studies that the cyanmet subunits in the deoxygenated hybrids apparently are in an equilibrium between different conformations and organic phosphates shift the equilibrium. Although this result also supports the MWC model, further studies are necessary to establish an unique model of cooperative oxygen binding in hemoglobin.

Finally, in $\alpha_2(O_2)\beta_2^+(CN)$ the response of the oxidized β subunits to DPG implies that the β subunits change the conformation with the oxygenation of the α subunits, *i.e.*, there is a propagation of conformational changes from the α subunits to the β subunits. Otherwise, the oxygen affinity of $\alpha_2(O_2)\beta_2^+(CN)$ would be insensitive to DPG. The conformational changes propagating to neighboring subunits over a subunit where ligation occurs, would contribute to the cooperativity in oxygen binding of hemoglobin. The conclusion is consistent with recent physicochemical studies of hemoglobin (Hayashi *et al.*, 1967; Maeda and Ohnishi, 1971; Ogawa and Shulman, 1971; Ogata and McConnell, 1971; Asakura and Drott, 1971).

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A Requirement for Cytochrome b_5 in Microsomal Stearyl Coenzyme A Desaturation†

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ABSTRACT: The stearyl coenzyme A desaturase activity of hen liver microsomes was resolved into two fractions by gel filtration in the presence of deoxycholate. Both the large molecular weight fraction (P_3) and the smaller molecular weight fraction (P_4G) were required for desaturase activity. This activity was stimulated by addition of NADH–cytochrome b_5 reductase and deoxycholate. The P_4G fraction contained lipid and cytochrome b_5. Removal of the lipid destroyed desaturase activity which could then be restored by addition of lipid dispersions. Partially purified cytochrome b_5 was prepared by the standard trypsin procedure (trypsin–cytochrome b_5) and by ion exchange and gel chromatography of a detergent extract of acetone-extracted microsomes (detergent–cytochrome b_5). Only detergent–cytochrome b_5 could replace P_4G in the desaturase assay. Maximal desaturase activity was obtained with P_3, NADH–cytochrome b_5 reductase, detergent–cytochrome b_5, lipid, and deoxycholate. The requirement for these components is further evidence for the involvement of the NADH-specific electron transport chain of microsomes in stearyl coenzyme A desaturation.

The conversion of stearic acid into oleic acid has long been known to be catalyzed by the microsomal fraction of a liver homogenate but the mechanism of the reaction remains to be elucidated. Morris (1970) has investigated the stereochemistry of this conversion in animals and has suggested that the two hydrogens are removed from stearic acid in a simultaneous concerted reaction. This would indicate that formal oxygenated intermediates are not involved in desaturation. The same conclusion had been reached earlier from experiments with hydroxystearic acids in animals (Elovson, 1964) and *Euglena gracilis* (Gurr and Bloch, 1966). Attempts have been made to solubilize and purify the desaturase but to date only a fivefold purification has been obtained and this partially

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purified preparation was reported to have a particle weight of greater than 4×10^6 (Gurr and Robinson, 1970).

From the requirement for reduced nicotinamide–adenine dinucleotides and oxygen it was suggested that the microsomal electron transport chain might be involved (Wakil, 1964; Oshino *et al.*, 1966). Early results suggested that the NADH-specific, rather than the NADPH-specific, electron transport chain was involved (Oshino *et al.*, 1966; Jones *et al.*, 1969), and more recent evidence supports this suggestion. Two components of the NADH-specific electron transport chain: the NADH–cytochrome b_5 reductase (Holloway and Wakil, 1970) and cytochrome b_5 (Holloway, 1971; Oshino *et al.*, 1971) have been implicated in desaturation of stearyl CoA. The present report presents further evidence to support a role for cytochrome b_5 in desaturation and also reinvestigates the lipid requirement noted previously (Jones *et al.*, 1969) but recently questioned (Gurr and Robinson, 1970).

Experimental Procedure

Hen liver microsomes were prepared as described previously (Jones *et al.*, 1969) and were "solubilized" and manipulated as described previously (Holloway and Wakil, 1970) to yield a purified particulate fraction P_2.

NADH–cytochrome b_5 reductase was isolated from the postmitochrondrial supernatant of rat liver homogenates by the method of Takesue and Omura (1970) with an additional purification upon hydroxylapatite as described previously (Holloway, 1971). The reductase had an NADH–dichlorophenolindophenol reductase activity of 76 μmoles of dichlorophenolindophenol reduced per min per mg of protein and was estimated to be 50% pure when compared to that isolated by Takesue and Omura (1970). Trypsin–cytochrome b_5 was the same preparation described previously (Holloway, 1971).

Protein was estimated by the biuret method of Gornall *et al.* (1949). Lipid content of microsomal subfractions was estimated from the phosphate content (Chen *et al.*, 1956) of a chloroform–methanol extract (Bligh and Dyer, 1959). Deoxycholate was estimated by a modification of the method of Mosbach *et al.*, 1954). Two milliliters of 65% sulfuric acid was added directly to a small volume of sample. After heating at 60° for 15 min, the solution was read at 385 mμ and the concentration of deoxycholate estimated from a standard curve. The protein and lipid components of the microsomal membrane subfractions did not interfere with this assay up to 0.1 mg of protein.

