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Microbial Conversions of Alkanes and Fatty Acids

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ABSTRACT

Alkanes are attacked readily by a wide variety of microorganisms. The most frequently encountered mode of oxidation is for one of the terminal methyl groups to be oxidized, through the alkanol, then the alkanal, to the corresponding fatty acid. Alkanes may be attacked subterminally also, and various ketones as well as the corresponding secondary alcohols can be produced. Subsequent degradation of these ketones occurs via introduction of oxygen into the chain to give a corresponding ester, which is then hydrolyzed to give a primary alkanol 2 carbon atoms shorter than the original alkane. The fatty acids arising by either route of oxidation, or by gratuitous introduction to the microbial system, may be oxidized by: (a) β-oxidation to give a number of acetyl-CoA units–intermediates of the process cannot be isolated from this pathway due to the tightly coupled nature of the substrates to the enzymes; (b) α-oxidation; or (c) oxidation at the other end of the molecule. In the latter case, ω- and $\omega-1$-hydroxyfatty acids can be produced. ω-Hydroxyfatty acids are subsequently oxidized to give dicarboxylic acids, which can be isolated, sometimes in high yield, by use of appropriate microbial mutants lacking in certain of the key metabolizing enzymes. With some yeasts, the fatty acids, including the ω-hydroxyfatty acids, can be esterified to various sugars to give a series of glycolipids. In some cases, wax esters are formed between fatty acid and alkanol; these wax esters can include diunsaturated molecules having a close chemical similarity to those of sperm whale and jojoba oils. Various recent innovations have occurred using isolated enzyme systems which can be used in transesterification reactions to convert cheap triacylglycerols into high value added commodities such as cocoa butter.

INTRODUCTION

Microorganisms, i.e., bacteria, yeasts and molds, can grow on a wide variety of hydrocarbons as sole sources of carbon and energy. They can partially oxidize an even greater range of such compounds. The list of compounds attacked is extensive and includes straight and branched chain alkanes, alkenes, alicyclic, heterocyclic and aromatic hydrocarbons. Indeed, there are probably few compounds that cannot be attacked, at least partially, by some microorganism; the most recalcitrant molecules are probably the macromolecular polymers such as polyethylene and polystyrene, where there are considerable difficulties for the microorganism to produce a solubilizing enzyme prior to oxidative degradation. Of course, there is no single organism which will utilize all hydrocarbons but, in general, each organism can utilize a range of hydrocarbons as sole source of carbon and energy.

The most readily assimilated hydrocarbons are the straight chain alkanes from C_{10} to C_{18}. Utilization of long-chain alkanes, e.g., plant paraffins of up to C_{35}, is less widespread, but some examples, particularly amongst the bacteria, have been reported (1). Isoalkanes with a single methyl side chain can be utilized for growth and, like the straight chain alkanes, be incorporated into cell components such as lipids. However, isoalkanes with branched chains at both ends of the molecule tend not to be utilized

as readily, and ones with multiple branching, such as pristane—2,6,10,14-tetramethylpentadecane, are even less readily utilized.

The problems which have to be overcome by an organism utilizing an alkane may be summarized as follows:

(a) *Uptake.* How is the insoluble hydrocarbon taken into the microbial cell?

(b) *Attack.* How is the initial oxidation of a hydrocarbon chain accomplished? Is more than one route of attack possible?

(c) *Degradation.* Can the compound produced by the initial oxidation step be degraded to provide metabolic intermediates to support subsequent cell growth or can the products be isolated without subsequent degradation?

(d) *Energy production.* The energy content of a hydrocarbon is considerable and is greater than the microorganism requires to convert the metabolites into cell materials. How then does the organism dispose of this energy which is surplus to its requirements?

(e) *Assimilation.* Can the alkane, or its oxidation products, be assimilated within the cell? In particular, can the cell take advantage of preformed long-chain acyl chains to produce various lipids which would be useful to it in the construction of its various membranous organelles?

With regard to this paper, we shall be particularly concerned with points (b) and (c), and to a lesser extent point (e), as it is from a discussion of these issues that we shall be able to highlight the possibilities of being able to produce a variety of chemically useful intermediates. For very much the same reasons, I have included a discussion of fatty acid oxidations. For details concerning points (a) and (d) the reader is referred to a recent review (2).

