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Properties of Rat Liver Microsomal Stearoyl-Coenzyme A Desaturase

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1. Rat liver microsomal stearoyl-CoA desaturase activity was shown to be stimulated by both bovine serum albumin and a basic cytoplasmic protein from rat liver. 2. Partially purified desaturase is unaffected by either of these two proteins. 3. Bovine serum albumin appears to exert its effect on the crude system by protecting the desaturase substrate, stearoyl-CoA, from the action of endogenous thiolesterases. 4. By using partially purified enzyme preparations, it was possible to establish the substrate specificity of the Δ^9-fatty acyl-CoA desaturase with the C_{14}, C_{15}, C_{16}, C_{17}, C_{18} and C_{19} fatty acyl-CoA substrates. Maximum enzyme activity was shown with stearoyl-CoA decreasing with both palmitoyl-CoA and nonadecanoyl-CoA, as reported previously for free fatty acids. 5. Both cytochrome b_5 and NADH–cytochrome b_5 reductase (EC 1.6.2.2) are required for these studies, and a method is described for the purification of homogeneous preparations of detergent-isolated cytochrome b_5 from rat liver. 6. From amino acid analyses, a comparison was made of the hydrophobicity of the membrane portion of cytochrome b_5 with the hydrophobicity reported for stearoyl-CoA desaturase. The close resemblance of the two values suggested that unlike cytochrome b_5 and its reductase, the stearoyl-CoA desaturase may be largely buried in the endoplasmic reticulum.

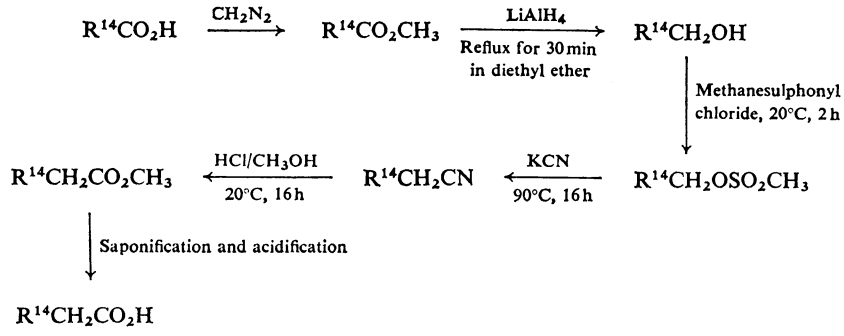
Mammalian Δ^9-fatty acyl-CoA desaturases, which catalyse the conversion of saturated fatty acid CoA esters into mono-unsaturated fatty acid CoA esters, are bound to the endoplasmic reticulum and have an obligatory requirement for NADH, molecular oxygen, cytochrome b_5 and NADH–cytochrome b_5 reductase (EC 1.6.2.2) (Holloway, 1971; Shimakata *et al.*, 1972; Strittmatter *et al.*, 1974). Catala *et al.* (1975) and Jeffcoat *et al.* (1976) have shown that cytoplasmic proteins are required for maximum activity of crude preparation of Δ^9- and Δ^6-fatty acyl-CoA desaturases. The enzymic conversion of stearoyl-CoA into oleoyl-CoA by microsomal preparations is stimulated by a protein which appears to be identical with ligandin (Litwack *et al.*, 1971). A second acyl-CoA-binding protein, which stimulates monoacylglycerol biosynthesis (Mishkin & Turcotte, 1974), has no effect on the stearoyl-CoA desaturation reaction. Homogeneous preparations of stearoyl-CoA desaturase have been prepared (Strittmatter *et al.*, 1974) and the activity has been re-constituted by the addition of NADH, cytochrome b_5 reductase and cytochrome b_5. Although the possible involvement of cytoplasmic proteins was not discussed by Strittmatter *et al.* (1974) it could be inferred that there was not an obligatory requirement for these proteins in the purified system. This has raised the question of the physiological significance of the apparent involvement of acyl-CoA-binding proteins in mammalian desaturase reactions.

