Desaturation of Saturated Fatty Acids by Rat Liver Microsomes

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ABSTRACT

A method was developed for the rapid determination of the initial velocity of the desaturation of saturated fatty acids. In the reaction, DPNH was a more efficient electron donor than TPNH. Fat-deficient rats have a 2.5-fold greater level of acyl desaturase per milligram of liver microsomal protein than did animals fed lab chow. Increasing the chain length of the acyl substrate from 10:0 to 18:0 increases the rate of monoene formation, but 19:0 is desaturated at a rate lower than that for 15:0. The energy of activation (Ea) for the overall desaturation reaction has been determined for 12:0 through 19:0. The Ea values for desaturation of 13:0 and 16:0 are markedly lower than for the other acids. An interaction between the alkyl chain of the substrate and polyunsaturated acids of the microsomal membrane-bound phospholipids is postulated to explain the recurring 3-carbon pattern of the relative reaction rates of the various acyl substrates.

INTRODUCTION

Studies of acyl desaturase in normal rat liver microsomes have focused on the desaturation of stearate and stearyl CoA. Oshino et al. (1) and Jones et al. (2) have reported methods for the study of the initial velocity of desaturase in liver microsomal systems using stearyl CoA. Other workers have investigated the desaturation of free stearate (3,4) and the saturated series 12:0-20:0 (5), apparently at equilibrium. Conclusions concerning the specificity and specific activity of acyl desaturase from non-first order reaction conditions (3-5) are open to question. Incorporation of substrate into lipids lowers the concentration of available substrate when low concentrations such as 10 to 20 μmolar are employed. Inhibition of the substrate desaturation by endogenous microsomal fatty acids has been demonstrated (4). Dilution of substrate by endogenous fatty acids of the same type would lead to false values. These problems can be best corrected by selecting a system in which the quantity of microsomes is low so as to provide a minimal contribution of endogenous fatty acid. The substrate concentration should be high enough to saturate the enzyme system, and the reaction rate should be proportional to protein concentration.

For this reason we developed an assay system for the measurement of the initial velocities of acyl desaturase from rat liver microsomes. The assay is linear with respect to time, directly proportional to protein concentration, and independent of substrate concentration over a threefold range above the point of saturation of the enzyme by substrate. Using this method, specific activities, substrate specificities, Arrhenius plots, and the energies of activation of the acyl desaturase reaction were determined. A novel hypothesis to explain the relative rates of desaturation of 10:0 through 18:0 is proposed.

Chemicals

The carboxyl-labeled acids, 12:0, 14:0, 16:0 and 18:0 were purchased from New England Nuclear Corp., Boston. The acids 15:0, 17:0 and 19:0, were synthesized in this laboratory by the method of Baumann and Mangold (6). Final purification of these odd-chain fatty acids was carried out according to the preparative GLC procedure of Schlenk and Sand (7). The acids 11:0 and 13:0, were purchased from Mallinckrodt Nuclear, St. Louis. The radio and chemical purity of all acids was found to be >98% by thin layer (TLC) and gas liquid chromatography (GLC). Nonlabeled fatty acids used as diluents and starting materials for syntheses were purchased from the Lipids Preparation Laboratory of The Hormel Institute, Austin, Minn. DPNH, TPNH, ATP and CoA were purchased from both Sigma Chemical Co., St. Louis, and P & L Biochemicals, Milwaukee, Wis. All other chemicals were of reagent grade quality and all solvents were redistilled.

Preparation of Microsomes

Male albino rats were maintained on either commercial lab chow or a synthetic diet containing no fat (8). The rats were killed under ether anesthesia by exsanguination; the livers were removed and placed in an ice cold solution of 250 mM sucrose and 5 mM $MgCl_2$. After weighing, the livers were minced and gently homogenized in 2 vol of sucrose-$MgCl_2$ (w/v).

