Lipid Biotechnology: A Wonderland for the Microbial Physiologist

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Many definitions of biotechnology have been advanced but all agree that biotechnology encompasses the use of microorganisms or their component parts for the manufacture of products or service systems. Plant and animal culture systems are included in most definitions, thus biotechnology can be seen as playing a vital part in some of the new advances that are occurring in agricultural and medical sciences. The application of biotechnology to the oils and fats industry, as just one sector of the very large agrochemical industry, is such that already in this decade we have seen the American Oil Chemists' Society (AOCS) sponsor several biotechnology sessions at annual conferences, a monograph on the subject (1), a major conference in 1985 that was devoted to emerging technologies (2), partially including the contributions of biotechnology, and now a major conference devoted exclusively to biotechnology.

This awakening of interest has stemmed from the rapid advances that occurred in the major biological disciplines and, in particular, in the advances that have occurred in molecular genetics.

The preceding contribution from Stumpf has set out the background and growth points for the contribution of the plant geneticist and biochemist to the oils and fats industry. Of course, plants are the cornerstone of the industry; the vast majority of oils and fats are produced by agricultural means. Animal fats constitute an important but nevertheless minor sector of the market, and microbial oils and fats contribute nearly nothing. Biotechnological applications to animal fat research and development are still very much in their infancy, though occasional thoughts have been raised about the possibility of changing the degree of unsaturation of the fatty acids in animals. However, such changes—even if nutritionally desirable (3)—would not be brought about by genetic engineering techniques but by more conventional means of animal breeding or even by control of the animals' dietary intake of fatty acids. Genetic engineering to modify the fat of an animal might be regarded as a project for the next century, though, this is only 13 years away.

Why then, if microorganisms currently contribute so little to the oils and fats industry, should we now be so enthusiastic about them? First, we can view microorganisms as potential sources of oils and fats, though for obvious economic reasons these oils should be of the higher value-added types rather than resembling the bulk low-priced commodities such as soybean oil, sunflower oil, etc. Second, microorganisms provide excellent means of carrying out numerous biotransformation reactions using either the whole microbial cell or one or more of its component enzymes. Third, microorganisms can provide useful models for studying the more intricate aspects of lipid biochemistry, metabolic control and function, thus giving invaluable leads to plant and animal lipid biochemists.

Each of the above three aspects will be covered in this short review; aspects of microbial biotransformations largely will be left to the succeeding presentation of Yamane for full coverage. However, it should be admitted that the study of microbial lipids has always been the "Cinderella" of the subject with regard to lipids and to microbiology. Even when microbiologists have chosen to address themselves to potentially interesting problems in this field, such as the biosynthesis of fatty acids, they have tended to use a very restricted range of microorganism: usually *Escherichia coli* for bacterial work and *Saccharomyces cerevisiae* for yeast work. Although much of this work has had considerable elegance, it has had only an indirect bearing on what happens in the oil-bearing seeds of plants or even in the adipose tissue of animals. Thus, the more applied aspects of lipid microbiology, what now would be called lipid biotechnology, often has been an "orphan" subject. Advances have failed to keep pace with developments elsewhere. We still do not know, for example, what controls the degree of unsaturation of a fatty acid; yet, almost every undergraduate biochemistry student is familiar with the intimate details of DNA organization with its introns and exons, TATA boxes and leader sequences. Thus, for a microbial physiologist such as myself to find a relatively unexplored area of biochemistry in the last quarter of the 20th century suggests that this is a wonderland just waiting for exploration. Therefore, I hope this review gives a reflection of how some of these perambulations have been proceeding in recent years.

MICROORGANISMS

Microorganisms range from bacteria without a defined nucleus, procaryotes, to yeasts and molds that have such a nucleus, eucaryotes. They also include the photosynthetic microorganisms, which may be both pro- and eucaryotic, thus covering the cyanobacteria, previously known as the blue-green algae, and the larger eucaryotic algae that range from the microscopic individual cells to the macroscopic large, brown algae recognized as seaweeds throughout the world.

Microorganisms can be seen to have the following advantages: a prodigious growth rate, 1 g of bacteria can generate a second gram of biomass in 20 minutes, a yeast cell takes a little longer—two to four hr; an omnivorous appetite in that almost any carbon-containing substrate can be used to sustain growth; and they can be manipulated by the geneticist or the physiologist to produce a wide range of products or to have the yield of a minor component amplified considerably. A chemist achieving a 0.1% yield for a reaction in the laboratory probably would give up and look in another

direction for the right process. That is not so with the microbiologist. As witnessed by most of the antibiotics, there are many examples in which the initial yield of a product was extremely low; by the application of genetical mutation and physiological control of the growth process, the yield has been multiplied many-fold.

The technology for sustaining large-scale cultivation of microorganisms has advanced over many years. Fermenters capable of producing up to 10^5 ton of biomass per year now are not uncommon. Microorganisms, according to the process, may be grown batch-wise or in a continuous mode. However, the real problem comes in deciding on what to grow the cells; for photosynthetic algae, the choice is obvious: CO_2. For other microorganisms, an organic source of carbon is necessary; for large scale fermentation processes, the price and availability of the starting substrate may be a key factor in determination of the process' economic viability (4).