Experimental

Materials

Biochemical reagents were from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. Fatty-acid-poor bovine serum albumin (Sigma) had been prepared from crystallized and freeze-dried albumin. Sodium deoxycholate was from BDH Chemicals, Poole, Dorset, U.K., and was further purified as described by Spatz & Strittmatter (1973). This was found necessary to avoid the formation of thixotropic solutions in the high-ionic-strength buffers used in gel filtration. [1-^{14}C]Stearoyl-CoA with a specific radioactivity of 50–60 Ci/mol was from New England Nuclear, South Wonston, Winchester, Hants., U.K.

Acyl-CoA esters were synthesized from their unesterified fatty acids via the *N*-hydroxysuccinimide esters (Lapidot *et al.*, 1967) by the method of Al-Arif & Blecher (1969). [1-^{14}C]-Myristic acid, -palmitic acid and -stearic acid were commercially available from The Radiochemical Centre, Amersham, Bucks., U.K. [2-^{14}C]-Pentadecanoic acid, -heptadecanoic acid and -nonadecanoic acid were synthesized from their respective precursor [1-^{14}C]fatty acids as shown in Scheme 1 (Harris, 1970).



Scheme 1. *Synthesis of* [2-^{14}C]*fatty acids*

The general scheme below outlines the synthesis of [2-^{14}C]fatty acids from their 1-^{14}C-labelled analogues and KCN. R = $CH_3(CH_2)_n$, where $n = 12$, 14 or 16.

The radiochemical purity of the acyl-CoA esters was determined after hydrolysis and methylation, by t.l.c. on silica gel G in the solvent diethyl ether/light petroleum (b.p. 40°–60°C) (1:1, v/v). Their chemical purities were determined from their A_{232} and A_{260} values, which were in good agreement with the reported values of other CoA esters (Seubert & Pappajohn, 1960).

Animals

Male rats of the Colworth–Wistar strain were fed on either a standard laboratory diet, consisting of (w/w) 44% starch, 6% cellulose powder, 25% casein, 10% groundnut oil, 4% minerals, 10% vitamin mix and 1% L-cystine, or a high-carbohydrate diet (Jeffcoat *et al.*, 1976) to increase the activity of the Δ^9-fatty acyl-CoA desaturase.

Subcellular fractionation

The preparation of the microsomal fraction, determination of the protein and the enzyme assays were as previously reported (Jeffcoat *et al.*, 1976).

Preparation of the electron-transport proteins

Cytochrome b_5, isolated by the partial trypsin digestion of rat liver microsomal fraction, was prepared in pure form by the method of Omura & Takesue (1970). Cytochrome b_5 and NADH–cytochrome b_5 reductase, solubilized from rat liver microsomal fraction with 1% (w/v) Triton X-100 and 1% (w/v) sodium deoxycholate, were partially purified by $(NH_4)_2SO_4$ precipitation and ion-exchange chromatography (Shimakata *et al.*, 1972). The reductase isolated in this way, although not pure, was free of both stearoyl-CoA desaturase activity and cytochrome b_5. The latter was resolved from residual NADH–cytochrome b_5 reductase by dialysis followed by rechromatography on a similar column. The cytochrome b_5 fractions were then pooled, dialysed against 10mM-Tris/1mM-EDTA, adjusted to pH8.1 with acetic acid, and applied to a column (20cm × 2cm) of DE-52 DEAE-cellulose equilibrated with the same buffer. The column was washed with 1 litre of this buffer to remove the Triton X-100 as monitored by its A_{275} (Spatz & Strittmatter, 1973). The cytochrome b_5 was then eluted from the DEAE-cellulose with 100ml of 0.2% sodium deoxycholate/1mM-EDTA/0.5M-KCl in 10mM-Tris, adjusted to pH8.1 with acetic acid. The pooled fractions were dialysed for 17h against 20mM-Tris/1mM-EDTA/0.4% sodium deoxycholate, adjusted to pH8.1 with acetic acid. They were then concentrated by ultrafiltration with Amicon PM-10 membranes and applied to a column (75cm × 2cm) of Sephadex G-75 equilibrated with the same buffer. Cytochrome b_5 was eluted just after the void volume of the column and was resolved from high-molecular-weight impurities. Triton X-100 was removed from NADH–cytochrome b_5 reductase by the procedure described above. Sodium deoxycholate was removed from both proteins by gel filtration on a column (20cm × 2cm) of Sephadex G-25 equilibrated with 0.1M-Tris, adjusted to pH7.5 with HCl.