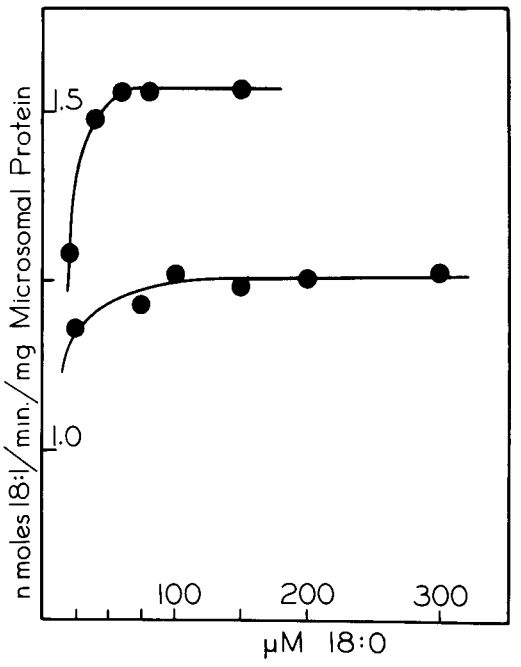


FIG. 1. The relationship of initial velocity to substrate concentration. The assay was performed at 40 C using two different microsomal preparations obtained from two EFA-deficient rat livers.

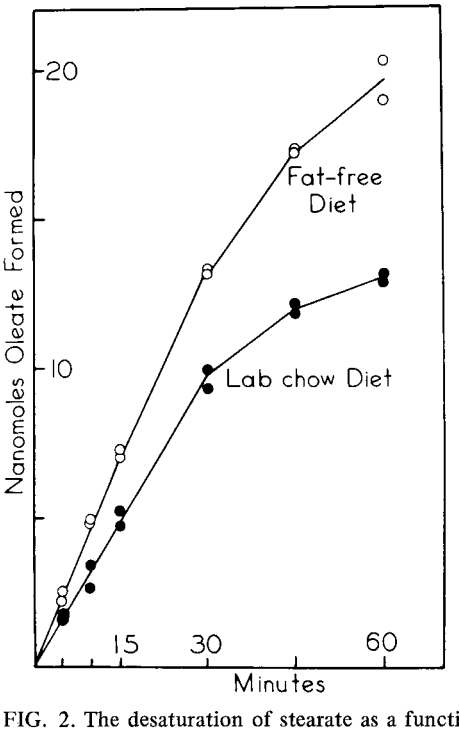


FIG. 2. The desaturation of stearate as a function of time by microsomes from rats fed fat-free diet and lab chow diet.

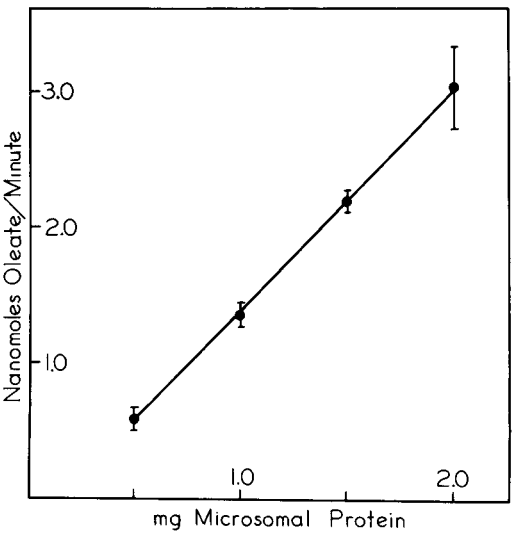


FIG. 3. The desaturation of stearate as a function of microsomal protein concentration. Microsomes were obtained from EFA-deficient rat livers. The bars represent the range of four determinations.

