Essays in Biochemistry

Volume 15 1979

Edited by

P. N. Campbell

and

R. D. Marshall

Essays in Biochemistry

Edited for The Biochemical Society by

P. N. Campbell

Courtauld Institute of Biochemistry
The Middlesex Hospital
Medical School
London W1P 7PN
England

R. D. Marshall

University of Strathclyde
The Todd Centre
31 Taylor Street
Glasgow G4 ONR
Scotland

Volume 15

1979

Published for The Biochemical Society by Academic Press
London, New York and San Francisco

ACADEMIC PRESS INC. (LONDON) LTD
24/28 Oval Road
London NW1

U.S. Edition published by
ACADEMIC PRESS INC.
111 Fifth Avenue
New York
New York 10003

British Library Cataloguing in Publication Data

Essays in biochemistry.
Vol. 15
1. Biological chemistry
I. Campbell, Peter Nelson
II. Marshall, R D
III. Biochemical Society
574.1'92 QH345
ISBN 0-12-158115-2 LCCCN 65-15522

Printed in Great Britain by
Spottiswoode Ballantyne Ltd.
Colchester and London

Biography

Roger Jeffcoat graduated in Biochemistry at the University of Manchester, Institute of Science and Technology and received his doctorate from the University of Leeds for his work on bacterial carbohydrate metabolism. He then spent two years as a Research Specialist at the University of Minnesota and two years as an I.C.I. Research Fellow at the University of Oxford studying the mechanism of enzyme–substrate interactions. Finally in 1972 he joined the Unilever Research Laboratories at Colworth House to work on the dietary control of mammalian lipogenesis.

Richard Denton graduated from the University of Cambridge and was then introduced to the problem of the hormonal control of metabolism in adipose tissue by Professor Philip Randle. After obtaining his Ph.D in 1967, his major research interest has remained in this area ever since with emphasis in recent years on the mechanism of action of insulin. His present position is Reader in Biochemistry at the University of Bristol.

Andrew Halestrap studied biochemistry at Cambridge University before going to Bristol in 1970 to work on the control of lipogenesis by insulin for his Ph.D. under the supervision of Richard Denton. Since that time he has been working on pyruvate transport across mitochondrial and plasma membranes and the control of hepatic gluconeogenesis. Formerly a Beit Memorial Research Fellow, he is now a lecturer in the Biochemistry Department at Bristol University.

Melvyn Greaves graduated in Zoology at University College London and received his Ph.D. training under Professor I. M. Roitt at the Middlesex Hospital Medical School. After spending a year at the Karolinska Institute in Stockholm he joined Professor Avion Mitchison's Experimental Biology Group at the National Institute for Medical Research, Mill Hill and subsequently moved with Mitchison to the new I.C.R.F. Tumour Immunology Unit at University College. In 1977 he transferred to the main laboratories of the I.C.R.F. to head his own department of Membrane Immunology. His main research interests centre around the immunology of cell surfaces and in particular the roles receptor molecules play in cell regulation, cellular interactions, differentiation and malignancy.

Conventions

The abbreviations, conventions and symbols used in these Essays are those specified by the Editorial Board of *The Biochemical Journal* in *Policy of the Journal and Instructions to Authors* (revised 1976 *Biochem J.* **153**, 1–21 and amended 1978 *Biochem J.* **169**, 1–27). The following abbreviations of compounds, etc., are allowed without definition in the text.