Microsomal pigments were estimated from reduced minus oxidized difference spectra. The sample was diluted to 2.5 ml with 10 mM potassium phosphate buffer (pH 7.2) and 7.4 μg of purified NADH–cytochrome b_5 reductase was added. The mixture was divided equally between two cuvets and a base line of equal light absorbance established in a Cary 14 recording spectrophotometer. To the sample cuvet was added 100 nmoles of NADH and the NADH-reduced minus oxidized spectrum was recorded. The ΔA between 424 and 410 mμ was a measure of cytochrome b_5. The extra ΔA between the maximum and minimum of the spectrum after addition of a few crystals of sodium dithionite to the sample cuvet was a measure of pigments reducible by dithionite but not by NADH. A ΔE mM of 185 mM^{-1} cm^{-1} for reduced minus oxidized between 424 and 410 mμ (Omura and Sato, 1964) was used for calculation of cytochrome b_5 concentrations. For the purpose of quantitation the dithionite-reducible pigment was assumed to have the same ΔE mM. The stearyl CoA desaturase was determined by use of [1-^{14}C]stearyl CoA, with the product, [1-^{14}C]oleate, being separated by thin-layer chromatography on $AgNO_3$-impregnated silica gel H (Jones *et al.*, 1969). The standard assay system contained 60 mM potassium phosphate (pH 7.2), 100 μM NADH, 20 μM [1-^{14}C]stearyl CoA, protein and other components, and water to a final volume of 0.5 ml. Incubations were at 37° for 15 min in air and desaturase activities are reported as nmoles of [1-^{14}C]oleate formed during this time. NADH–cytochrome *c* reductase was measured as described previously (Jones and Wakil, 1967), except that assays were performed at 37°.

The sonicated lipid used was prepared by dissolving 17.5 mg of egg yolk phosphatidylcholine and 4.7 mg of oleic acid in 3 ml of benzene. The benzene was removed by lyophilization, and the residue was taken up in 3.7 ml of 20 mM Tris-acetate–1 mM EDTA buffer (pH 8.2) and sonicated under nitrogen with a Biosonik II sonicator (Bronwill Scientific, Rochester, N. Y.) operated at 50% output with the 12.5-mm tip. The vessel was cooled in ice and after six 30-sec periods of sonication the lipid suspension had cleared to an opalescent liquid. The lipid dispersion was centrifuged at 105,000*g* for 15 min and the opalescent lipid dispersion was removed and stored under nitrogen at 5°.

Gel Filtration of the P_2 Fraction. The P_2 fraction (200–400 mg of protein) was diluted to 20 ml with 0.25 M sucrose. After homogenization in a Potter-Elvehjem Teflon homogenizer, to the suspension was added 1 ml of 1 M sodium Tricine buffer (pH 8.0)–0.1 ml of 0.2 M dithiothreitol–1 ml of 10% sodium deoxycholate. The mixture was rehomogenized, sonicated for 2 sec with a Biosonik II using the 4-mm tip at 20% output, and applied to a column (40 × 430 mm) of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) stabilized by a 0.5-cm layer of Sephadex G-75 on the top. The column was equilibrated with 20 mM sodium bicarbonate (pH 7.7) containing 0.1 mM dithiothreitol and 0.2% sodium deoxycholate. The column was eluted with the same buffer, and 16-ml fractions were collected. The eluate was usually combined into two fractions corresponding to tubes 10–15 and 16–23. Tube 10 coincided with the void volume of this column. The first fraction (tubes 10–15) was applied to a column of Sephadex G-25 equilibrated with 10 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM dithiothreitol (phosphate–dithiothreitol buffer) to remove the deoxycholate. The cloudy eluate was poured into an equal volume of stirred saturated ammonium sulfate solution, and the precipitate was collected by centrifugation at 20,000*g* for 15 min. The pellets were suspended in a small volume of phosphate–dithiothreitol buffer and stored at −20°. The fraction is designated the "P_3 fraction." Before it was assayed for desaturase activity the thawed P_3 fraction was clarified by a 2-sec sonication with a Biosonic II using the 4-mm tip at 20% output.

The second fraction eluted from the Sephadex G-200 column (tubes 16–23) was concentrated with an Amicon concentrator (Amicon Corporation, Lexington, Mass.) using a PM-10 membrane. This concentrated material was stored at −20° and is designated the "P_4 fraction." The thawed P_4 fraction was applied to column of Sephadex G-25, with a bed volume 20 times the volume of P_4, equilibrated with phosphate–dithiothreitol buffer. The excluded material was collected and designated the "P_4G fraction." The P_3, P_4, and P_4G fractions when assayed for deoxycholate contained 0.01, 5.0, and 0.02 mg of deoxycholate per mg of protein, respectively.

Acetone extraction of the P_4 fraction was performed by adding 1 ml of the P_4 fraction to 9 ml of cold acetone. The mixture was stirred for 10 min at 0°, and the precipitate col-

