Circadian Rhythm of Fatty Acid Desaturation in Mouse Liver

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ABSTRACT

A study was made of the diurnal changes in liver microsomal desaturation of labeled stearic, linoleic and α-linolenic acids to oleic, γ-linolenic and octadeca-6,9,12,15-tetraenoic acids, respectively. C3H-S mice were used and were exposed to light-dark cycles. A circadian rhythm was observed for stearic acid desaturation, and a different one for linoleic acid. Linoleic and α-linolenic desaturation had similar responses in the day cycle. This would indicate that different mechanisms control the oxidative desaturations of the fatty acids in the 9 and 6 carbons. The fatty acid composition of the whole liver and liver microsomes also showed variations. Remarkable oscillations were observed for stearic and oleic acids. Neither the total protein synthesis nor the free fatty acid concentration in the microsomes followed a rhythm parallel to the desaturation of the studied fatty acids. The injection of cycloheximide 4 hr before measuring the desaturation modified the circadian variation of both the 9 and 6 desaturations. The modification induced by cycloheximide was considered to indicate that both variations are related to the synthesis of specific proteins but not to that of a degradative or inhibitory protein.

Fatty acid olefination is one of the fundamental reactions in the biosynthesis of unsaturated fatty acids in the animal and is produced by an oxidative desaturation. The substrate of the reaction is acyl-CoA. The enzymes that take part in it are linked to the endoplasmic reticulum and catalyze the formation of a double bond in saturated and unsaturated fatty acids in the presence of NADH and 0_2 (1). Apparently there are various desaturases, and the information gathered up to the present indicates that the same enzyme desaturates oleic, linoleic and α-linolenic acid, while stearic acid is desaturated by a different one (2). These enzymes are called, respectively, 6 desaturase and 9 desaturase, according to the position in which the new double bond is formed (3).

Several factors influence the oxidative desaturation, contributing to the regulation of the production of unsaturated fatty acids. (2).

In the present work, and in order to contribute to the clarification of the regulation mechanisms, studies were made on the circadian rhythm of the oxidative desaturation of stearic, linoleic and α-linolenic acids by mouse liver microsomes. At the same time, the daily variations of the microsomal free fatty acid (FFA) content, the composition of the fatty acids of whole liver and liver microsomes, the synthesis of liver proteins and the effect of inhibitors on the protein synthesis, were also investigated.

METHODS

Treatment of Animals

Female C3H-S mice of 6 weeks of age were used. They were maintained since birth at 25 C ± 1 C on water and Purina chow ad libitum, and exposed to illumination (fluorescent white light 40 W) from 06:00 to 18:00 hr alternating with 12 hr of darkness. The fatty acid composition of the diet was: 2.6% myristic acid; 19.4%

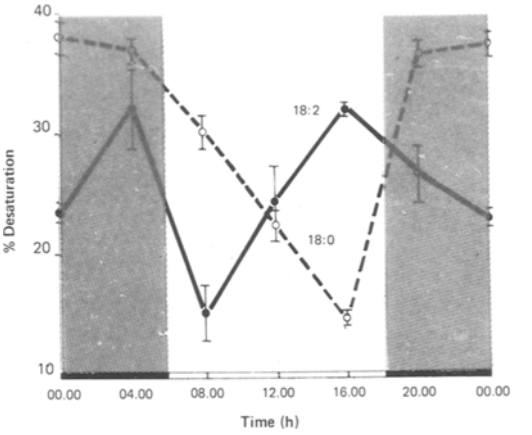

FIG. 1. Circadian changes of the desaturation of linoleic acid to γ-linolenic acid and stearic to oleic acid. Curve of 18:0/P between 00:00 hr vs. 16:00 hr <0.001; curve of 18:2/P between 04:00 hr vs. 08:00 hr $<0.005>0.001$; between 08:00 hr vs. 16:00 hr <0.001; between 16:00 hr vs. 00:00 hr <0.001.

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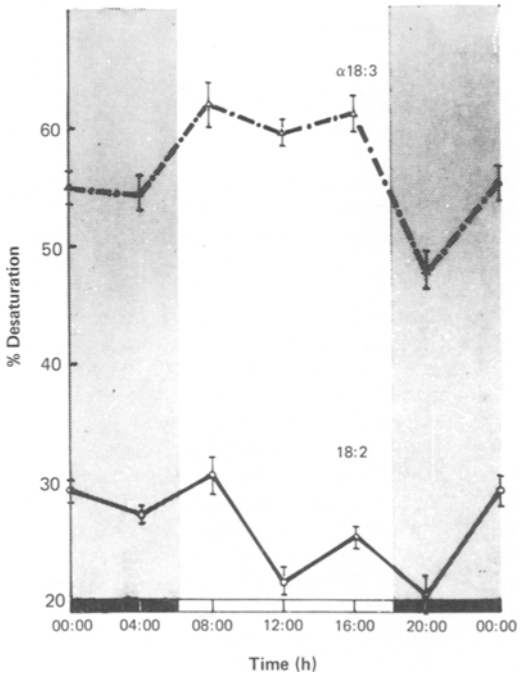