PROSPECTS FOR MICROBIAL OILS

Types capable of production. Probably the most expensive triacylglycerol oils being produced commercially today are those containing the essential γ-linolenic acid (6,9,12-octadecatrienoic acid). These oils are found in the seeds of *Oenothera* (Evening Primrose) and *Ribes* species (especially blackcurrant and gooseberries) (see Table 1). There is a small volume market (5200-500 ton/year) which is growing steadily for these oils. Although the exact nutritional value of exogenously supplied γ-linolenic acid is challenged, sometimes the role of this acid in vivo, which is to act as a precursor of the prostaglandins (PGE_1) series, is undisputed. As these oils command prices of $50/kg and beyond, there is little wonder that several groups have seen this as an obvious target for biotechnological innovation and exploration.

γ-Linolenic acid has been known as a fungal fatty acid since 1948 (5) and was established by Shaw (6) to be confined to the family of molds known as the *Mucorales.* Species of *Mucor* and other related organisms also have been known for many years to be capable of accumulating considerable amounts of lipid (7,8). Thus, by virtue of extensive screening for strains of fungi accumulating high amounts of γ-linolenic acid (9-12). Because of the prior publications in this area, these patent applications may not all be recognized or granted. Today, there are at least two commercial processes for the production of this oil. One in Japan is based on *Mortierella* and will be described later by Suzuki (13); the other uses *Mucor javanicus* and has been developed by J. & E. Sturge, Ltd., in the UK in conjunction with work in our own laboratories. This latter process is now at the 220,000 liter level of production with opportunities for expansion of up to four or five times by using similarly sized fermenters.

The fatty acid profile of this oil is distinct from that of the plant oils (see Table 1) but, interestingly, bears a strong resemblance to that of the milk fat from humans. The lower content of linoleic acid (18:2) in the *Mucor* oil than in plant oils makes it an attractive starting point for producing purified γ-linolenic acid; such as an oil is easier to fractionate than one containing many polyunsaturated fatty acids.

The prospects for producing other dietarily important polyunsaturated fatty acids (PUFA) including arachadonic acid (20:4), eicosopentaenoic acid (20:5) and docosahexaenoic acid (22:6) have been examined in microorganisms. Although these PUFA have been reported sometimes in high concentrations, they have been studied only in algae (18-20). Although algae require much less financial investment for their cultivation than do yeasts and molds, the yields of algae per liter of medium (or even expressed per m^2) are very much lower than yeasts or molds. Maximum yields of algae (*Chlorella, Spirulina* and *Scenedesmus*) under optimal outdoor conditions are unlikely to exceed 25 g/m^2/day (21). Thus, to produce 100 tons of biomass it would require four hectares of algal lagoons illuminated for 100 days with continuous sunshine and a temperature between 28-30 C. On the other hand, with yeasts and molds capable of reaching 80-100 g of cells/l in a 60 hr fermentation, a modest 200 m^3 fermenter would produce well over 1500 tons of biomass in one year, irrespective of sun, wind or rain and even allowing for a 30 hr turn-around between fermenter runs.

The oil produced in algae also is not as attractive as those of other microorganisms; it usually is comprised of numerous types in which the triacylglycerol fraction

TABLE 1

Spectra of Fatty Acids in Various Oils and Fats Containing γ-linolenic Acid

Source of oil	Oil content (% by wt)	Principal fatty acyl constituents (% W/W) (14-17)							
		16:0	16:1	18:0	18:1	18:2	γ-18:3	α-18:3	18:4
Oenothera biennis[a]	16	7	tr	2	9	74	8	0.2	–
Ribes nigrum[b]	30	6	tr	1	10	48	17	13	3
Ribes rubrum[c]	25	4	–	1	14	41	4	30	3
Ribes uvacrispa[d]	18	7	–	1	15	40	11	19	4
Borago officianalis[e]	30	10	–	4	18	42	23	0.4	–
Human breast milk		23	–	9	35	11	2*	0.2	–
Mucor javanicus[f]	18	23	tr	7	39	11	18	0.2	–

[a]Evening Primrose; [b]Blackcurrant; [c]Redcurrant; [d]Gooseberry; [e]Borage; [f]Production fungus (J. & E. Sturge, UK).

*Includes other polyunsaturated fatty acids.

TABLE 2

Effect of Sterulic Acid on Formation of Stearic in Selected Oleaginous Yeasts (31).