Purification of Δ^9-fatty acyl-CoA desaturase

Δ^9-Fatty acyl-CoA desaturase was partially purified by the stepwise solubilization of rat liver microsomal fractions with sodium deoxycholate as described by Strittmatter *et al.* (1974). The desaturase preparations used in the present studies [step 5 of the purification described by Strittmatter *et al.* (1974)] showed marked heterogeneity on polyacrylamide gels equilibrated with 0.1% sodium dodecyl sulphate, but were not contaminated with either cytochrome b_5 or NADH–cytochrome b_5 reductase. Enzyme assays were performed in a total volume of 1.0ml containing 100μmol of Tris, adjusted to pH7.5 with HCl, and the other components were added in the following order: 100nmol of [1-^{14}C]stearoyl-CoA (100nCi), 300nmol of NADH, 0.5nmol of cyto-

chrome b_5, 4 units of NADH–cytochrome b_5 reductase, 3mg of stearoyl-CoA-binding protein (peak II, Jeffcoat *et al.*, 1976) and 0–150μg of partially purified desaturase. Assays were peformed in duplicate and never differed by more than $\pm 0.5\%$ desaturation. Cytochrome b_5 was assayed by the method of Omura & Sato (1964) and NADH–cytochrome b_5 reductase by using potassium ferricyanide as the artificial electron acceptor, by the method of Takesue & Omura (1970).

Amino acid analysis

Samples (100μg) of trypsin-isolated and detergent-isolated cytochrome b_5 (see 'Preparation of the electron-transport proteins') were digested in constant-boiling HCl *in vacuo* at 110°C for 17, 48 and 72h. Corrections were made for those amino acids that were either destroyed or were slowly released during the hydrolyses. The cysteine content was determined as cysteic acid by performic acid oxidation (Hirs, 1956). The tryptophan content was determined from the 17h hydrolysis carried out in the presence of 3% (v/v) thioglycollic acid (Matsubara & Sasaki, 1969).

Fatty acid analyses

Acyl-CoA esters and unesterified fatty acids were separated by t.l.c. as described by Holloway & Holloway (1973).

Results and Discussion

Mammalian desaturases have been known for many years to be bound to the endoplasmic reticulum (Oshino & Sato, 1972) and to act on acyl-CoA esters derived from the fatty acids of the diet, of tissue lipids or by endogenous synthesis.

In the liver cell any saturated acyl-CoA can suffer a variety of fates, namely being either hydrolysed to the unesterified fatty acid, converted into lipid, β-oxidized, chain elongated or desaturated. In the period in which the acyl-CoA is being transported from the cytoplasm to the endoplasmic reticulum, where the desaturases are located, some protection would be required from these competing enzymic processes. Data obtained from crude desaturase systems, where competing reactions are possible, have shown a stimulatory effect by cytoplasmic proteins (Catala *et al.*, 1975; Jeffcoat *et al.*, 1976). The fatty-acid-binding protein that we described in the latter paper could not be shown to be obligatory, however. This might be explained from the kinetic data (Jeffcoat *et al.*, 1976) in terms of limiting amounts of the binding protein being already present in the membranes. To investigate this possibility, the stearoyl-CoA desaturase was partially purified and assayed as described in the Experimental section in the presence and absence of bovine serum albumin or of partially purified stearoyl-CoA desaturase-stimulating protein. The latter was prepared as described by Jeffcoat *et al.* (1976). Unlike the crude microsomal system, the partially purified desaturase shows no stimulation by either of these two fatty acid-binding proteins (Table 1). Further, the rate of desaturation of the stearoyl-CoA desaturase in the assay system was linear for 10min with up to 160μg of protein. The specific activity was 31.5nmol of oleate produced/min per mg of protein. The rate was independent of the presence of either binding protein.