FIG. 2. Circadian change of linoleic and α-linolenic 6 desaturation. Curve of 18:2/P between 08:00 hr vs. 12:00 hr <0.001; between 00:00 hr vs. 20:00 hr <0.001; between 12:00 hr vs. 16:00 hr <0.001; between 16:00 hr vs. 20:00 hr $<0.005>0.001$; curve of α-18:3/P between 08:00 hr, 12:00 hr and 16:00 hr vs. 20:00 hr <0.001.

palmitic acid; 2.7% palmitoleic acid; 3.2% stearic acid; 26.7% oleic acid; 45.4% linoleic acid; and traces of α-linolenic acid.

All the experiments were performed according to a transversal design (4). The animals were killed at 4 hr intervals during a 24 hr period, and each experiment did not last more than a week. To measure the fatty acid-desaturating capacity of liver microsomes, four pools of two mice each were used each time. To study the microsomal FFA content, the composition of the fatty acids of whole liver and liver microsomes, and the incorporation of leucine-C^{14} in liver proteins, six animals were analyzed independently. When the effect of cycloheximide on the desaturation of fatty acids was studied, male mice of 4 months of age were used under the same conditions. Four pools of two normal animals each were killed at 4 hr intervals. Likewise, 4 similar pools were intraperitoneally injected 4 hr before being killed with 0.5 mg cycloheximide in physiological solution per 100 g of weight.

Determination of Per Cent Desaturation

Stearic, linoleic and α-linolenic acids, labeled with ^{14}C in the carboxyl carbon were used.

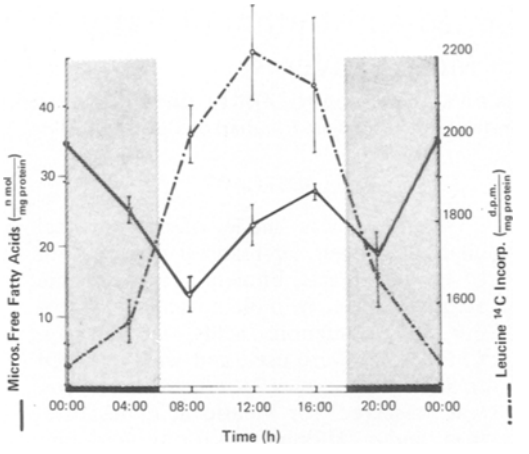

FIG. 3. Diurnal oscillations of leucine-1 ^{14}C incorporation in the liver proteins and FFA content of liver microsomes. Curve of leucine-1 ^{14}C incorporation: P between 00:00 hr vs. 12:00 hr <0.001. Curve of FFA content: P between 00:00 hr vs. 08:00 hr $<0.005>0.001$; between 08:00 hr vs. 16:00 hr <0.001.

They were purchased from The Radiochemical Centre, Amersham, England, and were 98% radiochemically pure. The acids were diluted with the same unlabeled acids (Hormel Institute, Austin, Minn.) to obtain a specific activity of 7.26 μC/μmol, 5.60 μC/μmol and 4.15 μC/μmol respectively.

After killing the animals, the livers were immediately excised and cooled at 0 C. They were homogenzied in the cold with a solution of 0.15 M KCl, 5 mM $MgCl_2$, 1.5 mM glutathione, 62 mM phosphate buffer (pH 7.0) and 0.25 M sucrose in 0.1 mM EDTA 3:1 v/w. The homogenate was centrifuged at 10,000 x g for 30 min, and the supernatant was recentrifuged at 110,000 x g for 1 hr. The pellet was resuspended in homogenizing solution (2:1 v/v) and proteins estimated by the buret method (5).

The desaturating capacity of the liver microsomes was determined by incubation of 10 nmol labeled acid at 35 C and pH 6.8 in air with 5 mg microsomal protein during 20 min. The incubation medium contained in μmol: ATP, 2.5; CoA, 0.2; NADH, 2.5; $MgCl_2$, 15: glutathione, 4.5; nicotinamide, 1; NaF, 125; phosphate buffer (pH 7.0), 125; in 0.15 M KCl and 0.25 M sucrose. The total volume was 3 ml. The 10 nmol acid were dissolved in 10 μl propyleneglycol. The incubation was stopped by the addition of 2 ml alcoholic 10% KOH. After saponification at 80 C for 45 min, the fatty acids were extracted and esterified with methanolic 3 N HCl for 3 hr at 63 C. The per cent of fatty acid desaturated was measured by

gas liquid radiochromatography in a Pye apparatus with proportional counter, using 10% diethylene-glycolsuccinate on Chromosorb W (80-100 mesh) at 180 C, as has been described previously (6). All determinations were performed in duplicate.