ADP, CDP, GDP, IDP, UDP, XDP, dTDP: 5′-pyrophosphates of adenosine, cytidine, guanosine, inosine, uridine, xanthosine and thymidine
AMP, etc.: adenosine 5′-phosphate, etc.
ATP, etc.: adenosine 5′-triphosphate, etc.
CM-cellulose: carboxymethylcellulose
CoA and acyl-CoA: coenzyme A and its acyl derivatives
Cyclic AMP etc.: adenosine 3′ : 5′-cyclic phosphate etc.
DEAE-cellulose: diethylaminoethylcellulose
DNA: deoxyribonucleic acid
Dnp-: 2,4-dinitrophenyl-
Dns-: 5-dimethylaminonaphthalene-1-sulphonyl-
EDTA: ethylenediaminetetra-acetate
FAD: flavin-adenine dinucleotide
FMN: flavin mononucleotide
GSH, GSSG: glutathione, reduced and oxidized
NAD: nicotinamide-adenine dinucleotide
NADP: nicotinamide-adenine dinucleotide phosphate
NMN: nicotinamide mononucleotide
P_i, PP_i: orthophosphate, pyrophosphate
RNA: ribonucleic acid (see overleaf)
TEAE-cellulose: triethylaminoethylcellulose
tris: 2-amino-2-hydroxymethylpropane-1,3-diol

The combination NAD^+, NADH is preferred.

The following abbreviations for amino acids and sugars, for use only in presenting sequences and in Tables and Figures, are also allowed without definition.

Amino acids

Ala: alanine	Asx: aspartic acid or asparagine (undefined)	Cys or Cys: Cystine (half)
Arg: arginine		
Asn*: asparagine	Cys: Cysteine	Gln†: glutamine
Asp: aspartic acid		Glu: glutamic acid

* Alternative, Asp(NH_2) † Alternative, Glu(NH_2)

Glx: glutamic acid or glutamine (undefined)
Gly: glycine
His: histidine
Hyl: hydroxylysine
Hyp: hydroxyproline
Ile: isoleucine
Leu: leucine
Lys: lysine
Met: methionine
Orn: ornithine
Phe: phenylalanine
Pro: proline
Ser: serine
Thr: threonine
Trp: tryptophan
Tyr: tyrosine
Val: valine

Sugars

Ara: arabinose
dRib: 2-deoxyribose
Fru: fructose
Fuc: fucose
Gal: galactose
Glc*: glucose
Man: mannose
Rib: ribose
Xyl: xylose

* Where unambiguous, G may be used.

Abbreviations for nucleic acid used in these essays are:

mRNA: messenger RNA
nRNA: nuclear RNA
rRNA: ribosomal RNA
tRNA: transfer RNA

Other abbreviations are given on the first page of the text.

References are given in the form used in *The Biochemical Journal*, the last as well as the first page of each article being cited and, in addition, the title. Titles of journals are abbreviated in accordance with the system employed in the *Chemical Abstracts Service Source Index* (1969) and its Quarterly Supplement (American Chemical Society).

Enzyme Nomenclature

At the first mention of each enzyme in each Essay there is given, whenever possible, the number assigned to it in *Enzyme Nomenclature: Recommendations (1972) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes, together with their Units and the Symbols of Enzyme Kinetics*, Elsevier Publishing Co., Amsterdam, London and New York, 1973: this document also appeared earlier as Vol. 13 (2nd edn, 1965) of *Comprehensive Biochemistry*, (Florkin, M. & Stotz, E. H., eds), Elsevier Publishing Co., Amsterdam, London and New York. Enzyme numbers are given in the form EC 1.2.3.4. The names used by authors of the Essays are not necessarily those recommended by the International Union of Biochemistry.

Contents

The Biosynthesis of Unsaturated Fatty Acids and its Control in Mammalian Liver

R. JEFFCOAT

Unilever Research, Colworth Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ, England