Fatty acids have the fundamental advantage over an alkane as far as microbial oxidation is concerned, since the carboxylic acid function confers a specific group on which subsequent degradation can be based. As all microorganisms of their own accord produce fatty acids, it follows that a greater number of microorganisms can utilize a fatty acid than can oxidize an alkane. The need for the specialized initial oxidative step has gone. All that is required is that the organism should possess a mechanism for taking the fatty acid into its cell. Fatty acids can be used by microorganisms to give selected products which have a higher added value than the starting material.

PRIMARY OXIDATION OF ALKANES

In the majority of organisms, initial oxidation of an alkane is at a methyl terminal. The reaction is catalyzed by a complex hydroxylase system which is notoriously very difficult to stabilize and isolate. As far as this author is aware, there have been no reports of its successful isolation and stabilization for a sufficient length of time to warrant consideration that it may be a commercially useful enzyme in the same way as many hydrolytic and oxidative enzymes are currently used in biotechnology.

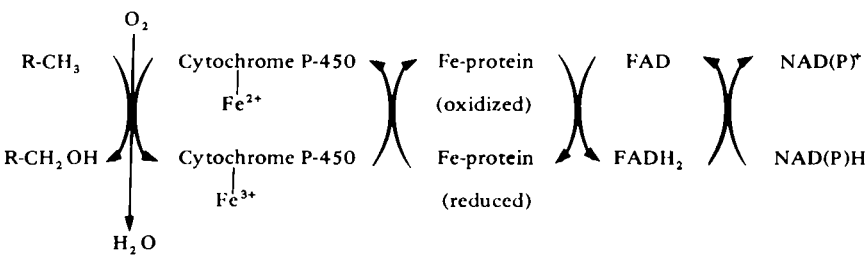
The alkane hydroxylase (Fig. 1) is linked to an electron carrier system, the components of which vary according to the microorganism used. In yeasts and most bacteria (as well as in man himself), cytochrome P-450 is used as the terminal oxidase and NAD(P)H-cytochrome P-450 reductase is the electron transfer component (Fig. 1). Recent work (3) has accomplished the purification of the first component from the yeast, *Lodderomyces elongisporus*, and has given a molecular weight of 79,000 for it.

In pseudomonad bacteria (4-6), the alkane hydroxylating system contains three protein components: a rubredoxin-like, nonhaem iron protein, an NADH-rubredoxin reductase and an ω-hydroxylase (Fig. 2). The enzyme system is coded for by DNA contained within a plasmid and thus opportunities exist for transferring the genetic information for hydrocarbon oxidation into organisms which do not possess this activity (7).

Also of importance for the primary oxidation of alkanes is the methane monooxygenase system. This is an enzyme system which can be purified and stabilized from bacteria capable of growing on methane, but these bacteria need not be grown on this substrate in order to achieve production of the enzyme. In other words, substrates which are easier to handle than methane can be employed, e.g., methanol. The enzyme system is capable of oxidizing a wide variety of substrate, from gaseous alkanes to aromatic and alicyclic compounds (8-11). It will also react with longer chain alkanes, and the oxidation of octane to octanol and octan-2-ol has recently been reported (12).

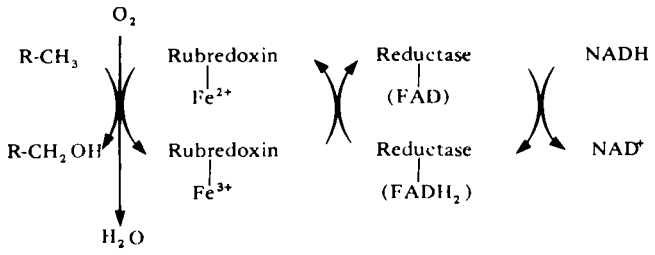
The enzyme is a multicomponent system involving NADH as an essential cofactor. Its mechanism of action is therefore similar to those depicted in Figures 1 and 2. The involvement of NADH precludes the ready large-scale application of the enzyme in an isolated form (and indeed the other alkane hydroxylases) as there is no easy way, as yet, to accomplish the reduction of NAD^+ back to NADH, which is needed to achieve recycling of this essential cofactor. The intact bacterial cells must therefore be used; their application can be very effective (11).

