lipid accumulation. Thus any change in its activity, either upward or downward, would then change the extent of lipid accumulation in an oleaginous cell. And so it has proved to be.

Wynn *et al.* (1999) followed the course of lipid accumulation in two filamentous fungi showing different extents of lipid accumulation: *Mucor circinelloides* has a ceiling of approximately 25% lipid of its biomass when grown under nitrogen-limiting conditions whereas *Mortierella alpina* can accumulate about 50% but no more. When the activity of various enzymes were followed throughout growth (see Fig. 9), that of malic enzyme was the only one that paralleled the course of lipid accumulation. Other enzymes examined (but not shown in Fig. 9) included fatty acid synthetase, acetyl-CoA carboxylase, ATP:citrate lyase, and three other enzymes generating NADPH: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and the cytolsolic isocitrate dehydrogenase ($NADP^+$ dependent). All these other enzyme activities remained detectable throughout the growth of both fungi. Malic enzyme activity, however, ceased after 40 h growth in *M. circinelloides* and thereupon lipid accumulation also ceased; in *Mort. alpina* malic enzyme activity continued for up to 90 h but, when it could no longer be detected lipid accumulation stopped (see Fig. 9). Only malic enzyme activity correlated with the pattern of lipid accumulation.

A somewhat similar investigation in *Cunninghamella echinulata* (Certik *et al.*, 1999) concluded that the activities of ATP:citrate lyase and acetyl-CoA carboxylase paralleled lipid accumulation and could have caused the eventual cessation of lipid biosynthesis. However, malic enzyme activity also diminished along with these other two enzyme activities. The decrease in all three enzyme activities came *after* lipid accumulation had ceased and were not in parallel with the slowing down of lipid synthesis. This work was carried out in shake-flask cultures where O_2 transfer is always a problem, and this may then lead to changes in gene expression and possible down-regulation of proteins for reasons other than the limitation of lipid accumulation. In the work described above with *M. circinelloides* and *Mort. alpina,* the work had been done in aerated, stirred tank fermenters with appropriate O_2 and pH controls.

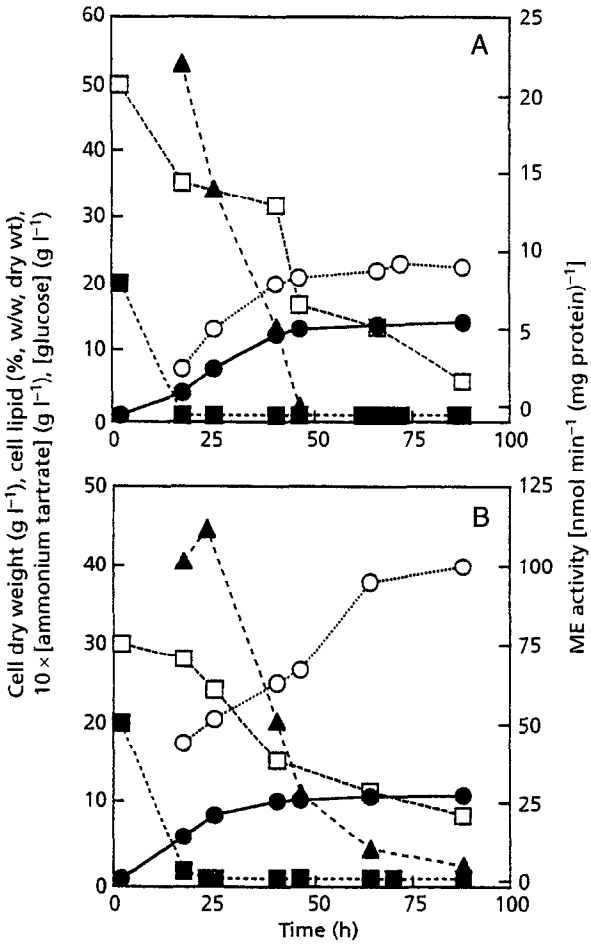

FIG. 9. The correlation of malic enzyme activity with the extent of lipid accumulation in *Mucor circinelloides* (A) and *Mortrerella alpina* (B). Each fungus was grown in a 4 liter stirred fermenter with a low amount of N in the medium but with glucose in excess throughout growth. Cell dry wt (●); cell lipid (○); ammonium tartrate (■), glucose (□) and malic enzyme activity (▲). (From Wynn *et al.*, 1999).

In the two filamentous fungi that we have studied, the cessation of malic enzyme activity is correlated with the onset of nitrogen limitation that is, of course, a necessary prerequisite for lipid accumulation to occur (see Fig. 3). If nitrogen (as ammonium tartrate) was added into the culture of *M. circinelloides* after the cessation of malic enzyme activity, then this resulted in the return of full activity within about 4 h (Wynn *et al.*, 1999). However, if an inhibitor of protein synthesis (cycloheximide) was added along with the extra nitrogen, the reinstatement of

malic enzyme activity was prevented. The conclusion was reached that malic enzyme activity was ceasing because of a down-regulation of the malic enzyme gene following nitrogen exhaustion from the medium.