A partial explanation for the lack of stimulation of the purified desaturase has come from a more detailed study of the effect of bovine serum albumin on the crude microsomal system. Stearoyl-CoA desaturase incubations were performed for 5, 15 and 45min with 0.5mg of rat liver microsomal protein in the presence and absence of 2.5mg of fatty-acid-poor bovine serum albumin. Portions of the assay mixture were assayed for the distribution of label in the acyl-CoA, unesterified fatty acids, phospholipid and triacylglycerol fractions. In all the incubations, less than 5% of the total radioactivity could be accounted for in the triacylglycerol fraction. A comparison of the rate of breakdown of acyl-CoA in the absence (Fig. 1*a*) and in the presence (Fig. 1*b*) of bovine serum albumin indicates that the albumin in some way protects the acyl-CoA from the action of endogenous acylthiolester hydrolases. The more rapid cleavage

Table 1. *Effect of fatty-acid-binding proteins on the activity of microsomal and of partially purified stearoyl-CoA desaturase*
Incubations were peformed at 30°C for 10min in a total volume of 1ml of 0.1M-Tris, adjusted to pH7.4 with HCl. Each incubation mixture contained 100nmol of [1-^{14}C]stearoyl-CoA (100nCi), 300nmol of NADH, 0.5nmol of cytochrome b_5, 4 units of NADH–cytochrome b_5 reductase and either 0.25mg of microsomal protein or 69μg of partially purified desaturase. Where appropriate, 2.5mg of either bovine serum albumin or of stearoyl-CoA-binding protein was added.

Addition	Specific activity (nmol of oleate formed/min per mg of protein)	
	Microsomal fraction	Partially purified desaturase
None	3.2	36.2
Bovine serum albumin	6.24	32.5
Stearoyl-CoA-binding protein	6.28	33.8