I. Introduction

For many years it has been known that fatty acids are important constituents of cellular membranes which serve not only as boundaries between individual cells but also to compartmentalize several major biochemical

processes within the cell. Furthermore although it is well established that membranes are not simply inert structures which contain the cellular components, it is not yet clear what are the important features of the fatty acids which contribute to the physical and biochemical properties of the membranes. Clearly the cell cannot tolerate an infinitely large spectrum of fatty acids within its membrane lipids and must therefore regulate the type, e.g. saturated and polyunsaturated, and proportion of fatty acids which are incorporated. Most saturated and monounsaturated fatty acids are derived either from the diet or from *de novo* synthesis by the condensation of acetate units followed by the direct oxidative desaturation of the long chain fatty acids. In contrast, polyunsaturated fatty acids can be derived only from dietary lipids since mammalian tissues lack the enzymes capable of synthesizing linoleic acid α-linolenic acids which are the precursors of all the polyunsaturated fatty acids found in animal cells. The initial observations of Burr & Burr[1], which led to the identity of these fatty acids, came from studies with animals which, when deprived of these fatty acids, developed what are now known as essential fatty acid deficiency symptoms, i.e. decreased growth rate, development of scaly paws, skin and tail, and fatty livers.

Apart from the well established role of fatty acids in membrane structure,[2] there is now increasing support for a different role of polyunsaturated fatty acids involving the control of cellular activity and metabolism. The review by Volpe & Vagelos[3] about the control of fatty acid synthetase and acetyl-CoA carboxylase underlines the complexity of this control from its simplest at the level of product inhibition to its most complex and as yet poorly understood control of gene expression by dietary polyunsaturated fatty acids. As well as other lipogenic enzymes such as glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and malic enzyme (EC 1.1.1.40), polyunsaturated fatty acids also control the activity of stearoyl-CoA desaturase which is responsible for the biosynthesis of monounsaturated fatty acids from saturated fatty acid precursors.

The central issue is therefore to understand at the biochemical level the mechanism of the control exerted by polyunsaturated fatty acids. By reviewing the literature on the control of the biosynthesis and metabolism of unsaturated fatty acids, it is anticipated that a judgement can be made to decide whether or not their effect on enzyme synthesis, prostaglandin synthesis, hormone binding sites and cell adhesion are all part of the same common phenomenon.

II. General Remarks

A. BIOSYNTHESIS OF SATURATED FATTY ACIDS

Saturated fatty acids are synthesized via a high molecular weight complex composed of two multi-active site polypeptide chains known collectively as the

fatty acid synthetase complex.[4] In mammalian systems the product is predominantly palmitic acid which serves as a substrate for the microsomal malonyl-CoA dependent elongase. Whether acceptor/carrier molecules of the type found in bacterial systems[5] are also present in mammalian cells, has not yet been established. However, palmitic acid can exert control over endogenous fatty acid synthesis, either by inhibiting the activity of the fatty acid synthetase complex or by reducing the availability of malonyl-CoA. This it does by binding to the acetyl-CoA carboxylase (EC 6.4.1.2) thus favouring the disaggregation of the active form into the inactive monomeric form. Citrate[4] may compete with palmitate for those binding sites and thus favour the reverse process, but at the present time there is some controversy as to whether the intracellular concentration of citrate is high enough to activate acetyl-CoA carboxylase as has been demonstrated *in vitro*. Perhaps of more importance is the phosphorylation and dephosphorylation of acetyl-CoA carboxylase which has been reported recently.[4]

B. BIOSYNTHESIS OF UNSATURATED FATTY ACIDS

In mammalian systems unsaturated fatty acids are synthesized by the direct oxidative desaturation of preformed long-chain saturated fatty acids. The substrates for these enzymes are therefore derived from dietary fat or the products of the cytoplasmic fatty acid synthetase and microsomal malonyl-CoA dependent elongase systems. Unlike saturated fatty acids, monounsaturated fatty acids are synthesized by a microsomal bound desaturase complex consisting of NADH-cytochrome b_5 reductase (EC 1.6.2.2.) cytochrome b_5 and the terminal desaturase enzyme, often referred to as the cyanide sensitive factor. The actual number of these terminal oxidases is not absolutely clear, but a recent review by James[6] provides a very strong case for the need and existence of only three or four enzymes with a broad chain length specificity. These enzymes are thus capable of synthesizing all known naturally occurring polyunsaturated fatty acids. The four desaturases, designated Δ^9-, Δ^6-, Δ^5- and Δ^4-fatty acyl-CoA desaturases although acting upon different substrates have many properties in common. They all catalyse the direct desaturation of fatty acids by the general mechanism shown below:

$$CH_3(CH_2)_xCH_2CH_2(CH_2)_yCO \sim SCoA + NADH_2 + O_2 \longrightarrow$$

$$CH_3(CH_2)_xCH{=}CH(CH_2)_yCO \sim SCoA + 2H_2O + NAD^+.$$

$(CH_2)_x$ can be a saturated methylene chain or can contain two or more methylene-interrupted double bonds

The enzymes all require molecular oxygen, a reduced pyridine nucleotide, are sensitive to cyanide but not carbon monoxide and act on a substrate usually in the form of a fatty acyl-coenzyme A. The one exception to this is the Δ^5-de-

saturase which exists in two forms in mammalian liver; one acting on the coenzyme A ester of eicosatrienoic acid (8, 11, 14, 20:3) and a second on a phospholipid containing this polyunsaturated fatty acid which gives rise directly to arachidonyl-phosphatidylcholine.[7]

C. RULES FOR DESATURATION

Although a vast spectrum of unsaturated fatty acids has been identified as naturally occurring in animal tissues, substrate specificity studies have enabled general guidelines to be formulated which govern their biosynthesis.[8] Most of these studies have been carried out with the Δ^9-desaturase which is responsible for the biosynthesis of monounsaturated fatty acids from their saturated fatty acid precursors. However, what has been found to be true for the Δ^9-desaturase is in general applicable to the other fatty acyl-CoA desaturases. Apart from the cofactor requirements and form of the substrate, it has further been established that the position of the double bond to be inserted is governed by (a) its position relative to existing double bonds and (b) its position relative to the carboxyl group; i.e. new double bonds are always inserted between existing double bonds and the carboxyl group, but never more than the C-9–C-10 carbons from the carboxyl (C-1). Thus the preferred product for the desaturation of stearic acid is 9c.18:1†, oleic acid and not 6c.18:1 although the latter has been demonstrated in low concentrations in preparations of young rat brain.[9] Similarly, the major desaturation product of 9c.12c.18:2, linoleic acid is 6c.9c.12c.18:3, γ-linolenic acid. For a review of the literature on substrate specificity and determination of double bond position, see James.[6] Under normal conditions where an animal is receiving an adequate diet with the full essential fatty acid requirement, the preferred substrate for the Δ^6-fatty acyl-CoA desaturase would be linoleic acid. In cases of essential fatty acid deficiency, the availability of oleic acid relative to linoleic acid is increased. This is because (a) there is a lower dietary intake of linoleic acid and hence less available to act as a substrate for the Δ^6-fatty acyl-CoA desaturase and (b) a reduction in dietary linoleic acid enables full expression of the fatty acid synthetase and stearoyl-CoA desaturase activities as will be discussed later. The net result is an enhanced desaturation of oleic acid as shown below:

$$9c.18:1 \xrightarrow{\Delta^6} 6c.9c.18:2 \xrightarrow{E} 8c.11c.20:2 \xrightarrow{\Delta^5} 5c.8c.11c.20:3.$$

Since the Δ^6-fatty acyl-CoA desaturase is the rate-limiting step in polyunsaturated fatty acid biosynthesis, there is an increase in tissue 20:3 which can be used as a biochemical marker for essential fatty acid deficiency long before the gross symptoms become apparent.

† The notation used above for characterizing fatty acids is as follows. The number before the colon denotes the number of carbon atoms and the second number refers to the number of double bonds.

The rules for mammalian desaturation may, therefore, be summarized as follows.

(1) All desaturases require molecular oxygen, a reduced pyridine nucleotide and catalyse the direct oxidative desaturation of preformed fatty acids usually in the form of coenzyme A esters.