When the malic enzyme gene has been identified, it should then be possible to place it under a constitutive promoter so that an active malic enzyme will continue to be synthesized after nitrogen depletion. Under such circumstances, it would then be expected that lipid accumulation would be substantially increased. Identification of the malic enzyme gene in *Mucor circinelloides* has been hampered by the identification of multiple forms of the enzyme, some of which are involved in anaerobic metabolism (Song *et al.*, 2001). The form of malic enzyme associated with lipid accumulation appears to occur in two isoforms: one form (Isoform III) is stable and occurs during the balanced growth phase of the organism. Upon the onset of nitrogen limitation, this isoform is converted (by some unknown posttranscriptional mechanism) into a second form (Isoform IV), which is unstable and then begins to lose activity so that eventually all activity is lost and lipid accumulation ceases. The stability of the malic enzyme, or the expression of the gene, determines the length of time over which lipid accumulation occurs and thus controls the ultimate content of lipid within the cell.

Based on knowledge of multiple forms of malic enzyme in *M. circinelloides,* Song *et al.* (2001) provided a biochemical explanation as to why acetate-grown cultures of this fungus accumulate about 35–40% more lipid than do glucose-grown cells (Du Preez *et al.*, 1997). In the acetate-grown cells, malic enzyme activity was consistently about 50% higher than in the glucose-grown cells throughout the lipid accumulation phase. The essential NADPH for fatty acid biosynthesis was thus provided at a higher rate and would explain why lipid accumulation continued for much longer when acetate was the growth substrate. The key isoform of malic enzyme (Isoform IV) apparently was more stable in acetate grown cells.

Interestingly, when sesamol, as the specific inhibitor of malic enzyme activity (see above), was added to cultures of *Fusarium moniliforme,* not only was there a substantial (35%) inhibition of lipid production but there was a simultaneous 20-fold increase in the formation of gibberellin, indicating that acetyl-CoA was being switched from fatty acid biosynthesis into the formation of the secondary metabolite (Jacklin *et al.*, 2000). Thus, it should be possible to control malic enzyme activity not only in a positive direction to increase lipid accumulation but also in a negative direction to divert essential acetyl-CoA units into preferred secondary metabolite products.

The correlation between malic enzyme and lipid accumulation therefore appears as *the* single event that controls the extent of lipid

accumulation in the oleaginous organisms studied. The absolute dependency of fatty acid synthetase for NADPH to be supplied directly from malic enzyme, means that there is no such thing as a "common pool of NADPH" at least as far as fatty acid synthesis is concerned (Wynn *et al.*, 1999). A coherent explanation can now be provided to explain not only the biochemistry of lipid accumulation in oleaginous organisms but also how the physical extent of lipid accumulation may be governed in different cells. This is discussed in Section IX.

VI. Desaturases

A. Background Information

Despite the key role fatty acid desaturases play in the biosynthesis of PUFA, and their regulatory importance in this process, relatively few biochemical studies of desaturases have appeared. The paucity of biochemical analysis of these systems reflects the difficulties these enzymes present for traditional purification/characterization (Lamascolo *et al.*, 1996; Michaelson *et al.*, 1998; Saito and Ochiai, 1999). Fatty acid desaturases are hydrophobic/membrane-bound proteins comprising at least three separate functions to yield the final desaturase activity (cytochrome b_5 reductase, cytochrome b_5 the terminal desaturase, and even perhaps malic enzyme—see Fig. 8). Furthermore, the substrates for these enzymes are either acyl-CoA or acyl residues attached to phospholipids. As the ^{14}C substrates required to assay these enzymes are either very expensive or simply not available commercially, many attempts to assay desaturases use labeled free fatty acids and rely on activating activities in the extracts to convert these into the required substrates for the desaturases.

The study of desaturase activities via the study of deficient mutants is likewise hampered by the fact that the deletion mutants (with the exception of mutants lacking $\Delta 9$ desaturase activity) often display no easily selectable phenotype (Das and Sen, 1983; Jareonkitmongkol *et al.*, 1992; Goodrich-Tanrikulu *et al.*, 1994). Often the mutants grow on defined medium as well, or nearly as well, as the wild type. Although the lack of cold tolerance has been used to select mutants deficient in the synthesis of PUFA in some systems (Jareonkitmongkol *et al.*, 1992), more often than not this approach is ineffective and many desaturase mutants have been identified by random screening of mutagenized populations for alterations in fatty acid profile (Jareonkitmongkol *et al.*, 1992). Using a range of techniques, various microorganisms, including cyanobacteria and fungi, deficient in $\Delta 9$, $\Delta 12$, $\Delta 6$, and $\Delta 5$ desaturases, have been isolated and studied (Jareonkitmongkol *et al.*, 1992; Certik and Shimizu, 1999).