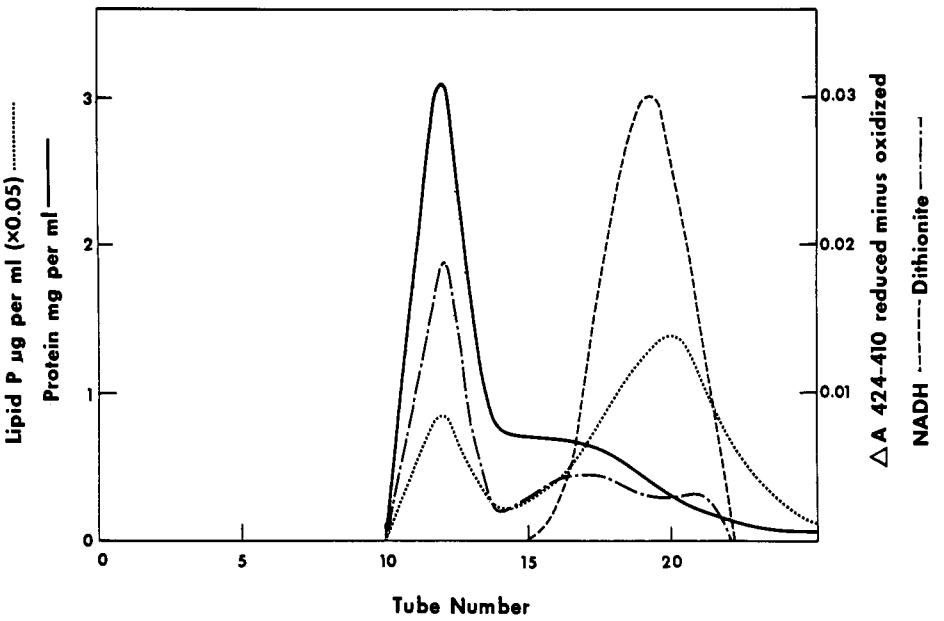


FIGURE 1: Resolution of the components of a microsomal subfraction (P_2) by gel filtration. The P_2 fraction was mixed with sodium deoxycholate and applied to a column of Sephadex G-200 equilibrated in 0.2% sodium deoxycholate as described in Experimental Procedure. Fractions were assayed for protein concentration per ml (——) and lipid phosphorus concentration per ml (.....). Cytochrome b_5 and pigments reducible by dithionite were determined after diluting 1 ml of each fraction with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.2) containing 7.4 μg of purified NADH–cytochrome b_5 reductase protein. The solution was divided between two cuvets and, after addition of NADH (100 nmoles) to the sample cuvet, the difference spectrum (reduced minus oxidized) was measured on a Cary 14 recording spectrophotometer. The ΔA between 424 and 410 mμ after addition of the NADH (– – – –) was a measure of the cytochrome b_5 content. A few crystals of dithionite were then added to the sample cuvet and the *additional* ΔA between 424 and 410 mμ (–·–·–) was a measure of pigments reducible by dithionite but not by NADH.

lected by centrifugation at 12,000*g* for 2 min. The pellet was suspended in 1 ml of phosphate–dithiothreitol buffer, sonicated for 5 sec with a Biosonic II using the 4-mm tip at 20% output, and subjected to the same gel filtration procedure used for making P_4G above. The excluded material was designated the "P_4A fraction" and contained less than 0.05 mM deoxycholate.

Preparation of Detergent–Cytochrome b_5. Hen liver microsomes (60 ml) were mixed with 1 M NaCl to a final volume of 300 ml and homogenized in a Potter-Elvehjem Teflon homogenizer. The microsomes were collected by centrifugation at 78,000*g* for 2 hr. The microsome pellets were resuspended in 10 mM sodium phosphate buffer (pH 7.2), homogenized, and centrifuged as before and the final pellets made up to 60 ml with 10 mM sodium phosphate. The washed microsomes were added to 600 ml of cold acetone and stirred for 10 min at 0°, and the precipitate was collected by centrifugation at 12,000*g* for 10 min. The acetone-extracted microsomes were suspended in 300 ml of 10 mM sodium phosphate containing 0.78 M NaCl. After centrifugation at 78,000*g* for 2 hr the pellets were collected and suspended in 300 ml of 10 mM sodium phosphate containing 2% Triton X-100. The mixture was stirred for 10 min at 0°; the Triton extract was collected by centrifugation at 78,000*g* for 2 hr and then applied to a column (25 × 200 mm) of DEAE-cellulose equilibrated in 50 mM Tris chloride (pH 7.7). The column was washed with 200 ml of 50 mM Tris chloride (pH 7.7) and the cytochrome b_5 eluted with a linear gradient of 150 ml of 50 mM Tris-HCl (pH 7.7) to 150 ml of the same buffer containing 0.5 M NaSCN. The cytochrome b_5 containing fractions were combined and treated with 1.5 volumes of saturated ammonium sulfate solution. The precipitate was collected by centrifugation, dissolved in a minimum of phosphate–dithiothreitol buffer, and applied to a column of Sephadex G-25 equilibrated in phosphate–dithiothreitol buffer. The excluded material was made 0.2% in deoxycholate and applied to a column (10 × 250 mm) of Sephadex G-200 equilibrated in 20 mM sodium bicarbonate (pH 7.7) containing 0.1 mM dithiothreitol and 0.2% deoxycholate. The column eluate was assayed for cytochrome b_5 and the tubes with highest specific activity were concentrated in an Amicon concentrator, using a PM-10 membrane. The solution was freed of deoxycholate by Sephadex G-25 gel-filtration and designated "detergent–cytochrome b_5."

Results

The P_2 fraction was prepared from hen liver microsomes by deoxycholate treatment as described previously (Holloway and Wakil, 1970). The P_2 fraction when treated with 0.5% deoxycholate formed a clear "solution" and was then subjected to gel filtration on a column of Sephadex G-200 equilibrated in 0.2% deoxycholate. The column eluate was assayed for protein, lipid phosphorus, cytochrome b_5, and other pigments reducible by dithionite but not by NADH. The results are shown in Figure 1. Although the majority of the protein was eluted shortly after the void volume (void volume corresponds to tube 10 in Figure 1), there was a clear resolution of other components of P_2. The cytochrome b_5 originally present in P_2 was eluted much later than the void volume; phospholipid was eluted in two distinct peaks, one associated with the excluded material and a later peak close to cytochrome b_5. A pigment reducible by dithionite but not by NADH plus NADH–cytochrome b_5 reductase was eluted with the excluded material. The recoveries of protein, lipid phosphorus, NADH–dichlorophenolindophenol reductase, and pigments are shown in Table I. Tubes 10–15 were pooled, the deoxycholate was removed, and the protein was precipitated by ammonium