The homogenate was centrifuged for 30 min at 15,000 x *g* at 0 C. The supernatant fluid was decanted into another set of 50 ml tubes and centrifuged at 50,000 x *g* for 2 hr. The resulting microsomal pellet was suspended in sucrose-$MgCl_2$ solution in a volume equivalent to the initial liver weight. Aliquots of the microsomal suspension were placed in screw cap tubes, flushed with N_2, capped and quick-frozen in dry ice-acetone. The preparations were stored at -20 C and were found to be stable for at least one month. Neither the supernatant nor the pellet of material sedimenting between 50,000 and 100,000 x *g* contained any desaturase activity according to the assay described below. Repetitive freezing and thawing, up to four times, had no significant effect on the enzymatic activity.

Enzyme Assay

The stored microsomes were thawed in an ice cold water bath as needed. Protein levels were measured by the biuret procedure (9). Appropriate dilutions of the microsomes were made with sucrose-$MgCl_2$ to yield 2.5 mg/ml of which 0.4 ml was used for each assay. In a total volume of 1 ml, the final incubation mixture contained 1.0 mg microsomal protein, 2.5 mM $MgCl_2$, 5 mM ATP, 0.25 mM CoA, 1 mM DPNH, 0.1 M sodium phosphate, 0.125 M sucrose, and 0.15 mM 1-^{14}C-labeled fatty acid (1.0 mc/mmole) prepared as previously described (10) at a pH of 7.0. The incubations were performed in 5 ml test tubes in a thermo-

TABLE I

Specificity Toward Reduced Pyridine Nucleotide

DPNH[a]	TPNH[a,b]
1.12	0.78
1.15	0.79
1.04	0.77
0.98	0.73
1.07[c] ± 0.077[d]	0.77 ± 0.026

[a]Nanomoles of oleate/min/mg microsomal protein.

[b]TPNH was added in the same molar quantity as DPNH.

[c]The means are significantly different ($p < 0.05$).

[d]Mean ± S.D. of four determinations from pooled liver microsomes from four EFA-deficient rats.

TABLE II

Desaturase Activity

Fat free diet[a]	Lab chow diet[a]
1.69	0.37
1.04	0.28
0.89	0.44
0.90	0.72
1.51	0.20
0.84	0.40
0.78	
1.09[b] ± 0.33[c]	0.40 ± 0.16

[a]Nanomoles of oleate/min/mg microsomal protein. Each value is the average of triplicate determinations from a single rat liver microsomal preparation.

[b]Means are significantly different ($p < 0.01$).

[c]Mean ± S.D.

regulated shaking water bath. The temperature of the bath was 40 C unless otherwise indicated since this was found to be very near the optimum for all the acids of the series. The reaction was terminated by the addition of 1.0 ml of 5% methanolic HCl. One half milligram of an equal mixture of 16:0 and 16:1 was added to serve as carrier. The total lipids were extracted by the method of Bligh and Dyer (11). The lipid extract was dried under a stream of N_2 with slight warming and then dissolved in 0.1 ml of $CHCl_3$-methanol (2:1 v/v) and esterified according to the method of Glass and Christopherson (12). An aliquot of the methyl esters in $CHCl_3$ was separated on glass fiber sheets impregnated with silicic acid (ITLC Type SA, Gelman Instrument Co., Ann Arbor) previously dipped in a 1% solution of $AgNO_3$ in methanol according to the method of Graff et al. (13). With the aid of 2,7-dichlorofluorescein, the bands of the saturated and monounsaturated esters were made visible, marked, cut from the paper and placed in scintillation vials. The vials were filled with 15 ml of a toluene-based scintillation solution containing 5.5 g of Permablend III (Packard Instruments Co., Downers Grove, Ill.) per liter. The radioactivity was determined in a Packard liquid scintillation spectrometer. The values for desaturation were first calculated as percentages and then converted to nmoles/min/mg of protein by use of appropriate factors.