The greatest success in studying fatty acid desaturases in microbial systems has come about using a molecular genetic approach. Indeed, the genes for all the desaturase activities involved with n-6 PUFA biosynthesis along with the elongases required for the synthesis of long-chain PUFA (LCPUFA), i.e., fatty acids with carbon chain lengths greater than 18, have been cloned. Although Δ15 (n-3) desaturases have been cloned from plant and animal systems (Kodama *et al.*, 1997; Meesapyodsuk *et al.*, 2000), and many fungi produce n-3 PUFA, the cloning of a microbial Δ15 desaturase has not been achieved to the authors' knowledge. The cloning of the desaturase genes has been greatly aided by the work of Stukey *et al.* (1990) and Shanklin *et al.* (1994), who identified key features that are common to all fatty acid desaturases. Desaturases from different species, and even different desaturases from a single species, often demonstrate a lack of overall similarity. However, certain key features, with structural and functional significance, are common to all, and are highly conserved between all desaturases. Most notable of these highly conserved areas is the histidine (HIS) boxes (Stukey *et al.*, 1990; Shanklin *et al.*, 1994). Three histidine-rich motifs, with a general structure HXXHH, are present in all desaturases characterized to date, although the exact sequence of the HIS boxes vary to some degree. The HIS boxes are separated by two long hydrophobic regions thought to be involved in anchoring the desaturases to the endoplasmic reticulum. It is envisaged that each hydrophobic region spans the endoplasmic reticulum (ER) membrane twice so that the three HIS boxes are positioned on the cytosolic face of the ER (Stukey *et al.*, 1990) where they are thought to play a crucial catalytic role in Fe^{2+} binding (Shanklin *et al.*, 1990).

The HIS boxes can be used to the design degenerate primers for PCR, to amplify a fragment of the desaturase gene to use as a probe for the cloning of intact desaturase genes from either cDNA or genomic DNA libraries. Likewise, searches of EST sequences for the presence of desaturase clones has often relied on the presence of sequences homologous to HIS box sequences for the identification of desaturase clones.

Despite the general lack of sequence homology between different desaturase genes, the structural similarity of the native proteins has been highlighted by the functional activity of heterologously expressed desaturases. The cross-species functionality of heterologously expressed desaturases applies not only to microbial desaturases but also to microbial desaturases expressed in plants and animals (Polashock *et al.*, 1992; Knutzon *et al.*, 1998; Kelder *et al.*, 2001). Indeed, heterologous expression of putative desaturase genes is one of the standard techniques employed to assign unambiguously a function to a cloned putative desaturase gene.

The heterologous functionality of microbial fatty acid desaturases provides the potential for the use of microbial systems as a reservoir of

genes for desaturases that could be transferred to plants to modify the fatty acid profile of seed oil crops to produce commercially attractive PUFAs that are not naturally synthesized by seed oil crops (Qiu *et al.*, 2001).

B. Δ9 Desaturases

The Δ9 desaturase catalyzes the insertion of the first double bond into saturated fatty acids between carbons 9 and 10 of the fatty acid chain. As such, it can be thought of as the first committed step in PUFA biosynthesis (see Fig. 2). The Δ9 desaturase can operate on either of the predominant saturated fatty acids in microbial cells, palmitate (16:0) and stearate (18:0), to produce palmitoleate (16:1n-7) or oleate (18:1n-9), respectively (Sakurdani *et al.*, 1999a). Although the preference for the Δ9 desaturase for these two potential substrates varies between systems, the Δ9 desaturase from microorganisms appears to utilize 18:0 preferentially (Sakuradani *et al.*, 1999b). The Δ9 desaturase is distinct from the other desaturases in a number of ways: it is the only desaturase that acts on saturated fatty acids and it is the only desaturase that has acyl-CoA rather than phospholipid-bound acyl groups as its substrate. In plant systems, the uniqueness of the Δ9 desaturase is further emphasized in that it is a soluble rather than a membrane-bound enzyme. In fungi, the Δ9 desaturase resembles the mammalian system with the Δ9 desaturase located within the ER. Nevertheless, the Δ9 desaturase from *S. cerevisiae* is functional when heterologously expressed in plant systems (Polashock *et al.*, 1992).

The Δ9 desaturase has been cloned from a number of yeasts and filamentous fungi, and several general features of these enzymes have been established. The gene has a ORF (open reading frame) of approximately 1500 base pairs (bp) and encodes a protein with between 440 and 500 amino acids with a molecular weight slightly greater than 50 kDa (Meesters and Eggink, 1996; Itoh *et al.*, 1998; Sakuradani *et al.*, 1999a; Lu *et al.*, 2000). The gene also encodes a cytochrome b_5 domain at the carboxy terminal of the native enzyme (Meesters *et al.*, 1997; Itoh *et al.*, 1998; Sakuradani *et al.*, 1999a; Wongwathanrat *et al.*, 1999; Lu *et al.*, 2000). This cytochrome b_5 is a prerequisite for activity, demonstrating that the Δ9 desaturase cannot accept electrons from an exogenous cytochrome b_5.