Yeast		Sterulic acid* added (ml l^{-1}) 0	0.02	0.10	1.0
Candida 107 (= NCYC 911)					
Lipid in cells		37	18	25	33
Rel. %	16:0	26	28	28	24
	18:0	5	24	33	44
	18:1	35	12	10	10
	18:2	21	30	23	14
Trichosporon cutaneum					
Lipid in cells		29	31	30	31
Rel. %	16:0	29	30	32	35
	18:0	7	19	24	23
	18:1	50	33	27	25
	18:2	13	16	14	13
Rhodosporidium toruloides					
Lipid in cells		30	30	34	32
Rel. %	16:0	16	15	15	13
	18:0	4	17	32	41
	18:1	42	19	23	18
	18:2	29	17	15	15
	18:3	5	9	7	6

*Added as sterculia oil, which contains 50% sterulic acid (Δ^9 cyclopropene 17:1) and 5% malvalic acid (Δ^9 cyclopropene 18:1).

may be only a minor component. Thus, extraction and processing of an algal oil becomes more complex than that of a microbial oil. There also is the problem of the co-extracted chlorophyll with which to deal, otherwise the algal oil will have an unwanted green color.

Nevertheless, in spite of these problems algae potentially are useful sources of arachadonic acid (20:4) (18) and eicosapentaenoic acid (20:5) (19), as other symposium presentations clearly indicate (22,23). The advantage of using algae to produce PUFA rather than to rely on fish oils is that the algae could be induced to produce high concentrations of a single fatty acid (18,19) rather than an array of fatty acid types. There also may be the possibility of producing these fatty acids as an adjunct to the algal production of carotenoids, especially β-carotene, which now is a commercial reality in several locations throughout the world (24).

Other high-value microbial lipids that are under current consideration include production of a cocoa butter fat or even a fat with superior specifications to cocoa butter. Cocoa butter currently sells for between $4,500–$5,000 per ton (25,26), although prices over the past six years often have exceeded this. Annual production of cocoa butter is about 800,000 tons (27), and a number of reformulated vegetable oils currently are produced as cocoa butter equivalents (CBE). The potential market for these CBE could be up to 10% of the total cocoa butter market.

Cocoa butter is characterized by a high content of stearic acid (30–35%) and has as its principal triacylglycerol 1-palmitoyl-2-oleoyl-3-stearoylglycerol. Unfortunately, most oleaginous microorganisms have stearic acid contents of 10% or less, and so efforts have been made to increase this level either by feeding stearic acid or its esters to yeasts (28–30) or by adding an inhibitor of stearate Δ^9-desaturase (31). This latter approach, using the naturally occurring cyclopropene fatty acid sterculic acid, has led to up to 40% increases of stearic acid in selected oleaginous yeasts (see Table 2). Comparisons of this yeast fat with cocoa butter have indicated a striking similarity between the two lipids (25).

Prospects for being able to delete by genetic mutation and selection the Δ^9-desaturase enzyme in oleaginous yeasts also should not be forgotten as a possible third route to achieving biotechnological production of CBE. However, as will be appreciated from Table 2, inhibition (or potential deletion) of the Δ^9-desaturase does not seem to greatly diminish the formation of linoleic acid (18:2), which would be necessary to achieve if the new cocoa butter substitute was to be entirely satisfactory.

Other lipids that currently are under consideration for biotechnological production include a number of glycolipids as potential surfactants, various carotenoids and, if we can include as a lipid the unusual bacterial polyester, poly-β-hydroxybutyrate. Such opportunities have been reviewed recently by several researchers (32–34). It seems unlikely, however, that wax ester substitutes for jojoba oil or spermaceti could be produced economically by microbial means.

COST

The increase in activity towards producing a microbial oil has led a number of the interested parties to suggest possible economies for potential Single Cell Oil (SCO) process.

Clearly, with a very high priced oil such as those containing γ-linolenic acid the costs look attractive. Prescott (35) has calculated that a single 220 m^3 fermenter can produce as much oil in four days as 30 acres of a good crop of Evening Primrose or Borage does in a year. Furthermore, the annual output of two such fermenters probably could satisfy the entire current world demand for this oil. Approximate cost for this process compared with agricultural costs are given in Table 3. It should be pointed out that these estimates are for average yield models for both plant and fungal oils; they do not take into account losses of oil or costs at the extraction stage. Even so, the lower costs of the biotechnological route are quite evident.

Costs for larger scale productions of oils and fats have been made by Davies (36,37), Moreton (25), Floetenmeyer et al. (38) and by Moreton and Norris (39) for fermentation processes in general. Interested readers are referred to these reports for further details as a complete discussion of the various points that must be considered are too many for a short review like this.

The important factors that have a bearing on the eventual costs include:

TABLE 3

Comparisons of Variable Costs of GLA from Different Sources (35).

Source	High margin	Low margin	Percentage of GLA in oil	Minimum cost of GLA
Evening Primkrose	£10,000	£5,755	9%	£64,000/ton
Borage	£9,333	£5,608	20%	£28,040/ton
M. javanicus	£2,000*		16%	£12,500/ton

*Cost per ton of oil in cells; i.e. does not include processing or extraction costs (but these are likely to be approximately equal in all three cases).