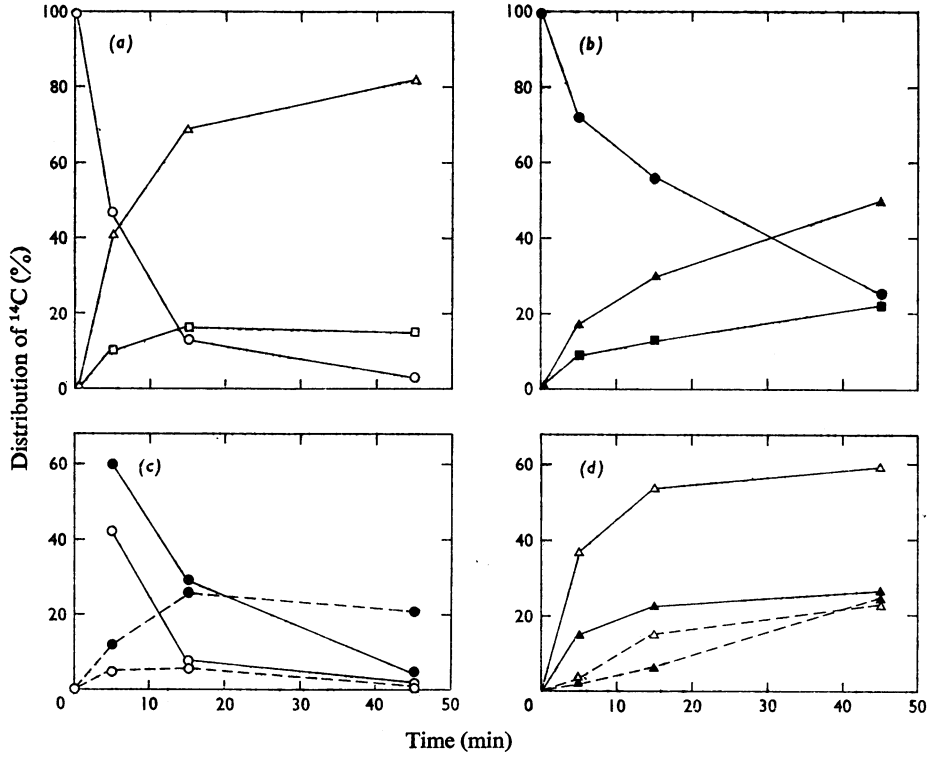


Fig. 1. *Distribution of radioactivity as a function of time during the microsomal desaturation of* [1-^{14}C]*stearoyl-CoA* In (*a*) and (*b*) the distribution of radioactivity in acyl-CoA (○, ●) unesterified fatty acid (△, ▲) and phospholipid (□, ■) is shown. (*c*) shows the labelling patterns for stearoyl-CoA (——) and oleoyl-CoA (– – – –), and (*d*) shows the labelling patterns for stearic acid (——) and oleic acid (– – – –). The open and closed symbols show the absence and presence of bovine serum albumin respectively.

of the acyl-CoA in the absence of bovine serum albumin is paralleled by an increased rate of production of unesterified fatty acid. These changes in rates of hydrolysis were also seen when the individual acyl-CoA esters and unesterified fatty acids were studied. For example, Fig. 1(*c*) shows that in the presence of bovine serum albumin the maximum oleoyl-CoA concentration is raised from 6 to 26% of the total radioactivity after a 15 min incubation. Similarly, there is a slower breakdown of stearoyl-CoA (Fig. 1*c*) and a slower release of stearic acid and oleic acid (Fig. 1*d*). The detailed mechanism of the stimulation by bovine serum albumin is not known. The following explanation, which is based on the known fatty-acid-binding properties of albumin (Goodman, 1958), seems to be most likely. Under optimum conditions for stearoyl-CoA desaturase stimulation, the bovine serum albumin reversibly binds stearoyl-CoA and protects it from the action of the acylthiolester hydrolases, but not from the desaturase. Therefore under these conditions the stearoyl-CoA desaturase competes more effectively than the acylthiolester hydrolases for the available substrate. In the purified enzyme system, competing enzymes have been removed and therefore there is no need for the binding proteins.

Substrate specificity of the Δ^9*-fatty acyl-CoA desaturase*

Previous substrate-specificity studies have been peformed by incubating unesterified fatty acids (Brett *et al.*, 1971) with mitochondrial supernatant fractions. With such a complex system it is difficult to control the many variables that could influence the rate of desaturation reaction under investigation. More recent studies with rat liver microsomal fractions (Jeffcoat *et al.*, 1976) have demonstrated the difficulties of establishing meaningful kinetic conditions for the desaturation of even one acyl-CoA. In the present studies, the use of partially purified Δ^9-fatty acyl-CoA desaturase has enabled many of these difficulties to be overcome. Such preparations exhibit linear relationships between enzyme activity and en-

Table 2. *Desaturation of acyl-CoA esters by partially purified Δ^9-fatty acyl-CoA desaturase*

Incubations were performed at 30°C for 10min in a total volume of 1ml of 0.1M-Tris/HCl, pH7.4, containing 4.5 units of NADH–ferricyanide reductase, 600pmol of cytochrome b_5, 300nmol of NADH, 120μg of partially purified microsomal protein and three concentrations of each acyl-CoA ester.