Of all the desaturases, the Δ9 appears to be the most conserved during evolution. The importance of this enzyme is confirmed by mutants lacking this activity, and are unable to grow in the absence of an exogenous supply of unsaturated fatty acids (Das and Sen, 1983; Goodrich-Tanrikulu *et al.*, 1994). Such unsaturated fatty acid auxotrophy is rare in mutants lacking other desaturases (Jareonkitmongkol *et al.*, 1992). The

Δ9 desaturase from *Cryptococcus curvatum* displays a high degree of identity, at the amino acid level, with both that of *S. cerevisiae* (72%) and rat (62%) (Meesters and Eggink, 1996).

Recently, multiple genes for the Δ9 desaturase in the oleaginous fungus, *Morti. alpina,* have been reported (Wongwathanrat *et al.,* 1999). One gene, Δ9-1, appears to be actively expressed in all strains of *Mort. alpina,* while expression and presence of the second gene varies between strains. Both genes encode a similar protein (86% identity) and contain a single intron whose position is conserved. Two clearly different genes encoding a functional Δ9 desaturase also have been cloned from *C. curvatum* (Meester and Eggink, 1996; Meesters *et al.,* 1997). A third Δ9 desaturase gene from *Mort. alpina* has been reported (MacKenzie *et al.,* 2002). Unlike the *ole1* and *ole2* genes (Wongwathanrat *et al.,* 1999), the third desaturase gene failed to complement the Δ9-desaturase (Δ9-3 gene) mutation in *S. cerevisiae.* The desaturase itself had a different substrate specificity than the other two Δ9-desaturases, and functioned only moderately on stearic acid (18:0) and not at all on palmitic acid (16:0). It was suggested that this desaturase may function with very long chain fatty acids (e.g., 26:0) though this remains to be substantiated.

The genetic regulation of the Δ9 desaturases still needs to be studied in detail, but expression of the gene(s) is repressed by exogenous fatty acids that have a Δ9 desaturation (Meesters and Eggink, 1996; Lu *et al.,* 2000). Furthermore, expression of the Δ9 desaturase in *Mucor rouxii* changes during cell growth and upon changes in cultivation temperature (Laoteng *et al.,* 1999), suggesting that the expression of this desaturase is involved in cold acclimation in microorganisms.

C. Δ12 Desaturases

The Δ12 desaturase catalyzes the conversion of oleic acid (18:1n-9), to linoleic acid (18:2n-6) by the insertion of a double bond between carbons 12 and 13 of the mono-unsaturated fatty acid chain. In the yeast, *L. starkeyi,* most of the Δ12 desaturase activity was in the cytosolic fraction after subcellular fractionation (Lomascolo *et al.,* 1996). Whether this is evidence of a truly cytosolic activity or an artifact caused by the disintegration of the endoplasmic reticulum during cell cleavage is uncertain, however. It is most likely that in this yeast, as in other systems, the Δ12 desaturase is a membrane-bound enzyme utilizing a phospholipid-bound 18:1 as its substrate.

The gene for the Δ12 desaturase has been cloned from number of microbial systems and, as with other desturases, the cloned gene is capable of expression and translation into a functional protein in other systems (Wada *et al.,* 1993; Huang *et al.,* 1999; Sakuradani *et al.,* 1999a). The gene

from the closely related oleaginous fungi, *Mort. alpina* and *M. rouxii*, have been cloned and found to possess ORFs encoding polypeptides of 399 and 396 amino acids, respectively (Huang *et al.*, 1999; Passorn *et al.*, 1999). Likewise, the ORFs of Δ12 desaturase genes from cyanobacteria appear to be relatively short, encoding proteins of approximately 350 amino acids (Wada *et al.*, 1993). As such, Δ12 desaturase genes encode smaller proteins than the Δ9 desaturases, since the Δ12 desaturase does not not possess a cytochrome b_5 domain (Sakuradani *et al.*, 1999b). Presumably, this desaturase (unlike desaturases with a cytochrome b_5 domain) can accept electrons from a discrete microsomal cytochrome b_5.

D. Δ6 Desaturases

The Δ6 desaturase converts linoleic acid (18:2n-6) to γ-linolenic acid as part of the n-6 fatty acid pathway and γ-linolenic acid (18:3n-3) to 18:4n-3 as part of the n-3 pathway (see Fig. 2). Although this enzyme is capable of taking part in two separate fatty acid desaturation/elongation pathways, in the vast majority of microbial systems only one of these pathways occur. The thraustochytrids appear to be an exception to this rule and are known to accumulate a cell lipid containing appreciable amounts of docosapentanoic acid (22:5n-6) and docosahexaenoic acid (22:6n-3) (Metz *et al.*, 2001). The genes encoding Δ6 deaturases from *Mort. alpina* and *M. rouxii* have been cloned, and their function confirmed by heterologous expression in yeast and *Aspergillus oryzae* (Huang *et al.*, 1999; Sakurdani *et al.*, 1999b, 1999c; Laoteng *et al.*, 2000) The *Mortierella* gene contains an open reading frame encoding a polypeptide of 457 amino acids, a similar size to the Δ9 desaturase, and possesses a cytochrome b_5 domain. However, in the Δ6 desaturases the cytochrome b_5 fusion protein is at the N-terminus of the expressed protein (Sakurdani *et al.*, 1999c; Laoteng *et al.*, 2000).