RESULTS AND DISCUSSION

The assay described above is coupled to acyl activation of the free acid to the CoA derivative. It was necessary, therefore, to demonstrate that desaturation rather than acyl activation was the rate limiting step. In our system, we found the value for acyl desaturation by normal rat liver microsomes to be 0.40 nmoles/min/mg which compares favorably with the value of 0.35-0.40 nmoles/min/mg for the desaturation of stearyl CoA (1). This indicates that the CoA derivative was generated in situ at a rate sufficiently rapid not to be the limiting factor. Indeed, the data of Pande and Mead (14) and of Graff and Holman (unpublished results) for the activation of 10:0, 12:0, 14:0, 16:0 and 18:0 to the CoA derivative suggest that the rate of acyl activation is 10 to 80 times that of desaturation. Nakagawa and Uchiyama (15) found the rates of acyl CoA formation from 16:0 and 18:0 to be nearly identical and therefore not responsible for the difference in the rates of desaturation of the two acids. From these data we conclude that acyl desaturation, not activation, is the rate limiting step in our assay, and we assume that the same holds true for the odd-chain length fatty acids tested.

Figure 1 reveals the relationship of initial velocity to substrate concentration. The rate is independent of substrate concentration from 100 to 300 μM 18:0. With minor variation this was found to be true for the other acids. The assays of initial rates at low substrate concentrations were not studied because of an unknown and uncontrollable level of endogenous free fatty acids. If the concentration of endogenous fatty acids were large in comparison to the added substrate, Michaelis constants derived from 1/[V] vs. 1/[S] would give anomalous results.

Figure 2 demonstrates the linearity of the assay of desaturation of 18:0 to 18:1 by microsomes from rats fed fat-free and lab chow diets. Although the rates were linear for all acids for 30 min, the data presented in the balance of this work were obtained from incubation times

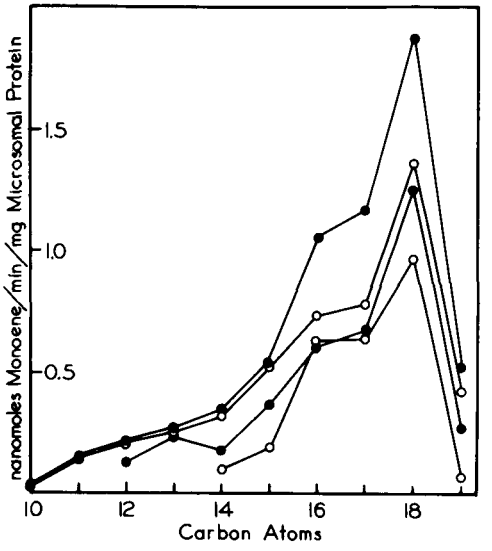


FIG. 4. The substrate specificity of desaturation as measured at 40 C in four separate microsomal preparations.

of 15 min for acids of 14 to 19 carbon atoms. For the acids 10:0 to 13:0, the reaction was linear to at least 1 hr. Longer incubation times were used when studying these acids in order to have sufficient monoene to be accurately determined. For the same reason, i.e., higher monoene production, EFA deficient rats were used for the balance of the work reported in this paper.

The rate of desaturation of 18:0 is directly proportional to the amount of added microsomal protein over the range of 0.5 to 2.0 mg shown in Figure 3. These data, together with those in Figures 1 and 2, clearly show that the assay described here gives a true measure of initial velocity under pseudo first order reaction conditions.

Oshino et al. (1) found DPNH to be more efficient than TPNH as a hydrogen (electron) donor for acyl desaturation. Table I shows that the same is true in our assay system.

The influence of diet on the level of acyl desaturase in liver microsomes is shown in Figure 2 and Table II. The maintenance of rats on a fat-free diet results in a significant increase in this enzymatic activity compared with normals maintained on lab chow. The activity does not vary significantly with the length of time on the fat-free diet from seven days to 18 months. Starvation of the fat deficient rats for 24 hr reduced the level of acyl desaturase below that of the controls. This is in agreement with the data of Uchiyama et al. (4).