(2) Double bonds are always introduced into the methylene chain at a fixed position from the carboxyl group.

(3) When the substrate is a saturated fatty acid, the first double bond is inserted between carbon atoms 9 and 10. Unlike plants, animals cannot introduce double bonds farther away than nine carbons from the carboxyl group.

(4) When the substrate is already unsaturated, subsequent double bonds are inserted between the double bond nearest the carboxyl group and the carboxyl group itself, in such a way as to usually maintain the methylene-interrupted distribution of double bonds.

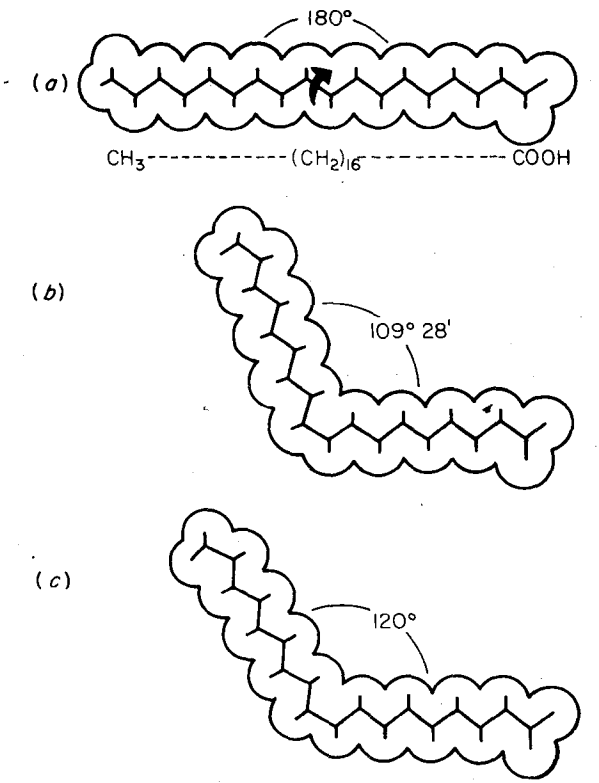


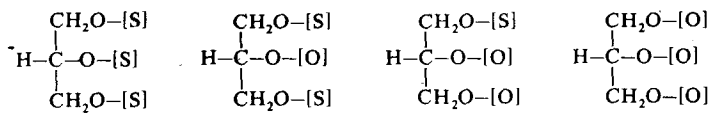
Fig. 1. Isometric projections of substrate and product of Δ^9-desaturase. (*a*) Extended chain of stearic acid, (*b*) Extended chain of stearic acid after rotation of the 9–10 C–C bond through 180° to form the eclipsed or gauche conformation and (*c*) oleic acid. (Diagrams modified from those published by Vandenheuvel.[112])

(5) In a metabolic pathway leading to the formation of polyunsaturated fatty acids, desaturation usually alternates with elongation.
(6) Although well established evidence exists for Δ^9-, Δ^6- and Δ^5-fatty acyl-CoA desaturases, the evidence for a Δ^4-fatty acid acyl-CoA desaturase is less convincing.[8]
(7) Unsaturated fatty acids with *trans* rather than *cis* double bonds are in general treated by the enzymes as saturated fatty acids.[8]