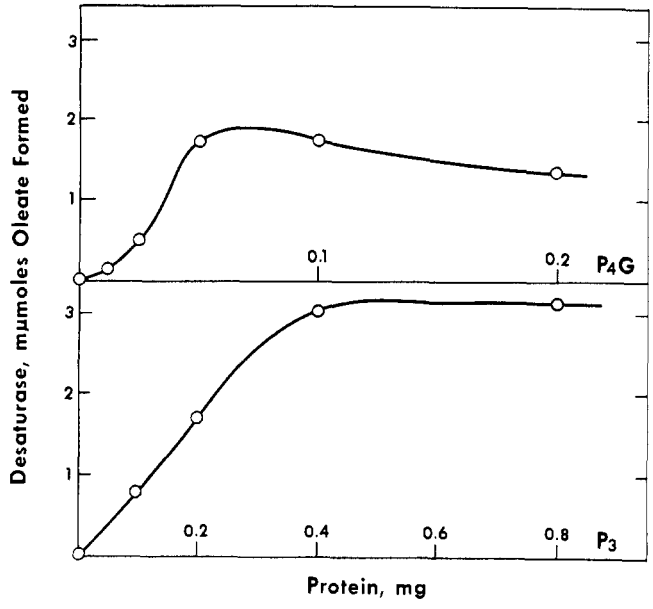


FIGURE 2: Dependence of desaturase activity on P_3 and P_4G fractions. The desaturase activity was measured by the standard assay described in Experimental Procedure with the addition of 3.7 μg of purified NADH–cytochrome b_5 reductase protein and 1.7 mM sodium deoxycholate. In the upper curve is shown the effect of adding increasing amounts of P_4G protein to incubations containing a constant level of 0.2 mg of P_3 protein. In the lower curve is shown the effect of adding increasing amounts of P_3 protein to incubations containing a constant level of 0.1 mg of P_4G protein.

sulfate to yield the P_3 fraction. The smaller molecular weight material (tubes 16–23) was concentrated in an Amicon concentrator to yield the P_4 fraction. Previous work (Holloway and Wakil, 1970; Holloway, 1971) had shown an absolute requirement for NADH–cytochrome b_5 reductase in desaturation. Table I, however, shows the NADH–dichlorophenolindophenol reductase activity is unstable to the chromatographic procedures used. Because of this instability it was feared that the amount of NADH–dichlorophenolindophenol reductase present in the P_3 or P_4 fractions (11 and 26 nmoles of dichlorophenolindophenol reduced per min per mg of protein, respectively) would be insufficient to support desaturation. Purified NADH–cytochrome b_5 reductase was, therefore, added to the standard desaturase assay. The amount of reductase added had an activity of 280 nmoles of dichlorophenolindophenol reduced per min and was a saturating level for desaturation.

TABLE I: Recoveries of Microsomal Components from Gel Filtration.[a]

Fraction	Protein (mg)	Lipid P (μg)	Cytochrome b_5 (nmoles)	Pigment (nmoles)[c]	DCIP Reductase (μmoles/min)
P_2	200	1470	13.5	2.7	11
10–15[b]	85	280	0	11	2.6
16–23[b]	35	610	6.0	0	1.9
P_3	55	186	0	4.9	0.6
P_4G	19	418	3.2	1.1	0.5

[a] The P_2 fraction and fractions isolated after Sephadex G-200 chromatography were analyzed for protein, lipid phosphorus (lipid P), cytochrome b_5, and dichlorophenolindophenol (DCIP) reductase as described in Experimental Procedure. [b] The fractions 10–15 and 16–23 refer to fractions obtained by pooling the indicated tubes of column effluent. [c] "Pigment" refers to cytochrome-like material which is reduced by dithionite but not by NADH plus NADH–cytochrome b_5 reductase. For the purpose of quantitation it was assumed to have the same ΔE mM reduced minus oxidized as cytochrome b_5.

TABLE II: Stearyl CoA Desaturase Activity of Hen Liver Microsomal Subfractions.[a]

Components	Stearyl CoA Desaturase (nmoles of Oleate Formed)
Microsomes	0.9
P_2	3.5
P_3 + reductase	0
P_4G + reductase	0
P_3 + P_4G + reductase	0.6
P_3 + P_4G + reductase + DOC	3.0
P_3 + P_4G + DOC	1.1
P_3 + reductase + DOC	0
P_4G + reductase + DOC	0

[a] The stearyl CoA desaturase activity was determined as described in the Experimental Procedure. The components added to the standard desaturase assay system as indicated were: 0.4 mg of microsomal, P_2, or P_3 protein; 0.08 mg of P_4G protein, 3.7 μg of NADH–cytochrome b_5 reductase (reductase) protein or 1.7 mM sodium deoxycholate (DOC).

When the P_3 and P_4 fractions were assayed individually, in the presence of NADH–cytochrome b_5 reductase, no desaturase activity was obtained (Table II). A slight restoration of desaturase activity was obtained with a mixture of P_3 plus P_4 fractions but this restoration was quite variable from preparation to preparation. As the desaturase had previously been shown to be inhibited by high levels of deoxycholate (Holloway, 1971) the deoxycholate concentration of the P_4 fraction was measured and found to be tenfold higher than the concentration of deoxycholate in the buffer used for the gel filtration of the P_2 fraction. Gel filtration of the P_4 fraction on Sephadex G-25 lowered the deoxycholate concentration from 50 mM to less than 0.1 mM. This material (P_4G) when assayed in the presence of the P_3 fraction gave a low but reproducible desaturase activity (Table II). This low activity could be stimulated by low levels of deoxycholate or Triton X-100 to a level almost equal to that exhibited by the P_2 fraction. The requirement for P_3 and P_4G fractions in desaturation is demonstrated in Figure 2. These, and subsequent assays, were performed in the presence of 1.7 mM deoxycholate, an optimal level. It can be seen that optimal activity was obtained with a ratio of P_3 to P_4G of four. If the value for cytochrome b_5 content of P_4G shown in Table III is used, this optimal ratio of P_3 to P_4G would correspond to a cytochrome b_5 content in the desaturase incubation of 0.09 nmole per mg of protein. This figure is close to that found for intact microsomes, 0.11 nmole per mg.