- *Cost of substrate.* Even the most efficacious oleaginous organism cannot convert substrate to lipid with a yield much better than 22%. Thus 4.5 to five ton of substrate are needed to produce one ton of oil. Year-round availability of substrate also is important.
- *Scale of operation.* The larger the process, the cheaper the unit costs become.
- *Fermenter design and operation.* Different economic costs depend on whether a batch or continuous process is chosen (or is desirable from a product viewpoint). Stirred tank reactors may not be as effective as airlift fermenters.
- *Down-stream processing and oil extraction.* This is the area that still requires the most work. There are obvious differences between a yeast-based process and a mycelial fungal process; the latter is easier to harvest and extract but poses more problems during the fermentation phase, and continuous operation almost is impossible. Extraction of cells with hexane is a standard practice with plant oilseeds, and a similar technology can be applied to yeast cells (40). Improvements in this process to avoid complete and expensive drying of the cells have been attempted (37), and the use of cross-flow filtration rather than centrifugation has been advocated (41).
- *Capital costs.* Returns on capital invested must take into account discount cash flow to provide a proper evaluation of the financial viability of the project (39).
- *Revenue.* There is no doubt that a good quality oil of almost invariable composition and properties can command a premium price. Single Cell Oils (SCO) can be produced year-round with high constancy of composition. Supplies of the oil are not influenced by the vagaries of the weather; agreed supplies at agreed costs are attractive propositions to potential customers of the SCO. The cell residue after oil extraction also is recognized as potential value as an animal fodder equivalent to soybean meal.

Davies (37), in a very detailed series of cost estimates, has calculated that it would cost approximately $3 million to build a process plant capable of producing from whey 1,000 tons of oil plus 1,800 tons of protein residue that is salable as animal fodder. To make the process attractive to potential financial companies, requiring a 20% rate of return after taxation, the oils would have to sell for more than $1,000/ton and the protein for $275/ton. However, costs do not include any credit for abatement of a potential pollution problem by using the whey.

Moreton (25) has indicated that the lowest reasonable cost of a yeast SCO is more likely to be $2,500/ton than $1,000/ton. Floetenmeyer et al. (38), however, have suggested that given a zero-cost substrate this value could be as low as $680/ton of lipid, but they did not take into account the extraction costs of the oil. Moreton's calculations do include both the cost of substrate (at $240/ton) and extraction costs of about $1000/ton of lipid.

Thus, by a variety of calculations we probably can conclude that production of SCO will be limited only by economic considerations to the more expensive "up-market" oils and fats. However, given that a number of processes exist throughout the world for the production of Single Cell Protein (SCP), which utilize waste (zero- or negative-cost) substrates, it is fairly easy to show that production of SCO would be a better financial proposition than continuing with SCP (41). A fuller review than this of the microorganisms potential for oil production recently has been published (42). This covers bacteria, algae, yeasts and fungi, and it summarizes the literature from 1980 to 1985.

MICROBIAL TRANSFORMATIONS

The ability of microorganisms to carry out numerous transformation reactions is well-known and forms one of the cornerstones of microbial technology. Transformations with lipids may be carried out by using either whole cells or their component enzymes (Table 4). A brief outline of the current activities and future prospects of this topic follows.

The simplest of the transformation reactions attempted with lipids has been feeding fats or fatty acids to selected microorganisms in the hope that the microorganisms would upgrade the oil quality by desaturating, or even saturating, the component fatty acids. In most cases (43-45), the composition of the fatty acyl groups recovered from the microorganisms has proved to be similar to those on which it was obliged to grow. The reason for this conservation of lipid structure is that the yeast (it usually has been yeasts that have been tried) obviously is able to satisfy its own requirements for fatty acids, either for the structural phospho-

TABLE 4

Agents for Biotransformation Reactions

Order of complexity (and cost)		
	1. *Whole cells*	- growing - nonproliferating - permeabilized by organic solvents (two-phase liquid systems)
	2. *Mutant cells*	- blocked to cause product accumulation
	3. *Crude enzyme preparations* (extracellular or intracellular)	- free - immobilized: single phase or liquid two-phase systems
	4. *Partially purified enzymes*	- free - immobilized
	5. *Enzymes with co-factors*	- coupled enzymes for co-factor regeneration

lipids or storage triacylglycerols, with a wide range of acyl groups. Thus, there is no environmental pressure exerted upon the microorganism to change the fatty acid substrate with which it is presented. The growth of yeasts on various fats and oils has been reviewed recently (46). However, much of the current interest in this area appears to be towards complete utilization of the unwanted fat to produce yeast salable as animal fodder material.

If changes in the fatty acids of a substrate are required, it usually will be necessary to mutate the parent microbial cells. Mutation can be used to block degradation of the fatty acid via the β-oxidation cycle, thus preventing undue losses of the substrate, or it can block the further oxidation of the product before it is linked into β-oxidation. Consequently, if fatty acids are presented now to such cells for transformation, partial oxidation products of fatty acids should be found (47,48). Such products would include hydroxy fatty acids and dicarboxylic acids.