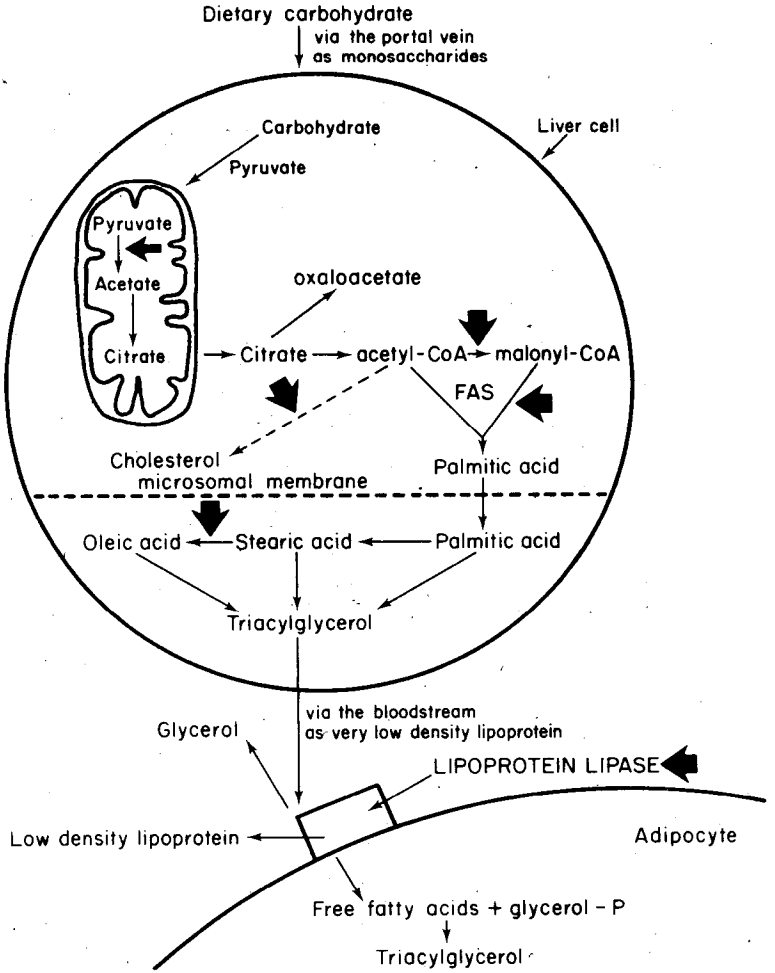
The enzymes which catalyse these desaturations would appear to have a co-enzyme A binding site which recognizes the fatty acyl-CoA substrate. From the results of Brett *et al.*[10] working with Δ^9-substrates and Do & Sprecher[11] working with Δ^5-substrates, it seems likely that the methylene chain of the fatty acid fits into a restrictive cleft of finite length. These conclusions have been reached from experiments using fatty acid substrates which have been methylated at several individual sites along the methylene backbone with the resultant loss of substrate potential. This close interaction of enzyme and substrate enables the latter to be "twisted" about the $-CH_2-CH_2-$ group to be desaturated and in so doing takes on an eclipsed or gauche conformation[10] (Fig. 1) which enables the two hydrogens to be removed in a concerted reaction.[12]

D. FUNCTION OF DESATURASES

Lipids have two major roles in the living system; (i) to form a major component of cell membranes and (ii) to act as a reserve of both carbon and energy. These requirements are met by a balance between endogenous synthesis and dietary intake. When the latter is insufficient to meet these needs, fatty acids are synthesized from dietary carbohydrate as outlined in Scheme 1. Under these circumstances saturated fatty acids would accumulate in lipids as tripalmitin, tristearin or mixed triacylglycerols containing both stearic and palmitic acids. The melting point of such lipids is approximately 65°C and they would, therefore, crystallize at physiological temperatures. The introduction of monoenoic acids, such as palmitoleic and oleic acids formed by the desaturation of palmitic and stearic acid respectively, causes a decrease in the melting point and would thus facilitate their biochemical processing, i.e. their incorporation into very low density lipoprotein and transport via the blood stream to the adipose tissue.



S—Stearic acid; O—Oleic acid



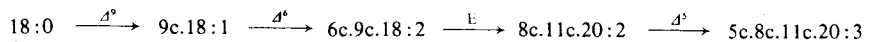
Scheme 1. The conversion of carbohydrate into triacylglycerols. The arrows indicate control points in the conversion of acetate into storage lipid.

