Assay for the terminal enzyme of the stearoyl coenzyme A desaturase system using chick embryo liver microsomes

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Abstract The NADH-dependent stearoyl CoA desaturase of hepatic microsomes (EC 1.14.99.5) is an enzyme system consisting of cytochrome b_5 reductase (EC 1.6.2.2), cytochrome b_5, and the terminal desaturase. We have developed a simple method for routine assay of the terminal enzyme based on complementation of the enzyme with chick embryo liver microsomes lacking desaturase activity. Desaturation of [1-^{14}C]stearoyl CoA by the enzyme–microsome mixture is then assayed by thin-layer chromatography of the reaction products and determination of the amount of oleate formed. Microsomes from the livers of starved-refed rats were used as the source of the stearoyl CoA desaturase. The enzyme alone, solubilized and free from cytocrome b_5 reductase and cytochrome b_5, was unable to catalyze the desaturation of stearoyl CoA. However, after preincubation with chick embryo liver microsomes in the presence of 1% Triton X-100, the enzyme was active. The enzyme activity was linear with time and desaturase protein under the conditions described and depended on the concentrations of Triton X-100 present in the preincubation and the assay. The optimum concentrations of Triton X-100 were 1% for the preincubation and 0.1–0.15% in the assay. The desaturation activity was dependent on NADH and O_2, and was inhibited 95% by 1 mM KCN. The use of chick embryo liver microsomes in this method eliminates the need to use purified cytochrome b_5 reductase, cytochrome b_5, and liposomes for routine assays and greatly reduces the complexities of timing and order of addition encountered in the existing assays.

Supplementary key words terminal desaturase · cyanide-sensitive factor · complementation · cytochrome b_5 reductase · cytochrome b_5 · Triton X-100 · rat liver microsomes

The NADH-dependent stearoyl CoA desaturase system of liver microsomes has been studied extensively (1–4). It has been established that the hepatic microsomal system is membrane-bound and consists sequentially of NADH–cytochrome b_5 reductase, cytochrome b_5, and a terminal cyanide-sensitive factor, the desaturase (5–10). Solubilization of the individual components of the system with reconstitution of active complexes has been attempted by several groups (5, 8, 10, 11). Assay of the terminal desaturase activity required lipid, purified cytochrome b_5 reductase, cytochrome b_5, and the desaturase all to be mixed sequentially in the proper concentration of detergent to achieve adequate dispersal while avoiding denaturation (11). Stearoyl CoA desaturase activity could then be determined from [^{14}C]oleate formation with ^{14}C-labeled substrate (12), or by the spectral method based on cytochrome b_5 reoxidation at 424 nm (6, 11), or by measuring NADH fluorescence disappearance (11).

In this report we describe a simple method for the routine determination of activity in isolated desaturase preparations. It is based on the substitution of hepatic microsomes from 20-day-old chick embryos or 1-day-old chicks for the cytochrome b_5 reductase, cytochrome b_5, and phospholipid used in the existing assays. We reported in a previous paper that the desaturase system in such microsomes specifically lacks the terminal desaturase enzyme (13) and have taken advantage of this phenomenon to establish conditions under which the desaturase enzyme will interact with the microsomes to form a catalytically active complex. The method avoids the complexity of the reconstituted system and can spare the purified enzymes for other investigations.

EXPERIMENTAL PROCEDURE

Preparation of microsomes

Embryonated eggs from White Leghorn chickens were incubated in a forced draught incubator at 37.5°C and 65% relative humidity. Chick embryo liver microsomes were prepared from 20-day-old embryos or 1-day-old chicks essentially as described in a previous report (13). Rat liver microsomes were prepared from Sprague-Dawley rats kept under a feeding regimen designed to increase the desaturase content (11, 14). Microsomal pellets could be stored under N_2 at −70°C for several weeks without loss

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of activity. Before use, the pellets were suspended in 0.02 M Tris acetate, pH 8.1, to give a concentration of 20 mg protein per ml as determined by the method of Lowry et al. (15).

Purification of desaturase

The method of solubilizing and purifying the terminal desaturase from rat liver microsomes described by Strittmatter et al. was employed (11). The purification procedure was followed through to a step where the enzyme was free from the other components of the desaturase system and exhibited no desaturation activity when assayed alone, usually after elution from a DEAE–cellulose column (Fraction 7). This desaturase preparation contained 1.5% Triton X-100 (Rohm and Haas Co., Philadelphia, PA) and 0.15% sodium deoxycholate.