Most of the metabolism for fatty acids have not been worked out using fatty acids; they have stemmed from extensive studies on alkane metabolism in a variety of microorganisms. These studies elegantly have demonstrated the power of using mutants to produce greatly elevated levels of intermediate products. Conversions of alkanes to α,ω-dioic acids in yield up to 70% and with amounts up to 60 g/l have been reported with mutants of *Candida cloacae* and *C. tropicalis* (49,50). 3-Hydroxyalkanedioic acids also have been recovered from such transformation experiments (50).

Beyond the use of whole cells lies the possibility of using cells held in solvents; this obviously would kill the cells but allows the intrinsic enzyme activity to be retained and even enhanced. Such a technique is practiced with some sterol transformations in which the solvent, often chloroform, allows penetration of the substrate into the cells and migration of the product out of the cells (51,52). The advantage of this technique is that it does not require the physical isolation of unstable enzymes, such as the hydroxylases, to be involved in the initial attack on alkanes or fatty acids. Furthermore, the enzymes can retain activity for a considerable time and can transform many times their own weight from substrate into product (52). Cells used in such systems may be the original parent (wild-type) strain or mutant produced to prevent further reactions of the product (53). Recent examples of biocatalysis occurring in two-phase systems include epoxidation of 1,7-octadiene by whole cells of *Pseudomonas putida* (54) and stereospecific hydrolysis of *d,l*-menthyl acetate by cells of *Bacillus subtilis* (55).

The next degree of complexity for biotransformation is the use of isolated enzyme systems, and these may be recovered from the extracellular growth medium or from within the cells. The less purification that has to be carried out, the cheaper the eventual product. Consequently, considerable effort currently is being spent trying to isolate new microorganisms with enhanced enzyme activity or, if this fails, even restorting to genetic engineering to enhance the enzyme complement of the cells. For lipids, isolated-enzyme technology usually means lipases that now are featured so prominently in this and previous symposia organized by the AOCS (56-58). Lipases, besides being either nonspecific or specific and being able to carry out transesterification reactions, may also act in a synthetic mode (57,58) but only if the water content of the reaction mixtures is kept extremely low. Once again, we see that enzymes are not to be thought of as just water-soluble agents; they can, and do, perform certain reactions better in a nonaqueous environment than in water.

Lipases have much to offer to the oils and fats industry (56,59), and one of their main potentials is catalyzing the formation of cocoa butter-type triacylglycerols from palm oil and stearic acid by interesterification (59). Unfortunately, commercialization of this has not been realized yet. The difficulty appears that although purified vegetable oils, such as palm oil, work extremely well with lipases in the laboratory, the

enzyme does not retain its longevity when operating with commercial grades of oil at pilot-scale levels (60). Thus, activity is lost prematurely; this adversely affects the overall economics of the process.

Lipases used in these interesterification reactions are immobilized to prevent their loss from the reactor. Unfortunately, the number of commercially successful immobilized enzymes still is extremely small (about four or five), in spite of considerable early promise that this would be a major activity of biotechnology by the 1980s. The lack of success is due principally to many enzymes requiring specific biological (and hence expensive) co-factors or requiring an input of energy into the reaction to make it proceed. These constraints mean that it is the hydrolytic or isomerizing enzymes that are potentially the most useful for commercial processes but there are only a limited number of such reactions that are interesting commercially. In spite of these drawbacks, considerable effort currently is underway to circumvent some of these problems; it is likely that the next decade will see coupled enzymes being used for co-factor recycling or even an energy input into reactions. However, these reactions probably will operate on a relatively small scale and will deal with high value-added products.

Because lipases catalyze the hydrolytic type of reaction and require neither co-factor nor energy input, they would seem ideal candidates for exploitation. However, applications of them are very narrow; it has to be remembered that chemists have been very successful in devising all of the reactions that are used in the oleochemical industry. Thus the biotechnologist has to devise some novel enzymological reactions that the chemist would find very hard to emulate. Biotransformation reactions undoubtedly will find a place in the oils and fats industry, but the biotechnologist must not expect the chemist to sit idle, waiting for his reactions to be superseded.

MICROORGANISMS AS MODEL SYSTEMS FOR STUDYING LIPID BIOCHEMISTRY

"I should like to have it explained," said the Mock Turtle. Alice's Adventures in Wonderland, Lewis Carroll.

Plants and animals are complex; microorganisms, in comparison are simple. Most biochemical pathways that are common to all living cells usually have been first recognized in microorganisms. Lipid biochemistry follows this generalization, though there are some exceptions.

Pathways for the biosynthesis of fatty acids and from fatty acids to triacylglycerols, phospholipids and glycolipids have been recognized in most microorganisms, and the complexity of the component enzymes has been analyzed in some detail (61,62). Even biosynthesis of some of the more complex lipids that often are thought of as animal lipids, such as the sphingolipids, which includes the cerebrosides and sphingomyelins, also have been clarified in yeasts (see ref. 63 for review).