Thus it would appear that one of the functions of the Δ^9-fatty acyl-CoA desaturase, in conjunction with the intracellular fatty acid binding proteins is to alter the physical state of fatty acids so that they can be processed by the cell.

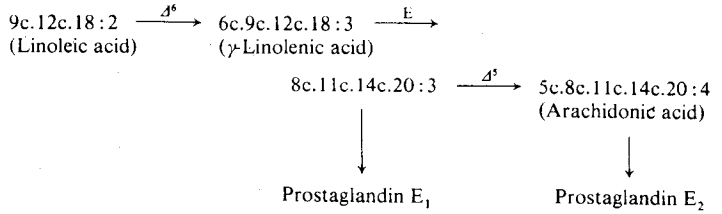
In contrast to the Δ^9-fatty acyl-CoA desaturase which synthesizes monounsaturated fatty acids, the Δ^6- and Δ^5-fatty acyl-CoA desaturases have a very different role as was suggested earlier.[13] Their primary function is to synthesize polyunsaturated fatty acids which are incorporated into membrane phospholipids. It is perhaps significant that the highest levels of Δ^6-desaturase activity

Palmitoleic acid series

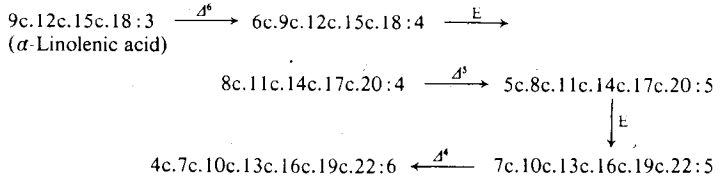
Oleic acid series



Linoleic acid series



α-Linolenic acid series



E—elongase

Δ^9, Δ^6, Δ^5, Δ^4 } fatty acyl-CoA desaturases

Scheme 2. Biosynthesis of unsaturated fatty acids.

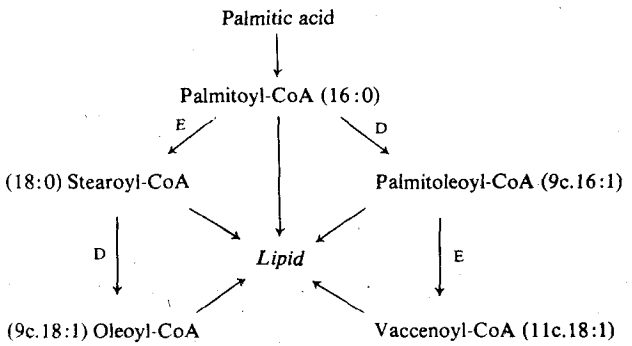
are in the unborn animal when there would be a high demand for phospholipids containing polyunsaturated fatty acids.[14] In particular, they are responsible for maintaining the tissue levels of arachidonic acid which in conjunction with di-homo-γ-linolenic acid, 8, 11, 14, 20:3 (Scheme 2) act as precursors of prostaglandin E_2 and E_1 respectively.

III. The Enzymology of Desaturation

A. INTRACELLULAR ORGANIZATION AND LOCATION

The detailed understanding of the biochemistry of unsaturated fatty acid biosynthesis eluded for many years even the most conscientious research worker. It was not until the early 1960s that real progress was made. Even then the problems of handling membrane-bound enzymes and defining meaningful