Although there have been various suggestions that an alk-1-ene may be an intermediate in the reactions of Figures 1 and 2, these claims have not been substantiated (13). However, Abbott and Casida (14) described isolation of a mixture of internal hexadecenes from hexadecane by *Nocardia salmonicolor* grown on glucose, indicating that under certain circumstances alkenes might be producible.



Net reaction: $R\text{-}CH_3 + NAD(P)H + O_2 \rightarrow R\text{-}CH_2OH + NADP^+ + H_2O$

FIG. 1. Alkane hydroxylase: the cytochrome P-450 monooxygenase system.



Net reaction: $R\text{-}CH_3 + NADH + O_2 \rightarrow R\text{-}CH_2OH + NAD^+ + H_2O$

FIG. 2. Alkane hydroxylase: the rubredoxin system.

SUBTERMINAL OXIDATION OF ALKANES

Subterminal oxidation of an alkane represents a radical departure from the usual mechanism of attack. Attack may be seemingly at any carbon atom of the alkane, and the property has been found in several bacteria and molds (13,15,16). In a detailed study of the oxidation of tridecane by *Pseudomonas* spp. (17-22), the major attack was at the 2-carbon atom (Fig. 3). The key reaction after the formation of the secondary alcohol is a Baeyer-Villiger type of rearrangement (17,18). The ensuing ester is then hydrolyzed by a specific esterase (19,20) to give the primary alcohol, undecanol, plus acetic acid.

There have been no detailed reports on the mechanism of the initial oxidation step of the alkane (23), although the next reaction has been shown to be catalyzed by a 2-tridecanone oxygenase which has been purified and characterized (17).

A variation to this scheme has been noted (24) in which a secondary alcohol was attacked by a *Pseudomonas* sp. to give a hydroxyketone (Fig. 4) that was further oxidized to a diketone, which was then hydrolyzed.

In a detailed examination of the voracious bacterium, *Acinetobacter* HO1-N, Finnerty has shown that subterminal oxidative attack occurs on the alkyl chain of various dialkyl ethers, $CH_3(CH_2)_nCH_2OCH_2(CH_2)_nCH_3$ where n = 5-8 (25). The resulting products are the alkoxy acetic acid, $CH_3(CH_2)_nCH_2OCH_2COOH$, plus the corresponding dicarboxylic acid $COOH(CH_2)_{n-2}COOH$. The scheme proposed is given in Figure 5. It is the dicarboxylic acid which is then subsequently degraded to allow the organism to grow. The alkoxy acetic acid accumulates during growth and is apparently not degraded further.

The same bacterium (26) may also attack alkanes subterminally, although this is probably a minor route. In this way, an alkane such as hexadecane is converted to decanedioic acid plus hexane. As other alkanes are also oxidized to the same dicarboxylic acid, the attack is presumed to be always at the C_{10}-C_{11} bond.

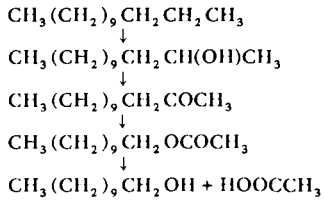


FIG. 3. Subterminal oxidation of tridecane by *Pseudomonas aeruginosa* and *Ps. multivorans* (21,22).

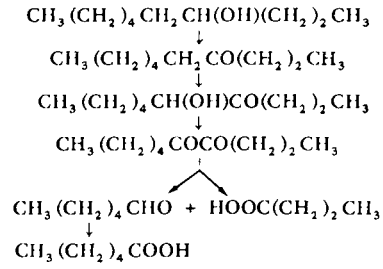


FIG. 4. Dissimilation of 4-decanol by *Pseudomonas* sp. (24).

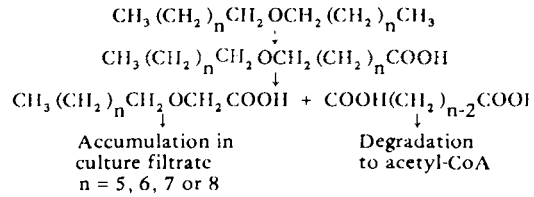


FIG. 5. Degradation of dialkyl ethers by *Acinetobacter* HO1-N (25,26).