TABLE III: Cytochrome b_5 Content of Microsomal Subfractions.[a]

Subfraction[b]	Cytochrome b_5 (nmoles per mg of Protein)
Microsomes (1)	0.11
P_2 (1)	0.17
P_3 (1)	0
P_4G (0.2)	0.46
P_4A (0.2)	0.32
Detergent–cytochrome b_5 (0.03)	5.4
Trypsin–cytochrome b_5 (0.01)	17

[a] The cytochrome b_5 content of microsomes and microsomal subfractions was determined as described in the Experimental Procedure. [b] The numbers in parentheses after each component refer to the concentration in mg of protein per ml at which the cytochrome b_5 assay was performed.

TABLE IV: Stearyl CoA Desaturase and Cytochrome *c* Reductase Activities of Microsomal Subfractions.[a]

Components	Stearyl CoA Desaturase (nmoles of Oleate Formed)[b]	NADH–Cytochrome *c* Reductase (nmoles per min)[c]
P_4G	1.2[d]	15
P_4A	0.2	23
P_4A + lipid	1.0	24
Trypsin–cyt b_5	0	31
Trypsin–cyt b_5 + lipid	0	33

[a] The stearyl CoA desaturase and cytochrome *c* reductase activities were determined as described in the Experimental Procedure. [b] To the standard desaturase assay system was added 1.7 mM sodium deoxycholate, 0.6 mg of P_3 protein, 3.7 μg of NADH–cytochrome b_5 reductase protein, and the components indicated in the following amounts: 0.05 mg of P_4G protein, 0.07 mg of P_4A protein, 1.4 μg of trypsin–cytochrome b_5 (trypsin–cyt b_5) protein or 0.12 mg of lipid. [c] To the standard cytochrome *c* reductase assay system was added 1.7 mM sodium deoxycholate, 3.7 μg of NADH–cytochrome b_5 reductase protein, and the components indicated in the following amounts: 0.5 mg of P_4G protein, 0.7 mg of P_4A protein, 14 μg of trypsin–cytochrome b_5 protein, or 1.2 mg of lipid. The amounts of P_4G, P_4A, and trypsin–cytochrome b_5 used were such that each desaturase assay contained 0.046 μM cytochrome b_5 and each cytochrome *c* reductase assay contained 0.23 μM cytochrome b_5. [d] When the amount of P_4G added was increased to 0.1 mg of protein the stearyl CoA desaturase was 2.3 nmoles of oleate formed.

As shown in Figure 1 and Table I, the fraction P_4 is enriched in both lipid and cytochrome b_5, and attempts were therefore made to elucidate the role, if any, of these two components in stearyl CoA desaturation. As shown in Table IV, acetone extraction of P_4 resulted in almost complete loss of desaturase activity which could then be restored by a sonicated mixture of egg lecithin and oleic acid (Jones *et al.*, 1969). This suggests the lipid of the P_4G fraction is required for desaturase activity. If, however, the P_4G fraction was replaced by a solution of cytochrome b_5 made by the standard trypsin procedure (trypsin–cytochrome b_5) (Omura *et al.*, 1967), no desaturase activity was observed even when lipid or deoxycholate or both lipid and deoxycholate were added. Similar results with trypsin–cytochrome b_5 were reported previously (Holloway, 1971). In Table IV, it is also shown that the cytochrome b_5 present in P_4G or P_4A was able to function in the NADH–cytochrome *c* reductase assay. The amounts of P_4G, P_4A, and trypsin–cytochrome b_5 assayed with NADH–cytochrome b_5 reductase for NADH–cytochrome *c* reductase activity were chosen such that each incubation contained 0.23 μM cytochrome b_5. Under these conditions P_4G gave 49% and P_4A gave 75% of the activity of trypsin–cytochrome b_5. This similarity demonstrates that both types of cytochrome b_5 are almost equally effective at reducing cytochrome *c*. Attempts were made to isolate the cytochrome b_5 from the P_4 fraction by various chromatographic procedures without success. Attempts were, therefore, made to isolate detergent–cytochrome b_5 by the method of Spatz and Strittmatter (1971). This was hampered by the low concentration of cytochrome b_5 in hen liver microsomes (Table III). A preparation designated "detergent–cytochrome b_5" was obtained which was 50-fold purified over the original microsomes but which was still only approximately 8% pure. This material was assayed for desaturase activity as shown in Table V, with all incubations containing cytochrome b_5 at a level of 0.086 μM. Addition of detergent–cytochrome b_5 plus reductase to P_3 restored 50% of the activity shown by P_4G. This was stimulated by deoxycholate and lipid to the same level exhibited by P_4G. The amounts of stimulation with deoxycholate and lipid were somewhat variable. In other experiments using different preparations of the P_3 fraction, detergent–cytochrome b_5, and lipid, the stimulation by deoxycholate was more pronounced but, in that instance, lipid alone gave little stimulation of desaturase activity. In all experiments, however, addition of both deoxycholate and lipid was required for optimal desaturase activity. These variations could be due to variable amounts of lipid in the P_3 fraction. The P_4G and detergent–cytochrome b_5 were assayed for cytochrome *c* reductase activity, in the presence of NADH–cytochrome b_5 reductase. When the assays contained 0.22 μM cytochrome b_5 provided either by P_4G or detergent–cytochrome b_5 preparations the activities were 33 and 31 nmoles per min, respectively, in the presence or absence of 1.7 mM deoxycholate.