Assay of stearoyl CoA desaturase

The method of Jones et al. (12) was used to measure stearoyl CoA desaturation. Assays were run at 37°C in open tubes in a shaking water bath. The reaction mixture contained 10 nmoles of [1-^{14}C]-stearoyl CoA (New England Nuclear, Boston, MA, 5 μCi/μmole), 100 nmoles of NADH (P-L Biochemicals, Milwaukee, WI), 30 μmoles of potassium phosphate pH 7.2, enzyme protein, chick embryo microsomes, 0.1% Triton X-100 (Rohm and Haas) and water to a final volume of 0.5 ml. In order to observe desaturation activity it was necessary for the desaturase enzyme to be preincubated with 400–500 μg of chick embryo liver microsomes at a final concentration of 1% Triton X-100. Routinely 20 μl of solubilized desaturase (12 μg protein from fraction 7) was preincubated with 30 μl of chick embryo liver microsomes (400 μg protein) in the presence of 2μl of 10% Triton X-100. The preincubation was for 5 min at 4°C; increasing the time or temperature did not result in any improvement. At the end of this time the remaining components of the reaction mixture were added and the final volume was made up to 0.45 ml with water.

The reaction was started by the addition of 0.05 ml of [1-^{14}C]stearoyl CoA (1 μCi/0.2 μmole per ml) and stopped after 10 min by adding 0.1 ml of 20% methanolic KOH. After addition of stearic acid (0.15 mg) and oleic acid (0.15 mg), the sample was saponified for 15 min in a boiling water bath. The reaction mixture was acidified with 0.1 ml of 4 M H_2SO_4, and the fatty acids were extracted with 3 × 3 ml of redistilled pentane. The combined pentane extracts were taken to dryness in a stream of nitrogen and the residue was methylated by addition of excess ethereal solution of diazomethane. After 5 min, the residual diazomethane was removed in a stream of nitrogen. An aliquot of fatty acid methyl esters in distilled chloroform was resolved into saturated (R_f 0.65) and *cis*-monounsaturated (R_f 0.32) fatty acid esters by chromatography on a $AgNO_3$-impregnated silica gel HR (E. Merck, Darmstadt, Germany) thin layer plate (12). The silica gel from the areas corresponding to the saturated and monounsaturated esters was scraped from the thin-layer plate into counting vials containing toluene-based scintillation fluid and the radioactivity was measured in a Nuclear Chicago (Des Plaines, IL) liquid scintillation spectrometer. The amount of desaturation was calculated from the ratio of the radioactivity present in the *cis*-monounsaturated area to the total radioactivity recovered from both the saturated and the *cis*-monounsaturated areas on the thin-layer plate. A tube containing the same reaction mixture, except for soluble desaturase and microsomal protein, was used as a control.

Identification of the product of desaturation

The product of [1-^{14}C]stearoyl CoA desaturation catalyzed by the desaturase–embryo microsome complex was shown to be oleic acid by the following tests. The monoene separated by thin-layer chromatography was analyzed by radio–gas–liquid chromatography using a Varian 2700 gas chromatograph (Varian Assoc., Palo Alto, CA) equipped with a Nuclear Chicago 4998 flow counting system, and methyl octadecenoate was identified as the only radioactive component. An aliquot of the monene was oxidized with a mixture of periodate and permanganate according to the method of Scheurbrandt and Bloch (16). Analysis of the products of oxidative cleavage after methylation revealed a single radioactive component that cochromatographed with the methyl ester of authentic azelaic acid (Calbiochem, San Diego, CA). This finding indicated that the double bond was in the 9,10 position and established that oleic acid was the product of the desaturation reaction.

RESULTS AND DISCUSSION

It has been shown that excess solubilized cytochrome b_5 and cytochrome b_5 reductase can be incorporated into the microsomal membrane (17, 18), can accept electrons, and participate in catalysis. We tested whether the solubilized terminal enzyme of the stearoyl CoA desaturase system could interact similarly with chick embryo liver microsomes to form a catalytically active complex. As shown in **Fig. 1** the detergent concentrations required in the preincubation and during the reaction assay for activity are quite distinct and exhibit sharp optima, 1% for preincuba-