OXIDATION OF ALKENES

Alkenes, themselves, can be attacked either at their double bond, wherever it might be in the molecule, or at one of the terminal carbon atoms. Consequently, a variety of products may form (Fig. 6). The majority of work, understandably, has been with alk-1-enes, as other alkenes are not readily available. The hydroxylating system responsible for attacks at either end of the molecule is usually catalyzed by the same enzyme responsible for the initial oxidation of alkanes, i.e., the alkane hydroxylase (see Figs. 1 and 2). Epoxide formation has been reported in several instances and may, if the right conditions are attained, accumulate in some quantity from the oxidation of the alkene (11,27-29). The epoxide is then oxidized to the corresponding diol which then forms the α-hydroxycarboxylic acid (15). This is then decarboxylated to the next lower carboxylic acid which then undergoes β-oxidation (see below).

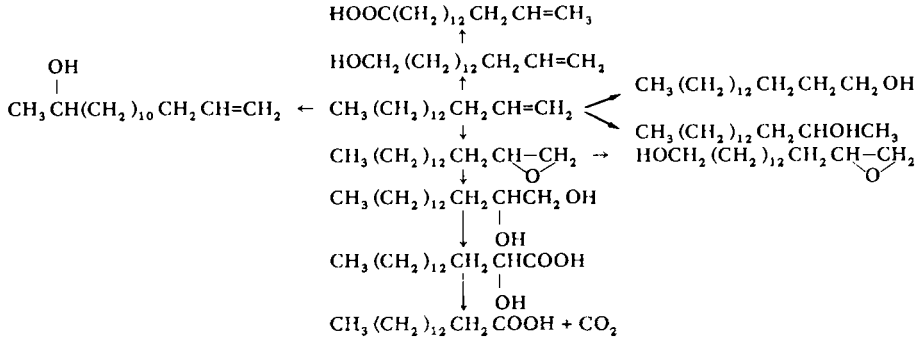


FIG. 6. Pathways of alkene dissimilation (adapted from ref. 15).

OXIDATION OF PRIMARY ALCOHOLS (FROM *n*-ALKANES)

Once the initial oxidation of the alkane has been accomplished, the functional group then becomes the focal point for subsequent oxidation. The alcohol is thus converted to the corresponding aldehyde and then to the fatty acid. The reactions are catalyzed by long-chain alcohol dehydrogenase and aldehyde dehydrogenase (Fig. 7).

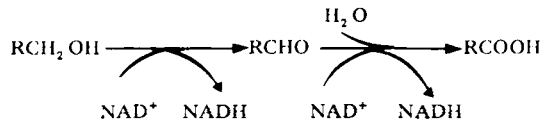


FIG. 7. Fatty alcohol and aldehyde dehydrogenases.

The specificity of these enzymes is broad in that substrates from C_{10} to C_{18} can be readily oxidized. There have been suggestions that these two enzymes, plus the alkane hydroxylase, may form into a multienzyme complex (30) and in certain bacteria all three activities are coded for by the DNA of transmissible plasmids (7).

OXIDATION OF SECONDARY ALCOHOLS

In two recent patents (11), it has been claimed that various methane-utilizing bacteria and methanol-utilizing yeasts each have a secondary alcohol-specific dehydrogenase (linked to NAD^+) which is capable of converting such secondary alcohols as propan-2-ol, butan-2-ol, pentan-2-ol and hexan-2-ol to the corresponding 2-ketone. The range of compounds which these organisms attack tend to be of the shorter chain lengths, but a wide range of substrate other than alcohols can be oxidized by these organisms. The secondary alcohols used in these reactions could be produced by the oxidation of the corresponding alkane, which is a reaction the methane bacteria can readily accomplish.

FATTY ACID DEGRADATION

Fatty acids may form by oxidation of alkanes or alkenes. Fatty acids, however, may be presented directly to the cells, either in their own right or in the form of triacylglycerols. A wide variety of microorganisms are capable of hydrolyzing oils and fats, enabling them to utilize and thus grow on the glycerol and fatty acids which would be released. Degradation of fatty acids, in the form of detergents or soaps, has ecological importance. Just as microorganisms can utilize a range of alkanes, so can they utilize a range of fatty acids, usually from C_{10} to C_{18}, although there are reports of fatty acids of both longer and shorter chain length being used.