The desaturation reactions catalyzed by P_2, P_3 plus P_4G, or P_3 plus detergent–cytochrome b_5 were all inhibited approximately 50% by 1 mM KCN. Cytochrome b_5 reduction in microsomes is reported to be unaffected by cyanide (Oshino *et al.*, 1971). Hence, these results suggest the "cyanide-sensitive factor," postulated by Wakil (1964) and Oshino *et al.* (1966) and isolated by Gaylor *et al.* (1970), is present in the P_3 fraction. The dithionite-reducible pigment present in the P_3 fraction may be involved in desaturation although it could not be reduced by NADH plus NADH–cytochrome b_5 reductase even in the presence of the P_4G fraction. The pigment had a dithionite-reduced minus oxidized spectrum typical of heme proteins with a broad peak at approximately 426 mμ.

The stimulation of desaturation by low levels of detergent has been reported previously (Holloway, 1971) and this

TABLE V: Regeneration of Stearyl CoA Desaturase Activity by Detergent–Cytochrome b_5 plus Lipid.[a]

Components[b]	Stearyl CoA Desaturase (nmoles of Oleate Formed)
P_3 + DOC + P_4G[c]	2.1
P_3 + P_4G	0.6
P_3 + detergent–cyt b_5	1.0
P_3 + detergent–cyt b_5 + DOC	1.4
P_3 + detergent–cyt b_5 + lipid	1.2
P_3 + detergent–cyt b_5 + DOC + lipid	2.0
Detergent–cyt b_5	0
Detergent–cyt b_5 + DOC + lipid	0

[a] The stearyl CoA desaturase activity was determined as described in the Experimental Procedure. [b] To the standard desaturase assay system was added 3.7 μg of NADH–cytochrome b_5 reductase protein and the components indicated in the following amounts: 0.4 mg of P_3 protein, 0.08 mg of P_4G protein, 8 μg of detergent–cytochrome b_5 (detergent–cyt b_5) protein, 0.12 mg of lipid or 1.7 mM sodium deoxycholate (DOC). The amounts of P_4G and detergent cytochrome b_5 used were such that each assay contained 0.086 μM cytochrome b_5. [c] When the amount of P_4G added was increased to 0.16 mg of protein the stearyl CoA desaturase was 3.3 nmoles of oleate formed.

phenomenon was investigated further. As shown in Figure 3, the optimal concentration for deoxycholate was independent of protein concentration and that for Triton X-100 was only slightly dependent upon protein concentration. The small difference between the deoxycholate optimum found for the routine assay (1.7 mM) and the optimum of 1.5 mM shown for deoxycholate in Figure 3 was due to the different order of addition of the components of the desaturase assay. The data shown in Figure 3 were obtained from desaturase assays where all the components were premixed (in a cocktail) and added to detergent. In the routine desaturase assay P_3 and P_4G were added after all the other components. Attempts were made to rationalize the coincidence of the curves for deoxycholate and Triton X-100 by measuring the critical micelle concentrations by the method of Herries *et al.* (1964) under the conditions of salt and temperature used for the incubations. The critical micelle concentration for Triton X-100 was found to be 0.27 mM (using an average molecular weight of 628 for Triton X-100) at 37° in 60 mM potassium phosphate buffer (pH 7.2) whereas that for sodium deoxycholate could not be measured but was greater than 4 mM. There is apparently no relation between these values and the curves in Figure 3 but it must be emphasized that the desaturase incubation also contained stearyl CoA, protein, and lipid which may modify concentrations at which micelles form.

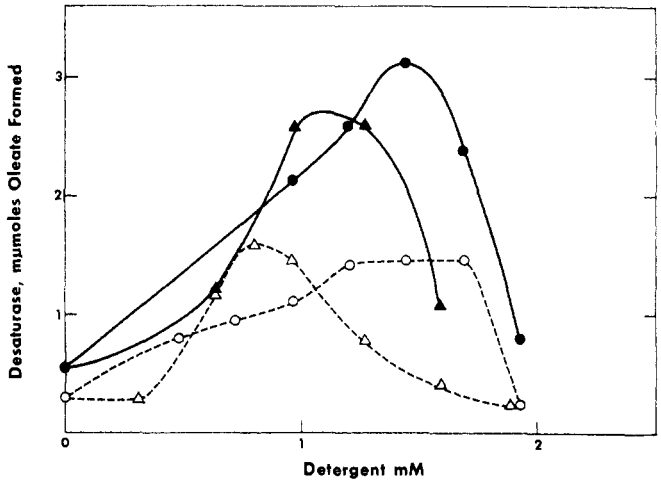
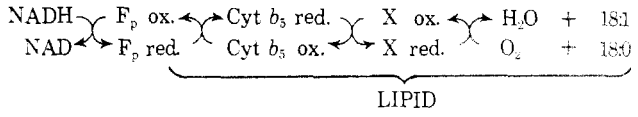


FIGURE 3: Effect of detergents on the desaturase activity of the P_3 plus P_4G fractions. The desaturase activity was measured by the standard assay described in Experimental Procedure with the addition of 3.7 μg of purified NADH–cytochrome b_5 reductase protein. The additional components in the first experiment were 0.4 mg of P_3 protein, 0.14 mg of P_4G protein with sodium deoxycholate (●——●) or Triton X-100 (▲——▲) at the concentrations indicated. In the second experiment the additional components were 0.2 mg of P_3 protein, 0.14 mg of P_4G protein with sodium deoxycholate (○- - -○), or Triton X-100 (△- - -△) at the concentrations indicated.