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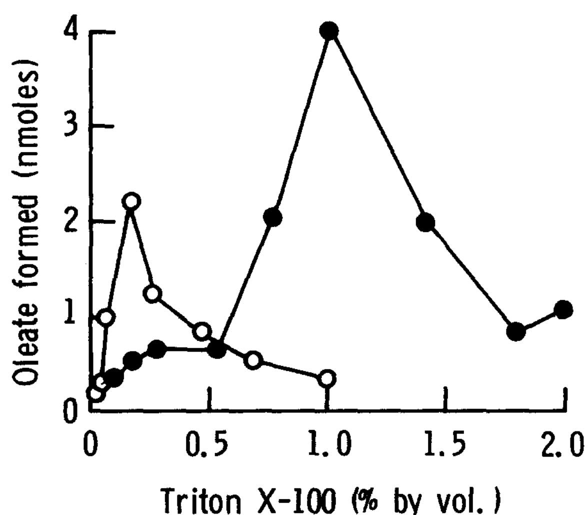

Fig. 1. Effect of Triton X-100 on stearoyl CoA desaturation activity by the isolated desaturase plus chick embryo liver microsomes. The desaturase activity was determined by the assay described in Experimental Procedure except that the detergent concentrations were varied as indicated. ●, Triton X-100 concentration present during preincubation; the final concentration of Triton X-100 in the assay was 0.06%. ○, Triton X-100 concentration present during assay; the preincubation mixture contained 0.6% Triton X-100.

tion and 0.1–0.15% for the reaction. These conditions are easily met by first performing the preincubation in a volume of 50 μl and then diluting with the assay components to a final 0.5 ml volume.

Having established the detergent concentration conditions, we investigated the dependence of the method on microsomal protein and desaturase protein. Desaturation activity is absent without chick microsomes (**Fig. 2A**) and increases as increasing amounts of microsomes are added to a constant amount of desaturase protein. A maximum is reached at between 400 and 500 μg of microsomal protein; the subsequent decrease in desaturation activity may be due to destruction of the substrate stearoyl CoA by acyl-CoA hydrolases in the chick microsomes. Likewise, desaturation activity is absent without the desaturase enzyme (Fig. 2B). Oleate formation increases linearly with added protein up to 15 μg, after which further addition of desaturase enzyme gives no increase in activity. This result suggests that, under the conditions of protein and detergent concentration in the preincubation, there is a limit to the catalytically active interactions that can be formed between the desaturase enzyme and the chick embryo microsomes. Therefore, activity determinations should be made at more than one level of desaturase protein to ensure proportionality. The amount of [^{14}C]oleate formed was linear with time for at least 10 min and then continued to increase more slowly up to 20 min at the protein concentration used in the standard assay (**Fig. 3**). No lag phase was observed.

Properties of reconstituted system

The stearoyl CoA desaturase system reconstituted by the interaction of chick embryo liver microsomes and the solubilized desaturase enzyme had the same general properties and cofactor requirements as hen liver microsomes (12). The reconstituted desaturating system required both oxygen and a reduced pyridine nucleotide, as shown in **Table 1**. NADH was a better electron donor than NADPH, an observation similar to that reported for hen liver microsomes (12).

The desaturase activity of the reconstituted system was inhibited by KCN (1×10^{-3} M) to a greater extent than that reported for hen liver microsomes