Substrate	Concn. (μM)	Desaturation (%)	Product (nmol/10min)
$C_{14:0}$ fatty acyl-CoA	48	15.2	7.3
	96	7.7	7.4
	144	4.2	6.1
$C_{15:0}$ fatty acyl-CoA	52.5	7.7	4.0
	105.0	4.7	5.0
	157.5	2.3	3.6
$C_{16:0}$ fatty acyl-CoA	52.5	20.1	10.6
	105.0	10.4	10.9
	157.5	5.2	8.2
$C_{17:0}$ fatty acyl-CoA	55.5	26.3	14.6
	111.0	12.8	14.2
	166.5	9.3	15.5
$C_{18:0}$ fatty acyl-CoA	55.5	37.9	22.0
	111.0	20.7	22.9
	166.5	12.3	20.5
$C_{19:0}$ fatty acyl-CoA	52.8	17.6	9.3
	105.6	8.9	9.4
	158.4	5.4	8.6

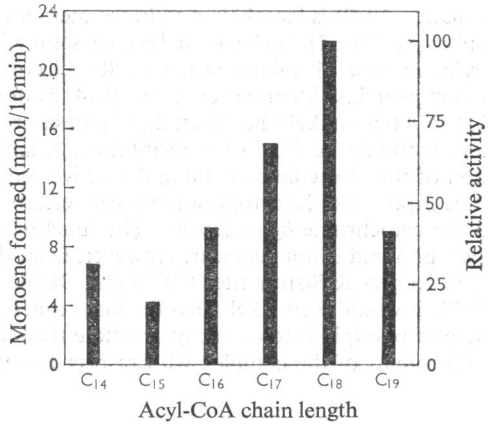


Fig. 2. *Substrate specificity of Δ^9-fatty acyl-CoA desaturase (expressed as nmol of product formed/10min) as a function of the chain length of the acyl-CoA ester*

Incubations were peformed as described in the text and a value for the specific activity calculated from the desaturations showing no substrate inhibition.

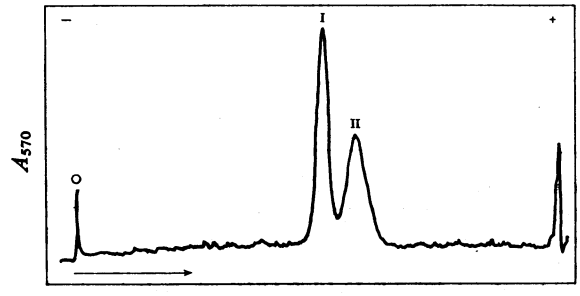


Fig. 3. *Densitometric scan of detergent (I)- and trypsin (II)- isolated cytochrome b_5 subjected to polyacrylamide-gel electrophoresis at pH7.0 in the presence of 0.1% sodium dodecyl sulphate*

When the proteins were run separately single bands were obtained. The above trace of the two proteins run together compares directly their different mobilities. The arrow shows the direction of migration. O, origin.

zyme concentration and are no longer susceptible to substrate inhibition as was found with crude microsomal preparations. Incubations were performed at 30°C for 10min with three different substrate concentrations in the absence of added binding proteins. Full details of the desaturations are given in Table 2 and the specificity of the Δ^9-fatty acyl-CoA desaturase is shown in Fig. 2. The small rise in desaturase activity with myristoyl-CoA may represent the presence of a second Δ^9-fatty acyl-CoA desaturase, as has been suggested for other systems (Brett *et al.*, 1971).

The electron-transport proteins necessary for these specificity studies were isolated and purified from rat liver instead of from rabbit, as used by other workers. Cytochrome b_5 was purified to homogeneity by using detergents as described in the Experimental section. Its molecular weight was compared with that of cyto-

Table 3. *Amino acid composition of detergent-isolated (a) and trypsin-isolated (b) cytochrome b_5*

Full details of the determinations of the amino acid compositions are given in Experimental section. The column headed ($a - b$) is obtained by difference of the previous two columns and the mol.wt. (5100) calculated from values for residue mol.wt. of amino acids. Polarity is determined as described by Capaldi & Vanderkooi (1972).