By the mid 1970s, it seemed the whole of lipid biochemistry in microorganisms probably had been elucidated and that microbial systems having played their part could be relegated to a minor position while more interesting projects involving plant or animal tissues could proceed. Since then, however, we have had to revise completely our ideas of how the fatty acid synthetase complex is organized in yeast and in animals. We now are faced with the enormous task of trying to understand how such a complex process as fatty acid assembly is carried out by only two proteins when eight separate reactions, some repeated seven times with different substrates, are required for the conversion of acetyl-CoA to palmitoyl-CoA. The approach to this problem now requires a combination of genetics and biochemistry, as eloquently described by Schweizer (64). Ultimately, this work should unravel not only the process in *Saccharomyces cerevisiae* (baker's yeast) but also in animal systems that share properties with the eucaryotic microbial cell. For assistance with plant lipid biochemistry, we again can look to microorganisms for guidance; although here it is the procaryotic bacterial cell that shows the greatest similarities.

It also was considered in the mid 1970s that yeasts and fungi would be the same in regards to lipid biosynthesis and that by understanding the process in one organism (*S. cerevisiae*), the process would be understood in all organisms. However, this ignored the real differences that obviously occurred between different microorganisms, namely that a few species had the ability to accumulate considerable amounts of lipid in their cells whereas other cells, even when placed under exactly the same conditions, did not. These fat-accumulating microorganisms, referred to as the oleaginous species (65), clearly had some unusual features of their biochemistry to account for this accumulation. A series of investigations (66) led us to the conclusion that it was not that these cells had a hyperactive system for synthesizing fatty acids but that they possessed a system for producing acetyl-CoA, the building units for fatty acid biosynthesis, which was not present in the non-oleaginous cells.

For once, the tables were turned on the microbial physiologist. It was the animal biochemists who had pioneered this area, though even here the animal biochemists considered their system for acetyl-CoA unique, thus completely absent from microbial cells (66).

The key to efficient acetyl-CoA production in oleaginous yeasts (and molds) was found to be ATP:citrate lyase (67), an enzyme catalyzing the cleavage of citrate in the presence of CoA and ATP to acetyl-CoA and oxaloacetate. However, the mere presence of this enzyme is not sufficient to explain oleaginicity, and one then must begin to work backwards to answer a series of questions:

Questions: How is the citrate provided for the ATP:citrate lyase, which is in the cytoplasm?
Answer: By transport out of the mitochondrion in exchange for malate (68).

Question: Why does the citrate accumulate and not get metabolized via the citric acid cycle?
Answer: Because its metabolism, at the level of isocitrate dehydrogenase in the mitochondrion, is blocked (67,69).

Question: How does the isocitrate dehydrogenase become blocked?
Answer: Because it requires AMP for activity (67,69,70)