Figure 4 presents the findings for the

TABLE III

Energies of Activation for the Desaturation of 12:0 Through 19:0

Fatty Acid	Kcal/mole
12:0	18.8
13:0	15.5
14:0	19.2
15:0	20.8
16:0	16.3
17:0	19.8
18:0	21.8
19:0	24.1

specificity of acyl desaturase toward the saturated fatty acid series, 10:0 to 19:0. Each of the curves represents a different microsomal preparation obtained from the livers of EFA-deficient rats. It is clear that desaturase activity increases with increasing carbon number from 10:0 through 18:0. Desaturation of 19:0 occurs to a lesser extent than 15:0 in all cases. Possibly 19:0 is too long for the enzymatic site. Of the acids tested, 18:0 is clearly the best substrate for desaturation. This is consistent with the fact that 18:1ω9 is the predominant monoene in the rat. In fact, the mass per cent of 18:1 exceeds that of 18:0 in triglycerides and phospholipids. This is not the case for 16:1/16:0 (16). The results for desaturation of 18:0 and 16:0 are in disagreement with the data of Nakagawa and Uchiyama (15). The difference in results may be due to the non-first order assay conditions which they used. Their substrate concentration (12 μM) is well below the optimum for our system (>100 μM). The system described by Johnson et al. (5) used hen liver as the source of microsomes. In their system, the desaturation of 14:0 was greater than or nearly equal to that of 18:0 and greater than all other acids tested. Their system employed very little substrate and an undefined level of protein, and no evidence was given that their system was pseudo first order. It is not clear whether their data represents the rate of desaturation or the equilibrium attained for each acid. This uncertainty precludes making a decision as to whether their is a true phylogenetic difference in acyl desaturase specificity.

Rate studies performed as a function of temperature (28 to 40 C) were graphed in the form of Arrhenius plots ($\log_{10}$ velocity versus 1/T absolute). The slopes of the lines were determined by the formula of the least squares using 30 to 60 data points for each acid. Figure 5 presents the results of these determinations. According to the Arrhenius equation in which $\log_{10}$ has been substituted for ln, the product of the slope and 2.303 R when the value of R is

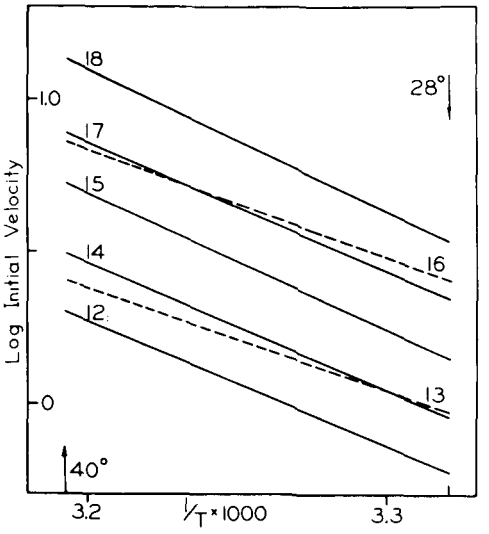


FIG. 5. An Arrhenius plot of the desaturation of saturated fatty acids of differing chain length. For the sake of clarity, the data points have been omitted. See text for details.

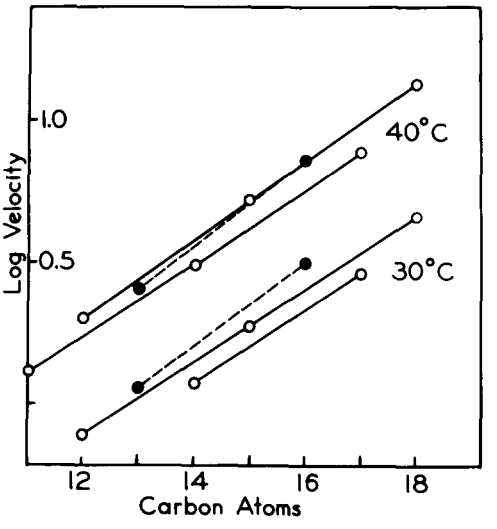


FIG. 6. The logarithm of velocity of desaturation versus substrate chain length as determined at two temperatures.