kinetic experiments with micellar substrates were only just beginning. Since then it has become clear that all desaturase systems from *Euglena gracilis*[15] to the rat[16] are bound to some kind of membrane structure. The early experiments using crude liver homogenates soon established that as well as fatty acid and a reduced pyridine nucleotide, it was necessary to have a source of ATP, Mg^{2+} and coenzyme A. These experiments coupled with those of Raju & Reiser,[17] who demonstrated the lack of desaturation of lipid-bound stearate, established the fatty acyl-CoA as the true substrate for stearoyl-CoA desaturase and the same has since been demonstrated to be true for the Δ^6- and Δ^5-fatty acyl-CoA desaturases.[14,18] Subsequent studies have revealed that the site of the desaturation is the microsomal fraction where it has been shown by Oshino & Sato[19] that during the induction of stearoyl-CoA desaturase in mammalian liver, the enzyme is first located (12 h after induction) on the rough endoplasmic reticulum and finally resides on the smooth endoplasmic reticulum. The fatty acids derived from endogenous synthesis therefore, are transported to the endoplasmic reticulum, by a mechanism which is not yet understood, activated by fatty acid thiokinases and the fatty acyl-CoAs then undergo one of several possible fates, as exemplified by palmitic acid below:



D—desaturation; E—elongation

Largely from the work of Bernert & Sprecher,[20] it has been shown that the rate of elongation is appreciably faster than the desaturation step and as a consequence the route exemplified by the formation of oleoyl-CoA would be preferred. This is also a reflection to some extent that stearoyl-CoA rather than palmitoyl-CoA is the preferred substrate for Δ^9 desaturation.[21]

B. PURIFICATION OF DESATURASES

It has already been mentioned that all desaturases are bound to membrane-like structures[19,22–24] and this one fact more than any other has been responsible for the slow characterization of the desaturase enzyme complex.

The earlier work of Nagai & Bloch[15] gave the first hint of the complexity of the system, when they fractionated the *Euglena gracilis* complex into three components which they called NADH-oxidase, ferredoxin and the very labile desaturase, $t_{1/2} < 10$ h. Later work by Sato's Japanese group[16] and Holloway's group[25] in America, established that this complexity was also to be found in mammalian liver, where the active system consists of three proteins (i) NADH-cytochrome b_5 reductase, (ii) cytochrome b_5 and (iii) the desaturase or cyanide sensitive protein. Since Bloch had shown that the individual "soluble" proteins of the Euglena system could be fractionated by gel filtration and ion-exchange chromatography, the rationale that was adopted for mammalian microsome systems was to first "solubilize" the membranes with detergents and then apply the standard protein chemical separation techniques. Unless great care is taken in defining these conditions, by choosing not only the right detergent, but also the right detergent:protein ratio, this rationale can lead to large irreversible losses of enzyme activity. Some of these problems, with particular reference to the microsomal stearoyl-CoA desaturase system, have been reviewed.[26,27]

Recently Strittmatter *et al.*[28] adopted an approach which was based on the solubilization of non-essential proteins, thus leaving the Δ^9-fatty acyl-CoA desaturase in the depleted membrane for as long as possible. We adopted a similar approach at about the same time by solubilizing non-essential proteins with a stepwise increase in concentration of a non-ionic detergent, Nonidet P-40.[26] Strittmatter took his studies, using sodium deoxycholate and Triton X-100, to their logical conclusion and purified the stearoyl-CoA desaturase to homogeneity as judged by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate. The specific activity of the final preparation was approximately 150-fold higher than in the initial microsomes which reflects a desaturase concentration of approximately 0·7% (w/w) even when the enzyme has been induced to super-normal levels by feeding the rats a carbohydrate rich/fatty acid free diet.

C. THE ELECTRON TRANSPORT COMPONENTS

The requirement for three protein components in the stearoyl-CoA desaturase has already been described. Since their identification, much effort has been focused on (i) the detailed characterization of the individual proteins, (ii) the way in which these proteins interact to catalyse the desaturation reaction and (iii) the role played by the lipid component of the membrane.

For some time firm evidence for the obligatory involvement of cytochrome b_5 remained elusive, but the immunochemical data of Oshino & Omura,[29] and the reconstitution studies of Strittmatter *et al.*[28] provide unequivocal evidence for the involvement of cytochrome b_5. The function of this haem–iron protein in the desaturation reaction as a carrier of electrons from a reduced pyridine