E. Δ5 Desaturases

Like the Δ6 desaturase, the Δ5 desaturase can potentially operate in either the n-3 or the n-6 fatty acid pathway, converting either dihomo-γ-linolenic acid (20:3n-6) to arachidonic acid (20:4n-6) or 20:4n-3 to eicosapentaenoic acid (20:5n-3—see Fig. 2). Furthermore, like the Δ6 desaturase it introduces a double bond into the fatty acid chain to the carboxyl-end relative to the initial Δ9 desauration site. As such, both the Δ6 and Δ5 desaturases belong to a subgroup of desaturases that have been designated the "front-end" desaturases. The front end desaturases have similar sequence homology and share distinct features that separate them from the desaturases that insert double bonds to the

methyl end of the fatty acid relative to the initial Δ9 desaturation. In particular, these desaturases possess an N-terminal cytochrome b_5 domain (Michaelson *et al.*, 1998; Saito and Ochiai, 1999; Saito *et al.*, 2000) and seem to differ slightly from other desaturases in that they have a H to Q substitution in the third HIS box to give a QXXHH consensus (Knutzon *et al.*, 1998; Michaelson *et al.*, 1998; Saito and Ochiai, 1999).

The gene from *Mort. alpina* coding for the Δ5 desaturase has been cloned. It encodes a sequence of 446 amino acids (Knutzon *et al.*, 1998). Duplicate genes for Δ5 desaturase occur in the slime mold *Dictylostelium,* one encoding a polypeptide of 464 amino acids and the other 467 amino acids (Saito and Ochiai, 1999; Saito *et al.*, 2000). In the slime mold, it appears that the Δ5 desaturase possesses an unusually long interval between the second and third HIS boxes (Saito and Ochiai, 1999).

F. A Δ4 Desaturase?

The existence of a Δ4 desaturase that desaturates docosapentaenoic acid (22:5n-3) as the final step in docosahexaenoic acid (DHA; 22:6n-3) (see Fig. 2) has been debated. In mammals it has been suggested that this conversion is carried out via elongation of 22:5n-3 to 24:5n-3 which is then desaturated by a Δ6 desaturase to 24:6n-3 and this subsequently chain shortened to yield DHA (22:6n-3) (Voss *et al.*, 1991). Furthermore, in DHA-producing marine microorganisms (both bacterial and eukaryotic) the synthesis of DHA is thought to proceed along a "polyketide" pathway rather than via a fatty acid route (Metz *et al.*, 2001), obviating the need for a Δ4 desaturase.

A gene encoding a protein with a Δ4 desaturase activity (in yeast and plants) has been cloned from *Thraustochytrium* sp. (Qiu *et al.*, 2001). The existence of this Δ4 desaturase gene reignites the arguments as to the exact route for DHA biosynthesis, at least in eukaryotic marine microorganisms. That a Δ4 desaturase gene appears to be present and encodes a functional protein suggests a fatty acid route to DHA biosynthesis is possible, although the fact that EPA biosynthesis in the related *Schizochytrium* was uneffected by anaerobic growth is not compatible with a truly fatty acid route reliant on O_2-requiring desaturases (Metz *et al.*, 2001).

VII. Fatty Acid Elongases

The other conversion required for the synthesis of the long-chain PUFA (LCPUFA) that are of current biotechnological interest is the

elongation of the fatty acid chain by a two-carbon extension at the carboxyl end catalyzed by fatty acid elongases. Fatty acid elongases act primarily on 16:0 to form 18:0 and on either 18:3n-6 or 18:4n-3 during the production of ARA and DHA (or EPA) via the n-6 and n-3 routes, respectively (see Fig. 2).

Like the fatty acid desaturases, the elongases are membrane-associated proteins and rely for activity on the cooperation of several catalytic subunits. As a result, the fatty acid elongases are as recalcitrant to conventional biochemical study as the desaturases and remain relatively poorly characterized. Indeed the number, substrate specificity, and even the detailed mechanism of fatty acid elongation remain a matter of some debate.

The reaction sequence for the elongation of fatty acids is the same as that for their *de novo* synthesis (by the FAS complex), i.e., it involves a sequence of four linked reactions:

1. *Condensation* of an activated acyl chain with malonyl-CoA to give a ketoacyl product and evolving CO_2.
2. *Reduction* of the ketoacyl product to give a γ-hydroxyacyl species.
3. *Dehydration* to give the enoyl compound.
4. *A second reduction* to give the final two-carbon elongated fatty acyl chain.

The key differences from the FAS complex are the specificity of the initial condensing enzyme (which also appears to be the rate-limiting step for the entire elongation process) and the membrane-bound nature of the elongase complex.