SCHEME I



Discussion

Previous studies on the stearyl CoA desaturase of hen liver microsomes demonstrated a requirement for NADH–cytochrome b_5 reductase in desaturation (Holloway and Wakil, 1970). It was also shown (Holloway, 1971) that a particulate fraction derived from an *N*-ethylmaleimide-treated membrane fraction could be resolved into two components by gel filtration on a column of Sephadex G-200 equilibrated in a buffer containing 0.2% deoxycholate. One of these components, isolated by gel filtration, contained cytochrome b_5 and lipid, and some evidence was obtained that these were both required for desaturation. Further evidence for the involvement of cytochrome b_5 in desaturation was presented by Oshino *et al.* (1971). The accumulated data support the involvement of the NADH-specific electron transport chain of microsomes in desaturation according to Scheme I.

In Scheme I F_p is NADH–cytochrome b_5 reductase, cyt b_5 is cytochrome b_5, and X is an unknown component. The successful resolution of an *N*-ethylmaleimide-treated microsomal subfraction by gel filtration (Holloway, 1971), together with several other published examples of membrane fractionation by gel filtration in detergent (*e.g.*, Nakai *et al.*, 1969; Martonosi and Halpin, 1971; Philippot, 1971), suggested that the desaturase of microsomes themselves should be ammenable to this procedure. Chromatography of Blue Dextran, bovine serum albumin, and cytochrome *c* demonstrated that Sephadex G-200, equilibrated with a buffer containing 0.2% deoxycholate, was able to separate soluble materials according to size.

A microsomal subfraction, P_2 (Holloway and Wakil, 1970), was subjected to gel filtration in the presence of deoxycholate and yielded two fractions P_3 and P_4G. Both these fractions were required for restoration of desaturase activity and optimal activity required the addition of NADH–cytochrome b_5 reductase and detergent. Since the P_4G fraction contained both cytochrome b_5 and lipid, attempts were made to show

the requirement for the P_4G fraction was due to the presence of these two components. It may be noted that the marked sigmoidicity of the upper curve in Figure 3 is in agreement with the P_4G fraction providing more than one component. Previous studies (Jones *et al.*, 1969; Holloway, 1971), using enzyme preparations subjected to acetone extraction, demonstrated a requirement for lipid in desaturation. Accordingly, the P_4 fraction was extracted with acetone to yield the P_4A fraction with concommitant loss of desaturase activity. The desaturase activity could then be restored by addition of lipid. In contrast to the loss of desaturase activity after acetone extraction, this procedure did not appreciably affect the cytochrome b_5. The cytochrome b_5 in P_4A was readily reduced by NADH plus NADH–cytochrome b_5 reductase and could serve as an intermediate electron carrier between the reductase and cytochrome *c*. This confirms the lipid requirement previously observed for desaturation (Jones *et al.*, 1969) and furthermore suggests lipid is required between cytochrome b_5 and oxygen. The NADH-specific electron transport chain of microsomes may then have at least two sites where lipid is required: between NADH–cytochrome b_5 reductase and cytochrome b_5 to account for the dependence of NADH–cytochrome *c* reductase on lipid (Jones and Wakil, 1967), and between cytochrome b_5 and oxygen as explained above.

The protein component of the P_4G fraction could be replaced by a paritially purified preparation of cytochrome b_5 provided the cytochrome b_5 was isolated from hen liver microsomes by use of detergent rather than by trypsin. In contrast, both detergent– and trypsin–cytochrome b_5 were equally effective at mediating electron flow from NADH–cytochrome b_5 reductase to cytochrome *c*. These results strongly suggest that cytochrome b_5, when isolated by a detergent procedure, is a component of the desaturase and that trypsin–cytochrome b_5 failed to restore desaturase activity to the P_3 fraction because it is a partially degraded form (Ito and Sato, 1968; Spatz and Strittmatter, 1971).

The P_3 fraction is almost completely uncharacterized. It contains the majority of the original protein present in the P_2 fraction, a small amount of lipid and a pigment reducible by dithionite. If X is the cyanide-sensitive factor postulated by Wakil (1964) and Oshino *et al.* (1966) and isolated by Gaylor *et al.* (1970), then the pigment present in the P_3 fraction may be this factor. The desaturase reconstituted from NADH–cytochrome b_5 reductase, the P_3 fraction, and either the P_4G fraction or detergent–cytochrome b_5 was still sensitive to cyanide. However, the pigment present in the P_3 fraction was not reduced by NADH plus NADH–cytochrome b_5 reductase even with the addition of the P_4G fraction. Further, more of this pigment was isolated in the P_3 fraction than was present in the original P_2 fraction suggesting that the pigment in P_3 is a denatured form of either cytochrome b_5 or cytochrome P_{450}.

The regeneration of the desaturase activity requires detergent in common with other multicomponent systems (Tzagoloff *et al.*, 1967). However the coincidence of the Triton X-100 and deoxycholate curves in Figure 3 was surprising in view of the differences in charge and critical micelle concentration between the two detergents. Although the optimal concentration for deoxycholate appeared to be independent of protein concentration that for Triton X-100 showed a slight dependence upon protein concentration. Triton X-100 gave optimum stimulation at a ratio of 1.3 and 1.5 mg of detergent per mg of protein at the higher and lower levels of proteins, respectively. These values are similar to the value reported by Reynolds and Tanford (1970) for bindirg of sodium dodecyl sulfate to proteins and may indicate that Triton X-100, and perhaps even deoxycholate, must block all the hydrophobic sites of the proteins before the components of the desaturase can interact. In this way the detergent may be mimicking the original lipid mileau of the microsomes. It must be stressed, however, that sonicated lipid dispersions would not replace deoxycholate or Triton X-100.