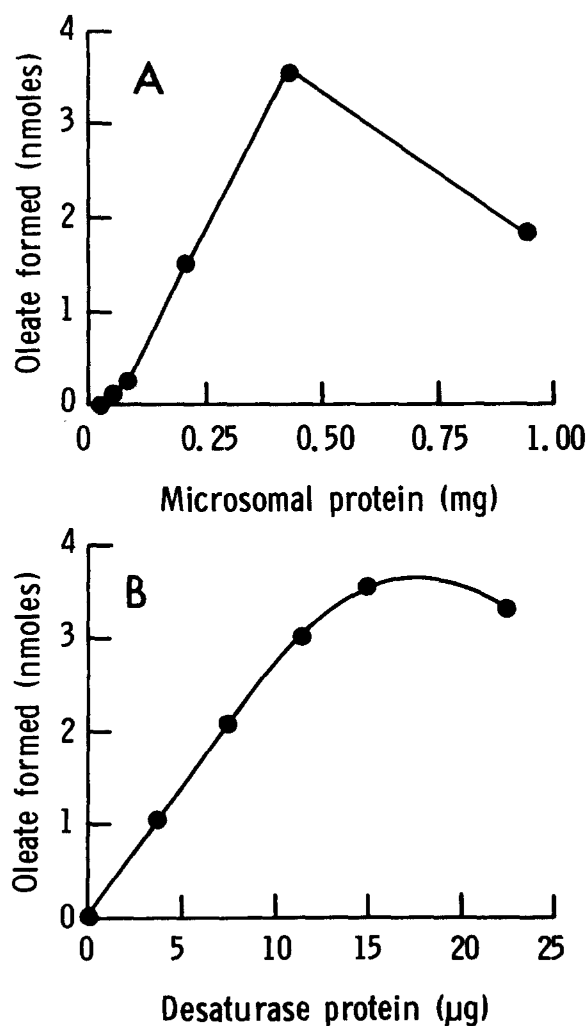

Fig. 2. Effect of increasing protein concentration on the reconstituted stearoyl CoA desaturation activity of the isolated desaturase plus hepatic microsomes from 1-day-old chicks. Desaturation activity was assayed as described in Experimental Procedure except that in A, the microsomal protein, and in B, the desaturase protein concentrations were as indicated.

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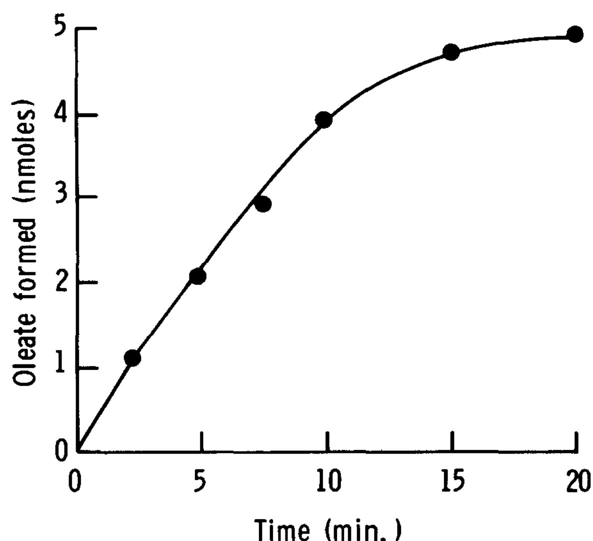

Fig. 3. Effect of increasing time of incubation on the stearoyl CoA desaturation by the reconstituted system. The desaturation activity of the isolated desaturase plus chick embryo liver microsomes was determined as in Experimental Procedure.

(12). It has been reported that stearoyl CoA desaturase activity in rat liver microsomes is more sensitive to cyanide inhibition (20) than that in hen liver microsomes. It thus seems likely that, since the desaturase enzyme used in this assay was solubilized from rat liver microsomes, the cyanide sensitivity is determined by the terminal enzyme of the desaturase system.

The desaturation activity of the reconstituted system is absent if the solubilized desaturase is boiled before preincubation (Table 1). In addition, if either the desaturase or the microsomes are omitted from the preincubation and then added later to the complete reaction mixture, desaturation activity is not reconstituted. These observations suggest that optimum concentrations of desaturase, detergent, and microsomes are required in the preincubation for reconstitution of the activity.

TABLE 1. Requirements and inhibitors of reconstituted stearoyl CoA desaturase activity

Assay Conditions	Formation of [1-^{14}C] oleate
	nmoles
Complete	3.96
$-O_2 + N_2$	0.27
−NADH	0.16
−NADH + 100 nmoles NADPH	1.70
+KCN (1×10^{-3} M)	0.21
Boiled desaturase	0.17
Desaturase added last	0.12
Microsomes added last	0.16

The complete system contained 0.4 mg of chick embryo liver microsomal protein, which was preincubated for 5 min at 0°C prior to the addition of the other components of the complete system, with 15 μg of solubilized desaturase protein in the presence of 1% Triton X-100. The reaction conditions and assay components are described under Experimental Procedure.

The desaturase activity of the reconstituted system in the presence of varying amounts of the substrate, stearoyl CoA, is shown in **Fig. 4.** Because the local concentrations of the substrate in the reaction mixture may be affected by binding to the membrane and formation of micelles, it is difficult to estimate a Michaelis–Menten constant. Nonetheless, it is possible to state that the substrate curve is generally similar to those reported for desaturase systems in intact microsomes (4, 12).