Although the biosynthesis of long-chain PUFA is often thought of as a membrane-associated process (in contrast to the *de novo* synthesis of saturated fatty acids up to C_{18}, which occurs in the cytosol), all the enzymes, both desaturases and elongases, are membrane proteins and the intermediates appear to be phospholipid-bound acyl chains. However, this cannot be the case for the fatty acid elongation as the initial condensation reaction requires access to the carboxyl end of the acyl chain, access that would be impossible if the acyl chain was bound as part of a phospholipid group. Also, the condensation requires that the acyl chain be activated (probably in the form of a CoA thioester). The details as to how the acyl chain is removed from the phospholipid and is replaced thereon remains obscure.

Until the recent success in cloning genes for the fatty acid elongases from *Mort. alpina,* little was known about these enzymes from oleaginous microorganisms. However, recent evidence has suggested that during long-chain PUFA biosynthesis, the elongation process may be a

crucial rate-limiting step (Wynn and Ratledge, 2000), and would be the key enzyme activity to target in gene cloning if one wished to increase the productivity of, say, arachidonic acid (20:4n-6).

In the past two years, two genes encoding discrete elongases (probably just the "condensing enzyme") with different properties and substrate specifies have been identified in *Mort. alpina* (Parker-Barnes *et al.*, 2000). The first, which was cloned using a protocol based on conserved regions in plant and yeast elongaes genes (i.e. from systems that do not accumulate PUFA), identified a gene possessing 40% similarity at the amino acid level with the yeast elongase. This elongase exhibited a preference for saturated and monounsaturated fatty acids, and is probably involved in the conversion of 16:0 to 18:0.

Subsequently, a second gene encoding an elongase that specifically acted on C_{18} PUFA was isolated. This gene encoded a polypeptide of 318 amino acids that demonstrated heterologous activity using either 18:3n-6 or 18:4n-3 as a substrate, despite *Mortierella* not possessing an active n-3 pathway. The gene encoding the PUFA-specific elongase was clearly distinct from the saturated/mono-unsaturated fatty acid specific enzyme, exhibiting <25% identity with the yeast elongase (Parker-Barnes *et al.*, 2000).

It is therefore now apparent that elongations of C18 to C20 fatty acids and of C20 to C22 fatty acids (see Fig. 2) are each carried out by a specific elongase encoded for by a distinct gene. These conversions are therefore not a result of a single elongase with broad specificity capable of elongation of both 16:0 and 18:3n-6. It would also appear that only one enzyme function, that of the 'condensing enzyme' (see above), needs to be cloned; the other three enzyme activities of the two reductases and a dehydratase must be supplied by the existing activities of the FAS complex.

VIII. Lipid Bodies and Their Role in Lipid Accumulation

For a long time, the formation of lipid bodies in oleaginous microorganisms was thought to involve the simple coalescence of the nonsoluble neutral lipid. As such, lipid body formation was thought to be a predominantly physical process, and lipid bodies were thought to be physiologically inert structures. This idea has now been largely discredited, and it is accepted that, far from being a biochemically inert structure, lipid bodies play a key role in the regulation of storage lipid formation and reutilization (Murphy and Vance, 1998). The formation of lipid bodies is beyond the scope of the current review, but has been reviewed in detail elsewhere with particular emphasis on lipid bodies in plants (Murphy and Vance, 1998).

That lipid bodies were not purely cellular "puddles" of accumulated storage lipid was first suggested due to the observation that most oleaginous cells maintain many lipid bodies (all of a specific size) rather than a single large lipid vacuole (Kamisaka *et al.,* 1999). Indeed, in *Mortierella ramanniana* lipid bodies "mature" during the lipid accumulation phase, starting with a diameter of ≈ 1 μm increasing to a maximum of 2–3 μm (Kamisaka *et al.,* 1999). Further lipid accumulation is achieved by formation of more lipid bodies rather than by the size of the existing lipid bodies increasing (Kamasaka *et al.,* 1999).

In addition to specific physical properties, lipid bodies possess a distinct enrichment of enzymes associated with triacylglycerol (TAG) assembly in the membranes that are around the lipid bodies (Kamisaka and Nakahara, 1994; Pillai *et al.,* 1998). The final enzyme in TAG assembly, diacylglycerol acyltransferase (DAGAT), is especially enriched in the membranes most closely associated with lipid bodies (Kamisaka and Nakahara, 1994; Kamisaka *et al.,* 1997; Pillai *et al.,* 1998). The other TAG assembly enzymes are likewise enriched in the membranes associated with the lipid bodies though to a lesser extent. Indeed, a correlation between the order of the enzyme in TAG assembly and their enrichment in the lipid body fraction from oleaginous microorganisms has been suggested (Pillai *et al.,* 1998).

As well as enrichment in the TAG assembly enzymes, microbial lipid bodies possess a specific set of membrane proteins, having molecular sizes of 24, 29, and 54 kDa (Kamasaka and Nakahara, 1994). The role of these proteins is probably structural, maintaining the integrity of the lipid bodies and avoiding coalescence with neighboring lipid bodies. They may be analogous to the oleosins of desiccation-resistant plant seeds (Murphy and Vance, 1998).