The first reaction for a fatty acid to undergo is the formation of a thiol ester, that with coenzyme A (Fig. 8). This reaction proceeds rapidly and serves to decrease the toxicity of free fatty acids within the cell. The reaction is catalyzed by a long-chain fatty acyl CoA synthetase. Such enzymes have been studied in detail in the yeast *Yarrowia* (= *Saccharomycopsis* = *Candida*) *lipolytica* (31).

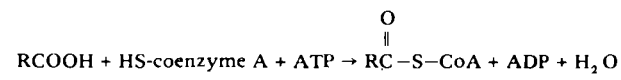


FIG. 8. Formation of fatty acyl-CoA from a free fatty acid and coenzyme A.

In this yeast, and possibly in others too, there are two distinct fatty acyl-CoA synthetases which have different roles according to whether the yeast is growing on a carbohydrate or an alkane. One enzyme (synthetase I) is involved in the direct transfer of fatty acyl-CoA esters into lipids; the other enzymes links the fatty acid arising from alkane oxidation to the β-oxidation used for subsequent degradation.

β-Oxidation

The β-oxidation system of fatty acid degradation follows the "classical" textbook pathway elucidated with animals and plant cells (see Fig. 9). There are four enzymes required to decrease the chain length of the fatty acyl CoA ester by two carbon atoms: (a) a dehydrogenase, (b) an enoyl-CoA hydrase, (c) a β-hydroxyacyl-CoA dehydrogenase, and (d) a thiolase. In yeasts, the process occurs in the peroxisome organelle and is not linked to the production of metabolic energy (2,32). No intermediates of this degradative sequence are ever released as all are tightly bound to the enzyme complex.

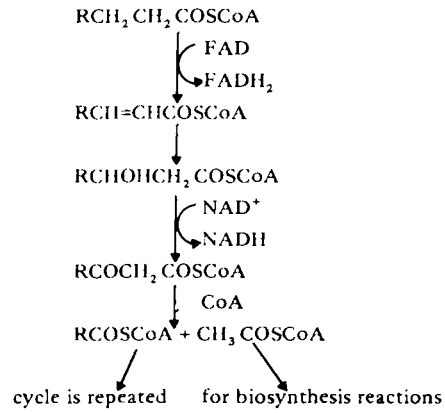


FIG. 9. β-Oxidation cycle for the oxidation of fatty acyl-CoA esters.

α-Oxidation

Evidence for the α-oxidation of fatty acids is very fragmentary, although the system does occur in plants. In this scheme, a fatty acyl-CoA ester is converted to the next lower homologue with the loss of CO_2. Reports of the occurrence of this system in the bacteria *Arthrobacter simplex* and in a yeast, *Candida utilis*, have been given (see ref. 2). This system of oxidation is usually invoked to explain the occurrence of fatty acyl groups (in the cell lipids) with an even number of carbon atoms arising from alkanes with an odd number of carbons. There are, however, alternative explanations for these occurrences (2). It is possible that intermediates might be isolated from this process as α-hydroxypalmitic acid was identified as a product of palmitate degradation by the above bacterium. The pathway, however, has not been extensively studied.

ω-Oxidation (Diterminal Oxidation)

In this mode of oxidation, end-products can be and have been isolated in some abundance. The oxidation, the scheme for which is shown in Fig. 10, occurs in both bacteria and yeasts. Both the α,ω-dioic acids and the ω-hydroxyfatty acids have been isolated from culture filtrates of various organisms; many patents covering various aspects of this process have appeared (2) and the subject has been extensively covered in two recent reviews (33,34). Using mutants of *Candida cloacae*, conversions of alkane to dioic

acid have been as high as 70% with the process being carried out on a 300-L scale.

The ω-hydroxylation of fatty acids (see Fig. 10) is done by the same alkane hydroxylase enzyme system used in the initial attack of an alkane (see Fig. 1). Alkenes may also be attacked by the same enzyme (see Fig. 6). However a fatty acid hydroxylase has been isolated from *Bacillus megaterium*, which is an organism unable to grow on alkanes (35-37). This hydroxylase does not oxidize alkanes but produces 13-, 14-, and 15-hydroxypalmitic acids when presented with palmitic acid (35), and 15-, 16- and 17-hydroxystearic acids from stearic acid (36) but produces epoxy acids from unsaturated acids (37).