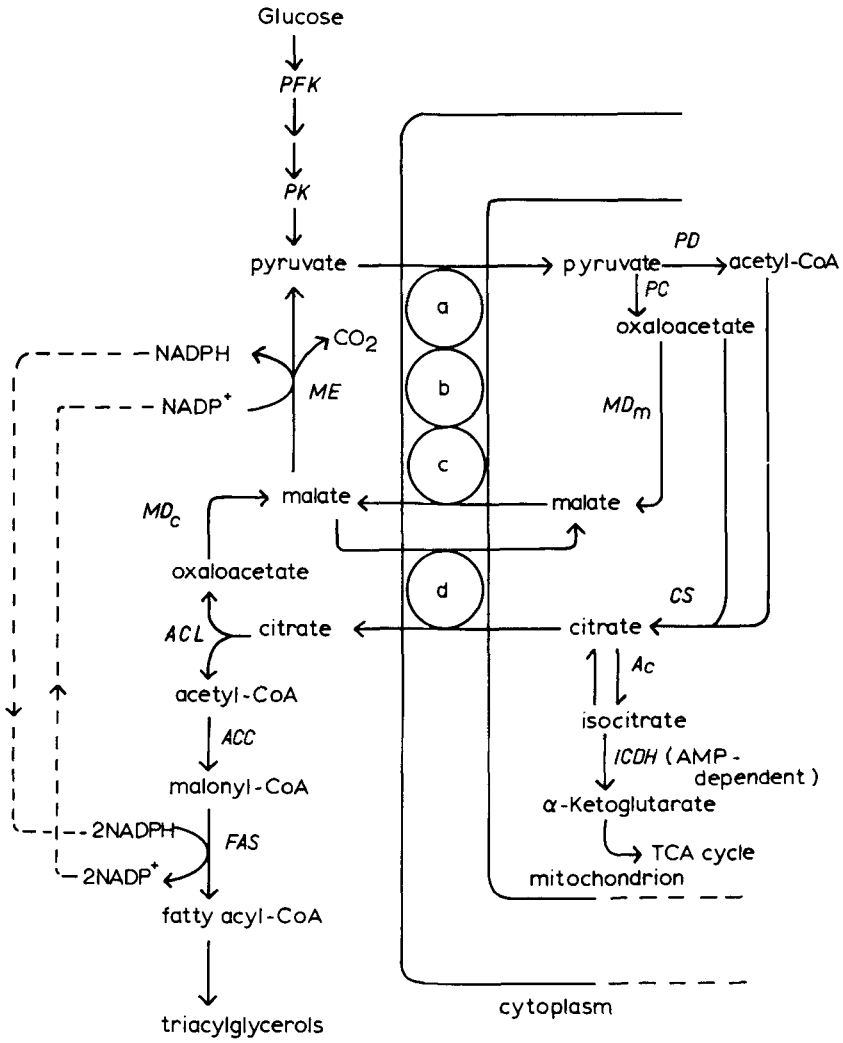

FIG. 1. Intermediary metabolism as linked to fatty acid biosynthesis in oleaginous microorganisms. Mitochondrial transport process: a, b, c, interlinked pyruvate-malate translocase systems; d, citrate -malate translocase. Enzymes: AAC, acetyl-CoA carboxylase; AC, aconitase; CL, ATP:citrate lyase; CS, citrate synthase; FAS, fatty acid synthetase complex; ID, isocitrate dehydrogenase; MD_c, malate dehydrogenase (cytosolic); MD_m, malate dehydrogenase (mitochondrial); ME, malic PC, pyruvate carboxylase; PD, pyruvate dehydrogenase; PFK, phosphofructo PK, pyruvate kinase.

this co-factor quickly disappears when the cells run out of nitrogen and are ready to begin the accumulation of lipid (71).

Question: How does the AMP deaminase become activated?
Answer: Not known.

Question: What makes the AMP disappear?
Answer: AMP deaminase, which becomes activated when the nitrogen supply is exhausted (72).

Question: How does the malate get out of the mitochondrion so it can exchange with the citrate, which is inside the mitochondrion?
Answer: Probably in exchange for pyruvate (72).

Question: How is the supply of pyruvate then ensured?
Answer: By the key enzymes of glycolysis phosphofructokinase (PFK) and pyruvate kinase (PK) being fully active (73,74).

Question: Where does the malate come from in the first place?
Answer: From oxaloacetate, which comes from the pyruvate entering the mitochondrion.

Question: When the citrate is cleaved, the acetyl-CoA goes for fatty acid biosynthesis but what happens to the other product, oxaloacetate?
Answer: This is converted to malate.

Question: Is this the malate, which then exchanges for citrate?
Answer: Only in part as more malate arrives outside the mitochondrion than is taken in exchange for citrate.

Question: What happens to this extra malate?
Answer: It is converted to pyruvate, which then can

begin the process over again by malic enzyme, an enzyme which simultaneously produces NADPH (72).

Question: What happens to the NADPH?
Answer: This is used for fatty acid biosynthesis.

Question: Is this a complicated scheme?
Answer: Look at Figure 1.

Question: How is the whole process coordinated to achieve the right balance of acetyl-CoA and NADPH production?
Answer: Not known.

The main aspects for a biochemical explanation for oleaginicity thus now are known (Fig. 1). It would seem more than likely that further study will answer some of the outstanding problems and, because the process is similar to that seen with lipid accumulation in animal cells, it may be possible to suggest a universal hypothesis. The accumulation of lipids in plant seeds probably is by the same mechanism. Certainly, ATP:citrate lyase is present in the cytosol of germinating endosperm (75), developing soybean cotyledons (76), ripening mango fruit (77) and pea leaves (78), though it has yet to be demonstrated in plant tissues synthesizing lipid reserves. The process used for NADPH production for fatty acid biosynthesis is less certain and though malic enzyme does occur (79), it may be linked only to NADH formation and not NADPH.

Microorganisms not only provide excellent models for studying pathways of biosynthesis and degradation but also for the regulation of these pathways. Techniques such as continuous culture are denied to animal and plant biochemists but such systems enable stringent control to be held over the environmental conditions and, most importantly, also produce cells being held at a constant growth rate in a steady state system. Therefore, it becomes impossible by using a chemostat (i.e. continuous culture fermenter) to calculate from observed enzyme activities if these are sufficient to account for the rates of cell growth and even for the overall rates of synthesis of individual components. In this way, it has been possible to show that ATP:citrate lyase in yeasts is probably the rate-limiting step in the whole of lipid biosynthesis (71). Thus, our view that this enzyme is probably the key regulatory enzyme because it is so sensitive to feedback inhibition by long chain fatty acyl-CoA esters is confirmed (80). However, the CoA esters, also inhibit equally strong the efflux of a citrate from the mitochondria (81), and it is possible that the enzyme could be coordinated and controlled, though this yet has to be established.