	Composition (residues/molecule)		
Amino acid	(*a*)	(*b*)	($a - b$)
Aspartate	15	12	3
Threonine	10	7	3
Serine	13	8	5
Glutamate	21	18	3
Proline	7	4	3
Glycine	13	11	2
Alanine	13	7	6
Cysteine	—	—	—
Valine	12	8	4
Methionine	1	—	1
Isoleucine	8	5	3
Leucine	15	9	6
Tyrosine	6	4	2
Phenylalanine	4	3	1
Histidine	6	6	—
Lysine	10	8	2
Arginine	6	4	2
Tryptophan	3	2	1
Mol.wt.	18000	13000	5100
Polarity	49.7	53.5	38.3

chrome b_5 that had been isolated from microsomal fractions by controlled proteolytic digestion with diphenylcarbamoyl chloride-treated trypsin. Both preparations appeared homogeneous on polyacrylamide gels equilibrated with 0.1% sodium dodecyl sulphate (Weber & Osborn, 1969) (Fig. 3). Amino acid analysis was performed on both forms of cytochrome b_5 and the data shown in Table 3 closely resemble the data already reported for cytochrome b_5 from other mammalian livers (Ozols, 1974). The amino acid composition of the hydrophobic tail has been derived by difference from the composition of cytochrome b_5 isolated by (*a*) detergent solubilization and (*b*) partial trypsin digestion of rat liver microsomal fractions. The polarities of the various polypeptide species have been calculated by the method of Capaldi & Vanderkooi (1972). As with other mammalian cytochrome b_5 molecules (Spatz & Strittmatter, 1971) the polarity of the low-molecular-weight tryptic peptide is considerably lower than that of the larger haem-containing tryptic polypeptide. It is now generally believed that cytochrome b_5 molecules (and NADH–cytochrome b_5 reductase) consist of two domains: an essentially globular, hydrophilic moiety containing the active site and terminating in a hydrophobic polypeptide tail. The latter serves to anchor

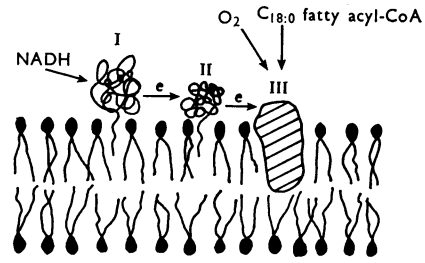


Fig. 4. *Diagrammatic representation of the stearoyl-CoA desaturase complex in phospholipid bilayer of the endoplasmic reticulum*

NADH–cytochrome b_5 reductase (I) and cytochrome b_5 (II) serve to transfer electrons (*e*) from NADH to stearoyl-CoA desaturase (III).

the cytochrome b_5 molecule on the surface of the endoplasmic reticulum. The striking similarity between the hydrophobicity of the cytochrome b_5 tail and the value of 38% reported for the stearoyl-CoA desaturase (Strittmatter *et al.*, 1974) would suggest that the latter, unlike cytochrome b_5 (Spatz & Strittmatter, 1971) and its reductase (Spatz & Strittmatter, 1973) is largely buried in the membrane phospholipid (Fig. 4). Such a model is consistent with the relative ease of solubilization of the electron-transport proteins (Strittmatter *et al.*, 1974; Safford *et al.*, 1975). It is unlikely, however, that the desaturase protein is totally buried in the membrane. A small portion of the molecule containing the active centre must be exposed to the cytoplasm to accept electrons from the cytochrome b_5 molecules. The function of the phospholipid is not yet clear. However, from the work of Rogers & Strittmatter (1973) and Okuda *et al.* (1972) it would seem likely that the main function of the phospholipid is to correctly orientate the individual proteins of the complex with respect to each other.

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