Degradation of α,ω-dioic acids is probably by β-oxidation (see Fig. 9) beginning at one of the termini (2). Shorter chain dicarboxylic acids, C_7 to C_9, have been recovered in certain instances, although in most cases degradation would appear to continue until succinic acid is reached, which can then be oxidized via the tricarboxylic acid cycle.

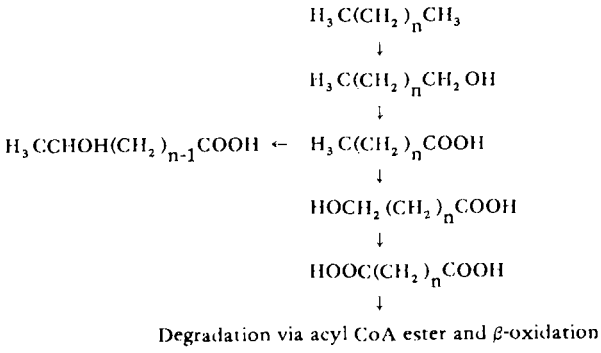


FIG. 10. Diterminal oxidation of alkanes.

FORMATION OF ESTERS AND WAXES

ω-Hydroxy and ω − 1-hydroxyfatty acids have been recovered as the fatty acyl components of a family of glycolipids produced extracellularly by *Torulopsis bombicola* and *Candida bogoriensis* in some yield (38). The glycolipid, a diacyl ester of the disaccharide sophorose (see Fig. 11), is a strong surfactant and could be considered to be a biological Tween. Applications for these sophorosides have yet to be identified, although conversions of hydrocarbon or fatty acid into the acyl moieties may be up to 80% with up to 40 g of glycolipid/L being attained.

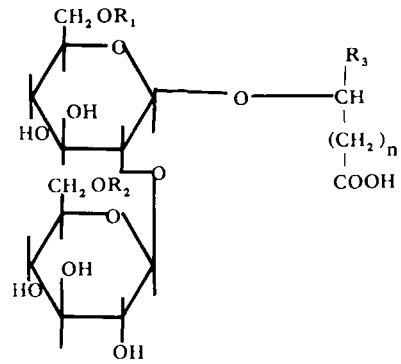


FIG. 11. Structure of sophorose glycolipid produced by *Torulopsis bombicola*. R_1, R_2 = H or acetyl; R_3 = H or CH_3; n = 14-16. (The terminal carboxylic acid may lactonize to the 4-O of the other glucose moiety of the sophorose).

Wax esters have long been known as products following the growth of *Acinetobacter* HO1-N (formerly known as *Micrococcus cerificans*) on long-chain alkanes (2,15,26). The main product following growth on hexadecane was cetyl palmitate (C_{16}-C_{16}) and 90% of this occurred extracellularly. However, when hexadecanol was used, the amount of wax ester doubled (to ca. 300 mg/g cell dry wt) with the wax being divided almost equally between the cell itself and its culture filtrate. Initial examination of the waxes produced by this bacterium indicates that they were being formed by an "ester synthase"—possibly with the fatty acyl-CoA ester and the alcohol. The presence of minor components indicated that the fatty acid may be shortened by 2 carbon atoms before esterification (39).

Recently more detailed examination of these waxes has been made by capillary gas chromatography, and a range of esters has been detected (40). Chain lengths of the wax esters were a function of the alkane used as substrate:

$$C_n \text{ alkane} \rightarrow C_{2n},\ C_{2n-2} \text{ and } C_{2n-4} \text{ wax esters}$$

More importantly, however, was the detection of unsaturated (monoene and diene) components whose ratio to the saturated components increased with chain length of the alkane substrate (C_{16}-C_{20} were used). With the diene components, further analysis showed that one double bond was in the acyl segment and the other in the alkoxy part. Table 1 summarizes results which were obtained and compares bacterial wax esters with sperm whale oil and jojoba oil.

TABLE I

Comparison of the Wax Esters Produced by *Acinetobacter* sp. HO1-N and Those Found in Sperm Whale and Jojoba Oils (40,41)

	Sperm whale oil	Microbial-produced wax esters	Jojoba oil
Carbon number of intact wax esters	28-40	32-40[a]	36-44
Acyl segments			
Carbon number	14-22	16-20[a]	16-24
Number of unsaturations	0,1	0,1	1
Alkoxy segments			
Carbon number	16-20	16-20[a]	18-24
Number of unsaturations	0,1	0,1	1
Predominant sites of unsaturation	ω7, ω9 and ω11	ω7 and ω9	ω9

[a]Carbon numbers dependent on *n*-alkane used as substrate.