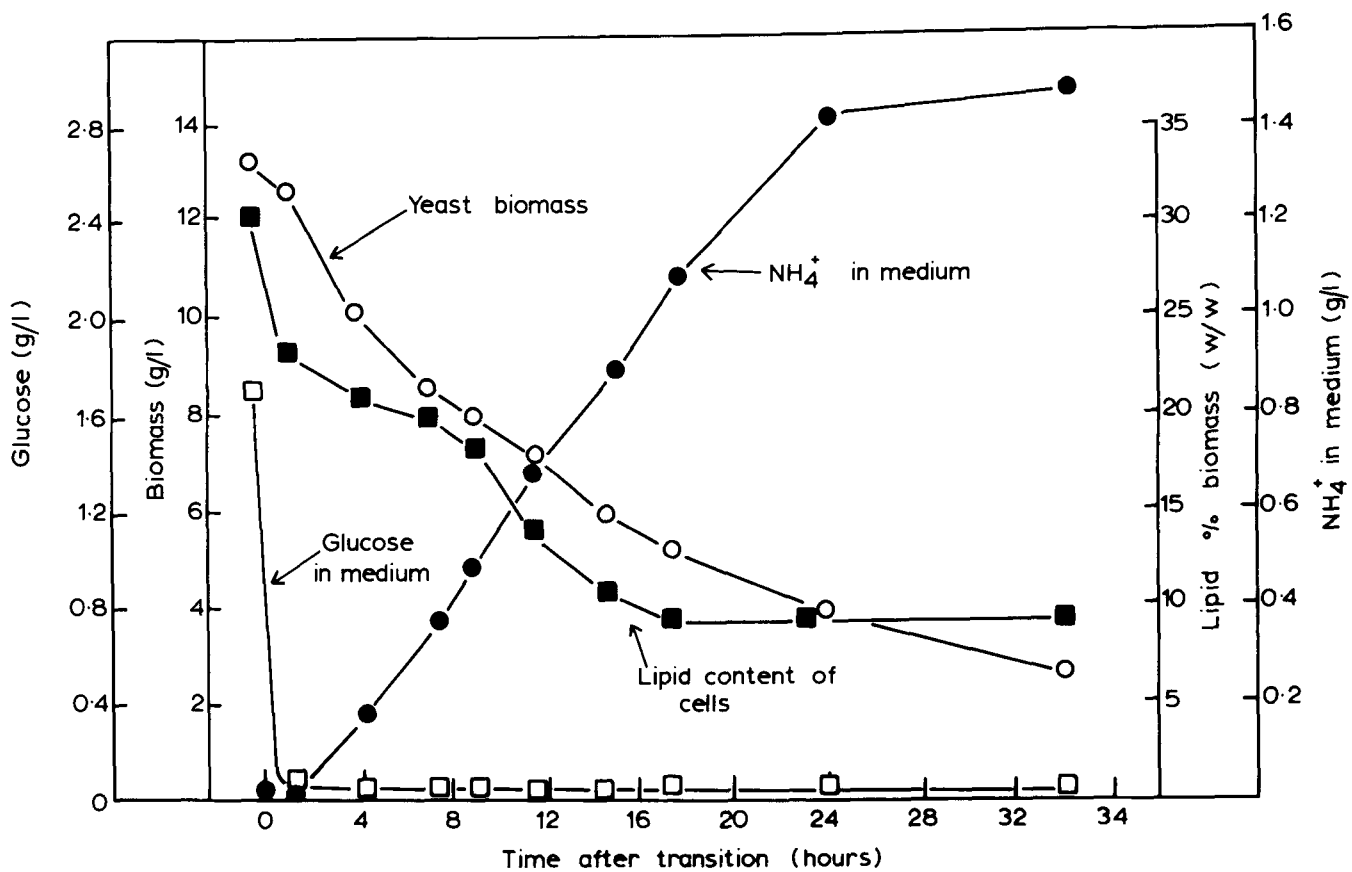

FIG. 2. Lipid degradation in an oleaginous yeast, *Candida curvata* D, on undergoing transition from steady-state lipogenic growth in a carbon-excess, nitrogen-limiting chemostat to a carbon-starvation environment while still in continuous culture at a dilution rate of 0.05 h^{-1}. Lipid mobilization immediately begins the transition is made (achieved by switching media entering the chemostat). (Taken from refs. 82 and 84.)

Just as we have been able to use chemostat cultures to follow the events leading up to lipid accumulation, we have been able to use the same techniques to follow lipid degradation (82,83). Again, the utilization of accumulated lipid reserves is an important process in animals, plants and microorganisms, and yet little is known about the events that trigger lipid mobilization and its subsequent degradation.

Initiation of lipid utilization can be a rapid event, as shown when steady-state, lipid-rich yeasts are switched from a high-fat state in carbon-excess growth to a carbon-starvation environment, a change that can be achieved in less than five minutes. The enzymes and organelles (peroxisomes) that are associated with the degradation of stored lipid then rapidly make their appearance. Utilization of lipid can be recognized readily following the switch to carbon starvation, as shown in Fig. 2 (82,83). It also has been relatively easy to show that the carbon within the stored triacylglycerol does indeed become incorporated into other cell components during starvation, thus proving that the lipid genuinely is used to generate new cells.

To achieve similar control over lipid degradation in other, more complex systems would not be possible. Though, by examining the microorganism as a model system, it should be possible to know what events might be investigated. Therefore, microorganisms can tell us much very quickly, and it is often prudent to study the simpler systems before trying to unravel the more complex.

"A likely story indeed!" said the Pigeon. Alice's Adventures in Wonderland, Lewis Carroll.

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