Incorporation of 1-^{14}C-Leucine in Liver Proteins

The animals received an intraperitoneal injection of 0.2 ml DL-leucine-1 ^{14}C in physiological solution (25 mC/mM) in a dosage of 10 μC/mouse 1 hr before being killed. Each of the excised livers was homogenized with 0.25 M sucrose 3:1 v/w. In an aliquot of the homogenate, proteins were precipitated with 10% trichloroacetic acid v/v. The precipitate was washed twice with 5% trichloroacetic acid v/v and then twice with 96% ethanol. It was stored overnight at 37 C and then dissolved in 1 N KOH. The concentration of proteins was determined by the buret method (5). Radioactivity was measured in a Packard scintillation counter. The results were expressed in dpm/mg protein.

Evaluation of FFA in Liver Microsomes

The livers were homogenized in 0.25 M sucrose 3:1 v/w. The microsomes were separated by differential centrifugation by the method already described. Each one of the pellets was resuspended in 2 ml 0.25 M sucrose, and the FFA were estimated in aliquots of 400 μl by the method of Dole and Meinertz (7). Then 2.5 ml of the Dole mixture (isopropanol-hexane-1 N H_2SO_4 4:1:0.1 v/v/v) was added, and after shaking, 1.5 ml hexane and 1 ml H_2O were added. After shaking and decanting, the upper phase was separated and an aliquot of it was titrated with 0.1 N NaOH. The results are expressed in nmol/mg protein.

Composition of Fatty Acids of Whole Liver and Liver Microsomes

Both 50 mg of the whole liver and 50 mg of microsomes of the previous experiment were separated and saponified in 10% KOH at 80 C during 45 min under N_2. After acidification, the total fatty acids were extracted with petroleum ether. After evaporation to dryness, they were esterified with methanolic 3N HCl during 3 hr at 63 C. The methyl esters were analyzed by gas liquid chromatography on diethylene-glycol-succinate on Chromosorb W (80-100 mesh) at 174 C, in a Pye chromatograph with an argon ionization detector.

Statistical Analysis

In all the figures the results are expressed as the mean ± standard error. The analysis of statistical significance of the results was made by Student's *t* test.

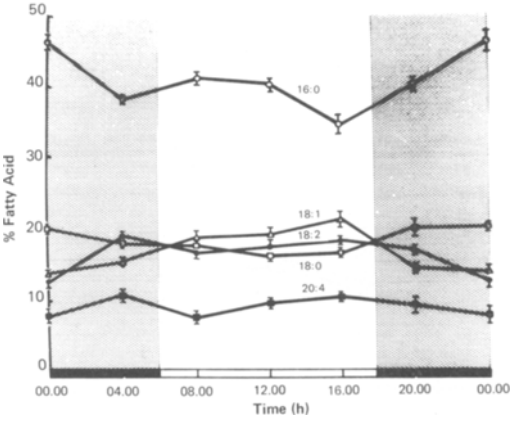

FIG. 4. Circadian changes of the fatty acid composition of the whole liver. Curve of 16:0/P between 00:00 hr vs. 16:00 hr <0.001. Curve of 18:0/P between 00:00 hr vs. 12:00 hr <0.001. Curve of 18:1 : P between 00:00 hr vs. 16:00 hr <0.001. Curve of 18:2/P between 00:00 hr vs. 04:00 hr <0.001. Curve of 20:4/P between 04:00 hr vs. 08:00 hr <0.01.

RESULTS

The changes in the microsomal desaturation of stearic to oleic acid and of linoleic to γ-linolenic acid in the course of the day are shown in Figure 1. In this figure the dark and light are indicated. As has been demonstrated repeatedly (8), the period of activity of mice is at night and they feed preferentially during this period, resting during the day. It can be observed that both studied acids undergo circadian rhythms in the desaturation. The stearic acid curve exhibits its maximal peak (38% desaturation) in the dark period (between 20:00 and 04:00 hr) and the minimum (15.5% desaturation) at the end of the light period. The linoleic acid curve, on the other hand, shows two maxima with 32% desaturation at different times than the stearic (at 04:00 and 16:00 hr) and minima at 08:00 and 00:00 hr with 15% and 23% desaturation, respectively.