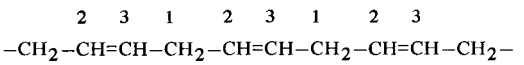
1.986 kcal/mole gives the energy of activation (Ea) for the overall reaction. Table III contains the results of these calculations. Despite the large differences in the rates of desaturation of the various acids, the energies of activation for the desaturase reaction are similar for 12:0, 14:0, 15:0, 17:0 and 18:0. The Ea values may be similar because the reaction produces Δ9 double bonds regardless of the acyl chain length (5). This implies that the rate of the overall reaction is dependent on some event other than the catalytic event leading to the formation of the double bond, assuming that the catalytic event has the highest energy of activation. The rate of desaturation may be controlled by the rate of substrate binding or of product release.

Fatty acids of chain length 13, 16 and 19 carbon atoms have greatly different Ea values. The Ea value for 19:0 → 19:1, which is much higher than that for other acids tested, is accompanied by a sharply lower rate of desaturation. On the other hand, the Ea values for the reactions 16:0 → 16:1 and 13:0 → 13:1 are lower than the other acids in the series but are not accompanied by sharply higher rates of desaturation. That is, the increments of one carbon atom beyond 12 do not yield simple proportional increments in Ea values. Chain lengths of 13:0, 16:0 and 19:0 seem to possess some unique characteristic in the reaction.

Figure 6 is a plot of the logarithm of velocity of desaturation versus the substrate carbon number for two different temperatures. For each temperature, the acids 12:0, 15:0 and 18:0 fall on a straight line; whereas, the values for 11:0, 14:0 and 17:0 form a line slightly below that of the former. As would be expected from their Ea values (Fig. 5 and Table III), the points for 13:0 and 16:0 shift their positions with respect to the other acids as a function of temperature. The same phenomenon was observed consistently for three preparations of microsomes from animals of different ages. These data suggest that the saturated fatty acids fall into three groups, and that the members of each group differ by three carbon atoms. The Arrhenius plots for desaturation of 13:0 and 16:0 likewise indicate that these acids are quite different from the other acids.

When the data of Abou-Issa and Cleland (17) for the acylation of α-glycerolphosphate by saturated acyl CoA thioesters are plotted as the logarithm of velocity versus carbon number of the substrate, a simple straight line relationship is found. This is in contrast to the 3-carbon rhythm of specificity in the desaturation of saturated acids. We speculate that this rhythm in reactivity shown by 11, 14, 17 vs. 12, 15, 18 vs. 13, 16 is the result of interaction of the alkyl chain with a binding site in a highly directed fashion, and that the binding site should itself possess a repeating 3 atom sequence. Binding an alkyl chain to the relatively polar peptide sequence which has a repeating C-C-N sequence, seems thermodynamically unlikely. Interaction of alkyl chains with nonpolar side chains of amino acids

is possible, but no 3 atom sequence is readily apparent. The desaturation of stearyl CoA is known to cease when the lipid components of the microsomes are partially removed, suggesting the role of lipid as a cofactor (2). Most of the microsomal lipid is phospholipid which contains a high proportion of polyunsaturated acids with a repeating 3 carbon unit:



The activated methylene groups (labeled 1) could explain a repeating 3 carbon rhythm of reactivities, if substrates were bound to a site containing one or more such systems. In contrast, the linear pattern in the reaction of saturated acyl CoA thioesters with α-glycerolphosphate suggests that in this case interaction of substrate is with saturated acyl groups in lipids of microsomes. Because microsomes carry such a large proportion of phospholipids, the association of the acyl CoA with the microsomal surface may be in the one case with the position 2 fatty acids, and in the other with the position 1 fatty acids of the phospholipids.

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