The role of lipid bodies in the organisation of TAG biosynthesis is now becoming more evident. In *Mort. ramanniana,* phosphatidic acid appears to be incorporated preferentially into the lipid bodies for TAG biosynthesis while phosphatidylcholine is incorporated into the ER membranes (Kamisaka *et al.,* 1999). Therefore, TAG biosynthesis may occur predominantly via a "cytosolic" route rather than via phospholipid (Fig. 10). If this is the case, then a PUFA-rich TAG, as is commonly produced by oleaginous microorganisms, must occur via "acyl-shuttling" between diacyl glycerol and/or TAG and the phospholipid where PUFAs are formed (Kamisaka *et al.,* 1999).

IX. The Metabolon Concept for the Integration of Lipid Biosynthesis

One of the most intriguing aspects of the whole process of lipid accumulation (as with any other metabolic process) is the intracellular

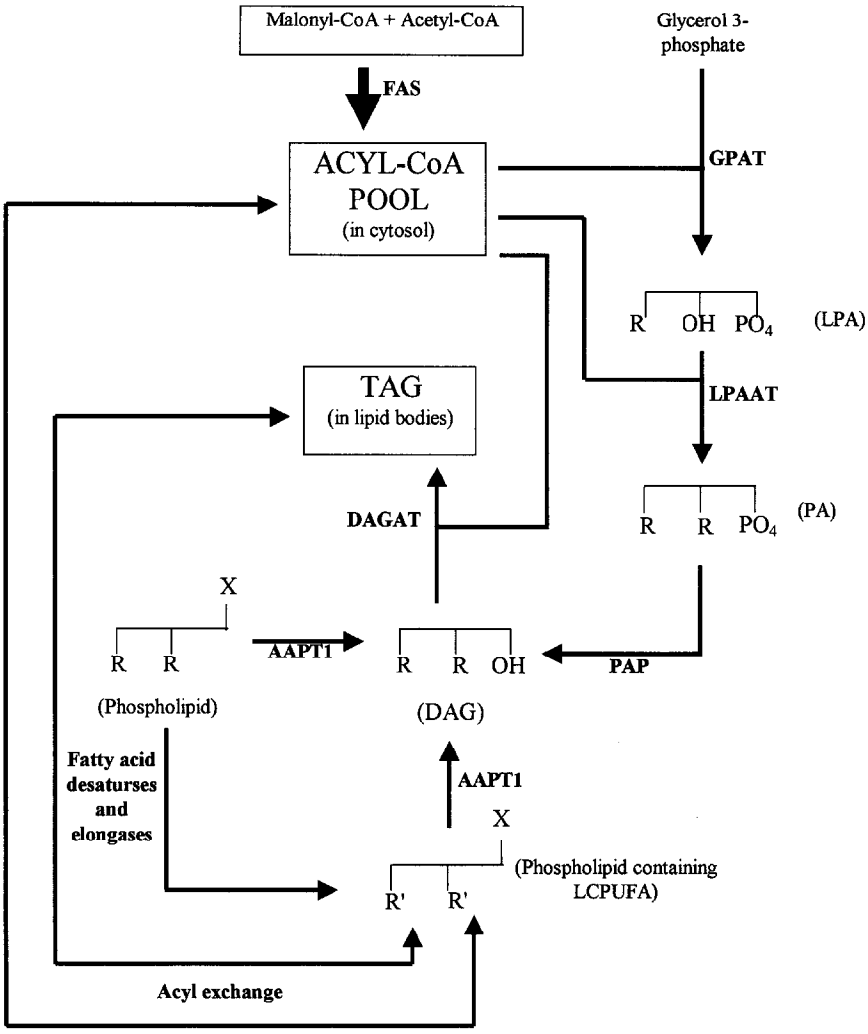

FIG. 10. The pathways for (PUFA)-rich triacylglycerol (TAG) biosynthesis. FAS: fatty acid synthase; GPAT: glycerol-3-phosphat acyltransferase; LPAAT: lysophosphatidic acid acyltransferase; PAP: phosphatidic acid phosphohydrolase; CDP-CPT: choline phosphotransferase; DAGAT: diacylglycerol acyltransferase; LPA: lysophosphatidic acid; PA: phosphatidic acid DAG: diacylglycerol; R: saturated fatty acid; R′: unsaturated fatty acid; X: phospholipid polar head group. Acyl exchange includes movement of acyl residues between phospholipid and TAG, by acyltransferases, and the activity of phospholipases to transfer unsaturated fatty acyl residues from phospholipid into the acyl-CoA pool.