The effect of different growth temperatures has been examined for the formation of wax esters (41). At 17 C there is more diunsaturated material produced than at 24 C or 30 C with both C_{16} and C_{20} alkanes. Optimum production would appear to be for *Acinetobacter* to be grown for 48 hr on eicosane at 17 C when 12 mg of wax ester is produced per liter, with 71% of the esters being of the diene type. As exciting as these results appear, there is still considerable progress to be made before these waxes could be considered as viable commercial alternatives to sperm whale oil and jojoba oil.

TRANSFORMATIONS AND TRANSESTERIFICATIONS

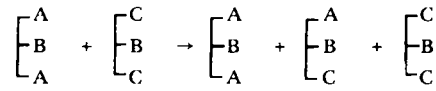
Transformation reactions of hydrocarbons, and various derivatives thereof, may be made using nongrowing cells, either in the form of a suspension or attached to, or entrapped within, a matrix. In the latter cases, such preparations are referred to as immobilized cells, and this allows ready separation of the product from the cells and then reuse of cells until they become completely degenerated. Various examples, based on the types of reaction detailed in the preceding sections, have been extensively reviewed and need not be repeated here (42,43). Many compounds, which may not in themselves support good growth of a microorganism, may nevertheless be attached and partially oxidized by a microorganism. Such a process is termed cooxidation (43) and can thus be used to effect the introduction of, say, a hydroxyl group into a molecule or to convert an alcohol to an aldehyde or carboxylic acid function. This type of approach has, of course, been extensively examined in the field of sterol transformation reactions (44) and finds ready application for some hydrocarbon oxidations (11).

Isolated enzymes, usually in an immobilized form, can be used in some cases in this field, but, of course, the prerequisite is one of stability. Immobilization of an enzyme will most often decrease the reactivity of the enzyme but will, though, confer on it long-term stability—stability which is then often measured in months rather than hours. However, such is the instability of many of the alkane hydroxylating enzyme systems that applications of isolated enzymes are extremely limited in this area of microbial biochemistry. The other limiting factor for the application of immobilized enzymes is that many of the reactions are dependent on the supply of a cofactor, such as NAD^+, which is not only very expensive but once reduced to NADH will not be reoxidized back to NAD^+. At best one must therefore resort to use of the whole microbial cell which can, though, be "permeabilized" (by treatment with a membrane-damaging solvent such as toluene) to allow better entry and exit of products and reactants into and out of the cell. Such cells, which may still be in an immobilized form, may be able to accomplish reoxidation of reduced cofactors by virtue of the other enzymes still within the cell. Applications of such approaches in this area of biotechnology are just beginning to be developed (see, e.g., ref. 11).

Where isolated enzymes have been successfully and effectively applied in this area has been with triacylglycerol transesterifications. Microorganisms have been known for a long time to be able to grow on oils and fats. The initial reaction is the hydrolysis of the triacylglycerol and this is accomplished by an extracellular lipase. These lipases can be readily isolated, are stable and will also carry out the interesterification of oils and fats (Fig. 12). Unlike other reactions mentioned above, there is no requirement for any cofactor and no net input of energy is required, thus obviating the need to supply any expensive cosubstrate. By exploiting the specificity of lipases from different microorganisms, it is possible to produce useful glyceride mixtures which cannot be obtained by conventional (chemical) interesterification methods (45,46).

Interesterification may be performed not only between different triacylglycerols (Fig. 12a) but also between a triacylglycerol and a fatty acid (Fig. 12b). It is thus possible to produce such triacylglycerols as *sn*-1-palmitoyl, 2-oleoyl, 3-stearoyl glycerol which, of course, is the major component of the commercially desirable cocoa butter. There are several patents for this process (47,48).

A. Triacylglycerol mixture interesterification



B. Triacylglycerol plus free fatty acid interesterification

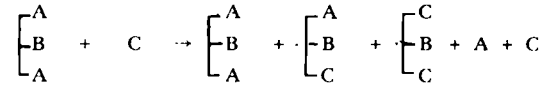


FIG. 12. Products formed by interesterification using 1,3-positionally specific lipases (45).

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