The data presently available suggest that the *in vitro* desaturation of stearyl coenzyme A proceeds according to Scheme I. This mechanism does not preclude the existence of other electron transport chains which may reduce the terminal oxidase, X, *in vitro* or *in vivo*. The above scheme does however have the advantage of employing three readily available stable components: NADH–cytochrome b_5 reductase, cytochrome b_5, and lipid, and it should enable the terminal oxidase, or intermediate components, to be identified.

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Effects of Oxygenated Cholesterol Derivatives on Adrenal Cortex Mitochondria†

Lowell D. Wilson

ABSTRACT: The effects of a variety of cholesterol derivatives on the optical properties of adrenal cortex mitochondrial P-450 have been examined. Included in the sterols studied are several suggested oxygenated intermediates in the oxidative side-chain cleavage of cholesterol (forming pregnenolone). Typical type I difference spectra were produced when 25-hydroxycholesterol, 24(*R*)-hydroxycholesterol, or 20α,22(*R*)-dihydroxycholesterol was added to aerobic adrenal cortex mitochondria. Type II spectra were produced by 22-ketocholesterol, 22(*S*)-hydroxycholesterol, and 22(*R*)-hydroxycholesterol. Titration of the sterol-induced spectral change demonstrates saturation behavior and the lowest apparent dissociation constants were found for 22(*R*)-hydroxycholesterol (8 μM) and 20α,22(*R*)-dihydroxycholesterol (3 μM). Unlike substrates for steroid 11β-hydroxylation (or the other sterols examined) 22(*R*)-hydroxycholesterol and 20α-hydroxycholesterol produce a decrease in absorbance at 420 nm in the presence of electron donors and an increase in absorbance at 415–420 nm in the absence of reducing sources. Oxygen consumption by mitochondria showing respiratory control (with malate) was greatly accelerated by these two sterols and by 20α-hydroxycholesterol. This effect, very similar to that produced by 11-deoxycorticosterone, is transient and can be repeatedly demonstrated by adding more sterol. Dual-wavelength studies indicate that the spectral change produced by these three sterols is also transient in the presence of reducing equivalents, suggesting that each can bind to cytochrome P-450 and be subsequently oxidized by this tissue.

The side-chain cleavage of cholesterol (producing pregnenolone[1]) is thought to be the slow step in the synthesis of adrenal steroids and the site of regulation by ACTH (Stone and Hecter, 1955; Karaboyas and Koritz, 1965; Koritz and Kumar, 1970). While it has been postulated that side-chain cleavage involves the sequential hydroxylation of the 20 and 22 carbons followed by oxidative scission between these atoms (Solomon *et al.*, 1956; Shimizu *et al.*, 1960, 1961, 1962; Constantopoulos and Tchen, 1961; Chaundhuri *et al.*, 1962), it has not yet been possible to identify the true intermediates or to convincingly dissect the overall process into its component steps.

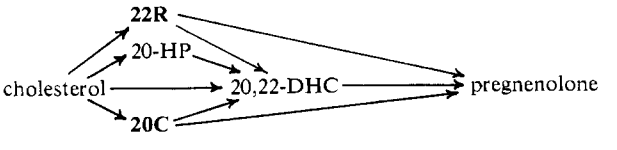
The difficulties arise from the rather slow rate of pregnenolone synthesis from cholesterol (Shimizu *et al.*, 1961; Kimura *et al.*, 1966), the inhibition by pregnenolone and 20α-hydroxycholesterol (**20C**) of their own synthesis (Koritz and Hall, 1964; Hall and Koritz, 1964; Ichii *et al.*, 1967; Raggatt and Whitehouse, 1966; Simpson and Boyd, 1967), and the general inability to isolate more than trace amounts of suspected intermediates after incubation of adrenal cortex with radioactive cholesterol (Hall and Koritz, 1964; Simpson and Boyd, 1967). Recently, Burstein *et al.* (1970a,b) have provided evidence that there are multiple routes from cholesterol to pregnenolone and that initial 20α-hydroxylation is probably a minor pathway. They suggest that 22(*R*)-hydroxylation is an important first step although evidence for a complex, concerted oxidation of the cholesterol side chain was also obtained.

It has been found that **20C**, 22(*R*)-hydroxycholesterol (**22R**), and cholesterol 20α-hydroperoxide (20-HP) are all effective precurors of pregnenolone in adrenal tissue (Shimizu *et al.*, 1961; Constantopoulos and Tchen, 1961; Chaudhuri *et al.*, 1962; Van Lier and Smith, 1970a; Burstein *et al.*, 1970a,b). There is some agreement that 20α,22(*R*)-dihydroxycholesterol (20,22-DHC) is an even better precursor of pregnenolone (Shimizu *et al.*, 1962; Constantopoulos *et al.*, 1962; Burstein *et al.*, 1970b), and, each of these sterols (except for the hydroperoxide) has been isolated from the adrenal cortex (Roberts *et al.*, 1969; Dixon *et al.*, 1970).

The suggested steps in the side-chain cleavage of cholesterol are summarized in Scheme I.

Cytochrome P-450 is known to be required somewhere in the overall process depicted above (Simpson and Boyd, 1967;

SCHEME I



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[1] The following trivial names and abbreviations are used: pregnenolone, 3β-hydroxypregn-5-en-20-one; **20C**, 20α-hydroxycholesterol; **22R**, 22(*R*)-hydroxycholesterol; **22S**, 22(*S*)-hydroxycholesterol; 20,22-DHC, 20α,22(*R*)-dihydroxycholesterol; **22K**, 22-ketocholesterol; 11-deoxycorticosterone (DOC), 21-hydroxypregn-4-ene-3,20-dione; 11-deoxycortisol, pregn-4-ene-3,20-dione-11α,17α,21-triol; Hepes, *N*-2-hydroxyethylpiperizine-*N'*-2-ethanesulfonic acid; 20-HP, cholesterol 20α-hydroperoxide.