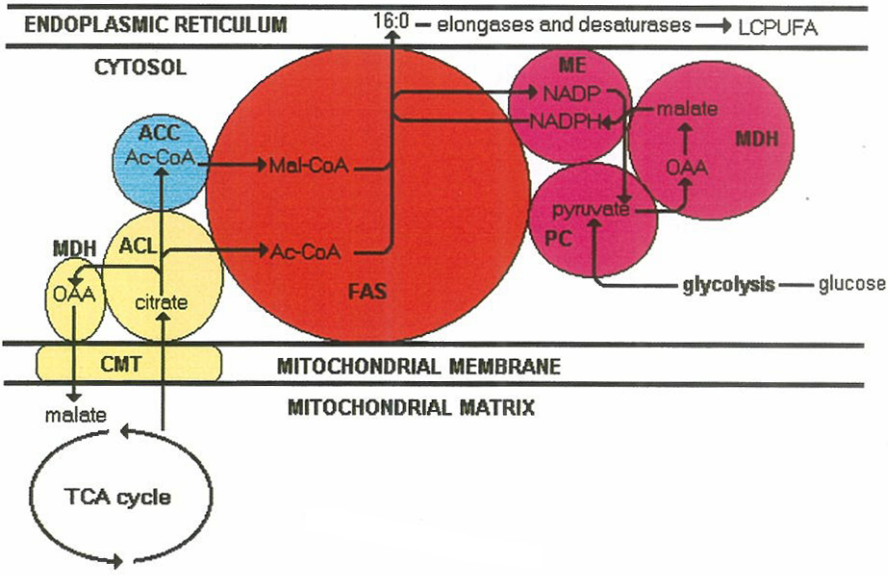

FIG. 11 A diagram of the hypothesized lipogenic metabolon with lines to demonstrate the routes of substrate channeling. OAA: oxaloacetate; Ac-CoA: acetyl-CoA; Mal-CoA: malonyl-CoA; FAS: fatty acid synthetase; ACL: ATP:citrate lyase; ACC: acetyl-CoA carboxylase; CMT: citrate/malate translocase; ME: malic enzyme; PC: pyruvate carboxylase; MDH: malate dehydrogenase. The "yellow" enzymes form the citrate/malate cycle while the "purple" enzymes represent the cytosolic transhydrogenase cycle.

organization that ensures the process continues in an efficient and closely regulated fashion. In this regard, we suggest that protein–protein interactions play a key role in the organization and regulation of lipid accumulation, and propose the existence of a "lipogenic metabolon" (Fig. 11, see color insert).

Protein interactions are clearly involved with the operation of the fatty acid desaturases (Fig. 8) and the clustering of triacylglycerol assembly enzymes in lipid body fractions as have been demonstrated by other authors (Kamisaka and Nakahara, 1994; Pillai *et al.,* 1998). Moreover, work in our laboratory has indicated that protein interactions may play a fundamental role in channeling substrates between lipogenic enzymes during *de novo* fatty acid synthesis.

It has long been apparent that malic enzyme plays a key role in the provision of NADPH for lipid synthesis (and desaturation) in fungal cells (Kendrick and Ratledge, 1992b; Wynn *et al.,* 1997, 1999; Wynn and Ratledge, 1997—see Section V.C). However, it is clear that malic enzyme is not the sole mechanism for NADPH generation in fungal cells (Wynn *et al.,* 1997; Wynn and Ratledge, 1997). Indeed, in the fungi studied the hexose monophosphate pathway appears the most active NADPH generating pathway (Wynn *et al.,* 1997; Wynn and Ratledge, 1997). As a consequence, it was hard to explain the specific role of malic enzyme in fatty acid synthesis unless a direct channeling of NADPH from malic enzyme to fatty acid synthase was assumed. Having become convinced of the interaction of malic enzyme and fatty acid synthase, it then seemed an obvious extension of our hypothesis to invoke other enzymes as potential members of the hypothesized lipogenic metabolon. This hypothesis, of course, is not completely novel. Srere, who originally developed the concept of the metabolon, had detected an apparent association between fatty acid synthase and another major lipogenic enzyme (ATP:citrate lyase) in animal cells (Finkelstein *et al.,* 1979; Linn and Srere, 1984) where this association was mediated via the endoplasmic reticulum.

Conclusive direct evidence of the hypothesized protein–protein interactions is still lacking. These interactions are likely to involve only weak noncovalent attractions between the various proteins, which would be readily disrupted by the physical stress and dilution effects that occur during cell breakage (Velot *et al.,* 1997). The metabolon concept is gaining ground in many areas of biochemistry. The energetic advantages of a high degree of intracellular organization (rather than separate enzymes floating around in a cytosolic substrate/product "soup") are obvious (Ovadi and Srere, 1996). Metabolon complexes are suggested to occur in the citric acid cycle (Verlot *et al.,* 1997), amino acid biosynthesis (Abadjieva *et al.,* 2001), pigment biosynthesis (Sugumaran *et al.,* 2000),

and β-oxidation (Eaton *et al.*, 2000). It is our firm assertion that fatty acid biosynthesis should (and will eventually) join this growing list of metabolons.

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Anonymous. (2001). *INFORM* **12,** 863–837; and see also Netlinks: Martek Biosciences DHA/ARA www.martekbio.com/dha.html and FDA response letter to Martek: www.cfsan.fda.gov/nrdb/opa-g041.html

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