The product of the desaturation of [1-^{14}C]stearoyl CoA catalyzed by the solubilized desaturase enzyme in the presence of chick embryo liver microsomes was shown to be *cis*-monounsaturated fatty acid by its behavior on thin-layer and gas–liquid chromatography. By oxidative cleavage and analysis of the cleavage products, the desaturation product was proved to be oleic acid, since the double bond was in the 9,10 position (See Experimental Procedure). The product of stearoyl CoA desaturation by the system reconstituted with solubilized desaturase from adult chicken liver has also been shown to be oleic acid (13).

The sensitivity of the desaturase system to inhibition by ionic detergent has been reported (9, 10, 19), and we investigated this effect in our system. As shown in **Fig. 5**, sodium deoxycholate at a concentration of 0.5% in the pre-incubation mixture caused

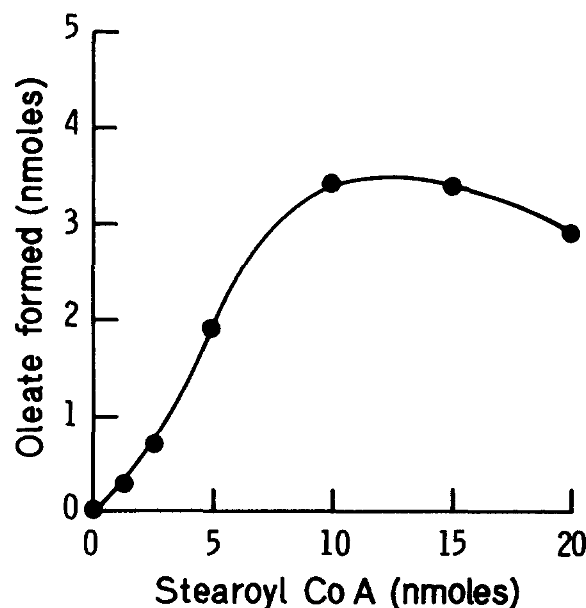

Fig. 4. Effect of increasing substrate concentration on the reconstituted stetaroyl CoA desaturase system. The stearoyl CoA desaturation activity was determined as in Experimental Procedure except that stearoyl CoA was present at the indicated levels.

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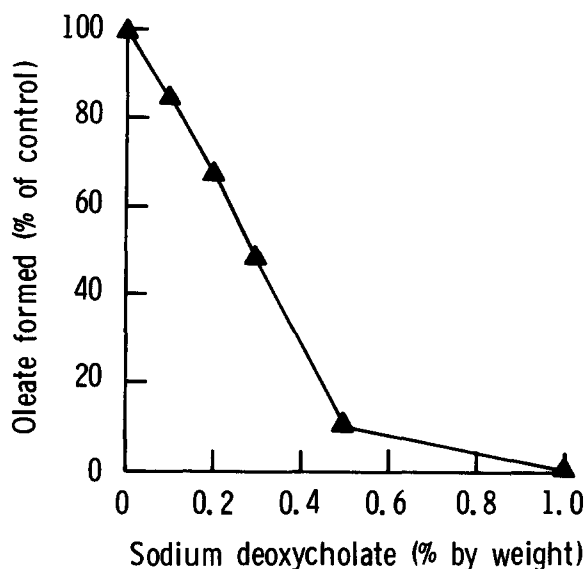

Fig. 5. Effect of increasing concentration of sodium deoxycholate on the reconstituted stetaroyl CoA desaturation activity. Incubations were carried out as described in Experimental Procedure except that sodium deoxycholate was present in the preincubation mixture at the indicated concentrations. The control assay, which did not contain deoxycholate in the preincubation mixture, gave a value of 3.9 nmoles of oleate formed.

a 90% inhibition of the activity. At lower concentrations, however, such as would result from assaying activity in column eluates containing 0.2% sodium deoxycholate, more than 70% of the activity remained.

The method described here for the assay of the terminal desaturase activity clearly offers considerable savings in time and effort. An important advantage of the use of chick embryo liver microsomes is that only three components are required for the preincubation: the microsomes, the desaturase, and detergent. In the existing assay (11), the order and timing of addition of the solubilized cytochrome b_5 reductase, cytochrome b_5, liposomes, and detergent to the desaturase create unavoidable and unwelcome variables. Much time and attention must also be devoted to the preparation of the solubilized components and liposome solutions. The embryo microsomes assay permits the routine monitoring of column eluates during the purification of the desaturase while the solubilized components can be reserved for use in detailed studies of mechanism, kinetics, and protein–protein or protein–lipid interactions.

This investigation was supported in part by grants HD 07516, GM 19091, and HL 17269 from National Institutes of Health.

Manuscript received 14 May 1976 and accepted 16 September 1976.

REFERENCES

1. Marsh, J. B., and A. T. James. 1962. The conversion of stearic acid to oleic acid by liver and yeast preparations. *Biochim. Biophys. Acta.* **60:** 320–328.
2. Holloway, P. W., R. Peluffo, and S. J. Wakil. 1963. On the biosynthesis of dienoic fatty acid by animal tissues. *Biochem. Biophys. Res. Commun.* **12:** 300–304.
3. Gellhorn, A., and A. Benjamin. 1964. The intracellular localization of an enzymatic defect of lipid metabolism in diabetic rats. *Biochim. Biophys. Acta.* **84:** 167–175.
4. Oshino, N., Y. Imai, and R. Sato. 1966. Electron-transfer mechanism associated with fatty acid desaturation catalyzed by liver microsomes. *Biochim. Biophys. Acta.* **128:** 13–28.
5. Holloway, P. W., and S. J. Wakil. 1970. Requirement for reduced diphosphopyridine nucleotide–cytochrome b_5 reductase in stearyl Coenzyme A desaturation. *J. Biol. Chem.* **245:** 1862–1865.
6. Oshino, N., Y. Imai, and R. Sato. 1971. A function of cytochrome b_5 in fatty acid desaturation by rat liver microsomes. *J. Biochem. (Tokyo).* **69:** 155–167.
7. Oshino, N., and R. Sato. 1971. Stimulation by phenols of the reoxidation of microsomal bound cytochrome b_5 and its implication to fatty acid desaturation. *J. Biochem. (Tokyo).* **69:** 169–180.
8. Holloway, P. W. 1971. A requirement for three protein components in microsomal stearyl coenzyme A desaturation. *Biochemistry.* **10:** 1556–1560.
9. Holloway, P. W., and J. T. Katz. 1972. A requirement for cytochrome b_5 in microsomal stearyl coenzyme A desaturation. *Biochemistry.* **11:** 3689–3696.
10. Shimakata, T., K. Mihara, and R. Sato. 1972. Reconstitution of hepatic microsomal stearoyl coenzyme A desaturase system from solubilized components. *J. Biochem. (Tokyo).* **72:** 1163–1174.
11. Strittmatter, P., L. Spatz, D. Corcoran, M. J. Rogers, B. Setlow, and R. Redline. 1974. Purification and properties of rat liver microsomal stearyl coenzyme A desaturase. *Proc. Nat. Acad. Sci. USA.* **71:** 4565–4569.
12. Jones, P. D., P. W. Holloway, R. O. Peluffo, and S. J. Wakil. 1969. A requirement for lipids by the microsomal stearyl coenzyme A desaturase. *J. Biol. Chem.* **244:** 744–754.
13. Wilson, A. C., S. J. Wakil, and V. C. Joshi. 1976. Induction of microsomal stearyl coenzyme A desaturase in newly hatched chicks. *Arch. Biochem. Biophys.* **173:** 154–161.
14. Oshino, N., and R. Sato. 1972. The dietary control of the microsomal stearyl coenzyme A desaturation enzyme in rat liver. *Arch. Biochem. Biophys.* **149:** 369–377.
15. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
16. Scheubrandt, G., and K. Bloch. 1962. Unsaturated fatty acids in microorganisms. *J. Biol. Chem.* **237:** 2064–2068.
17. Strittmatter, P., M. J. Rogers, and L. Spatz. 1972. The binding of cytochrome b_5 to liver microsomes. *J. Biol. Chem.* **247:** 7188–7194.
18. Rogers, M. J., and P. Strittmatter. 1974. The binding of reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase to hepatic microsomes. *J. Biol. Chem.* **249:** 5565–5569.
19. Safford, R., R. Jeffcoat, and A. T. James. 1975. Factors effecting the solubilization of stearyl-CoA desaturse of hen liver microsomes. *Biochim. Biophys. Acta.* **409:** 86–96.
20. Hiwatashi, A., Y. Ichikawa, and T. Yamano. 1975. Effect of cyanide on stearyl coenzyme A desaturase activities in microsomes from various mammalian tissues. *Biochim. Biophys. Acta.* **388:** 397–401.

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