The following experiment was to study simultaneously the diurnal changes in the desaturation of linoleic and α-linolenic acids. Figure 2 illustrates that the desaturation of α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid also undergoes circadian oscillations. α-Linolenic acid showed a higher desaturation than linoleic acid in the whole day cycle. However both cycles were rather similar. While the first experiment was done in winter, this experiment was done in summer. Therefore, although a general pattern of the rhythm of linoleic acid desaturation was similar to that shown in Figure 1, there was a displacement in the position of the maxima and minima of the

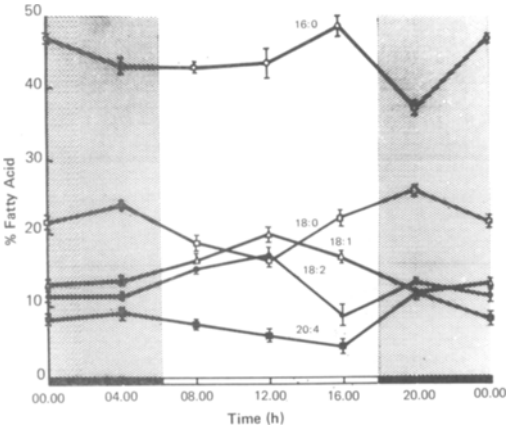

FIG. 5. Circadian changes of the fatty acid composition of liver microsomes. Curve of 16:0/P between 16:00 hr vs. 20:00 hr <0.001; curve of 18:0/P between 12:00 vs. 20:00 hr <0.001. Curve of 18:1/P between 00:00 hr vs. 12:00 hr <0.001; curve of 18:2/P between 12:00 hr vs. 16:00 hr $<0.010>0.005$; curve of 20:4/P between 16:00 hr vs. 20:00 hr <0.001.

curve. The existence of seasonal influences on circadian rhythms has already been described by other authors (9,10).

There may be several causes of a circadian rhythm in fatty acid desaturation that can be related to variations of enzymatic acitivy or changes in the microsomal composition, or both. Several experiments were performed to investigate this possible mechanism.

Figure 3 shows the incorporation of leucine-1-^{14}C in liver proteins in the course of the day. It may be considered that the curve presents the variation of the synthesis of liver total proteins. The incorporation of the radioactive amino acid changes according to the hour of the day in which the experiment is performed, showing a clear circadian rhythm. The maximal values are reached between 08:00 and 16:00 hr, during the light period that corresponds to the resting time of the animals. The minimum is at 00:00, during the period of active feeding. These results agree with those found by Rebolledo and Gagliardino (9) in muscle of mice of the same strain and age, but does not correlate with the curves of fatty acid desaturation.

Figure 3 also exhibits the changes of the microsomal FFA content in the course of the day. The FFA content increases during the dark period, reaching its maximal value at 00:00 and the mimimum at 08:00 hr. Then it begins to rise again during the light period up to 16:00 hr, when a new decrease begins. Nevertheless this curve is not correlated to those of desaturation (Fig. 2).

The changes in the fatty acid composition of the whole liver and liver microsomes in the course of the day are shown in Figures 4 and 5. This experiment was simultaneous with that described in Figure 3. For the sake of accuracy, only the most abundant acids were measured: palmitic, stearic, oleic, linoleic and arachidonic. The curves of Figures 4 and 5 illustrate rather similar variations in the fatty acid composition of whole liver and microsomes, although the oscillations are more pronounced in microsomes. Rather important and opposite changes for stearic and oleic acids are found. The stearic acid maximum is found in the dark period, whereas oleic acid is higher in the light period. In the whole liver there is a correlation between linoleic acid and arachidonic acid that is only partial in the microsomes.

The next step was to investigate the influence of cycloheximide--an inhibitor of protein synthesis--on the diurnal variations of the desaturation of stearic acid and α-linolenic acid. The animals used in this experiment were of a different age than those used in the other experiments, as indicated in Methods. Figure 6 shows the results for α-linolenic acid desaturation. In this experiment the desaturation curve for normal animals does not exhibit a very clear rhythm. In spite of this, the effect of cycloheximide at different moments of the day can be observed. In the rising parts of the curve, the cycloheximide diminishes or nullifies the increase. In the descending parts, the effect is seen in an accentuation of the decay. In Figure 7 the effect of cycloheximide in the desaturation of stearic acid proves to be similar, except for the increase produced at 00:00 hr, which is not inhibited by the cycloheximide.

DISCUSSION

Several works published during the last years have demonstrated the existence of circadian changes related to different biological processes (11,12). Within the lipid field, Shapiro and Rodwell (13) and Hickman et al. (14) have studied the diurnal variation of the hepatic synthesis of cholesterol. Scott and Potter (15) have demonstrated that circadian rhythms are produced in the incorporation of CH_3COO^- into CO_2 and in lipids of different rat tissues. The changes in the lipogenesis in the course of the day were investigated by Kimura et al. (16).

The results of the present work (Figures 1 and 2) demonstrate that the oxidative desaturation of the stearic, linoleic and α-linolenic fatty acids is also modified in the course of the 24 hr of the day. The data were obtained with low substrate concentration. Consequently the de-

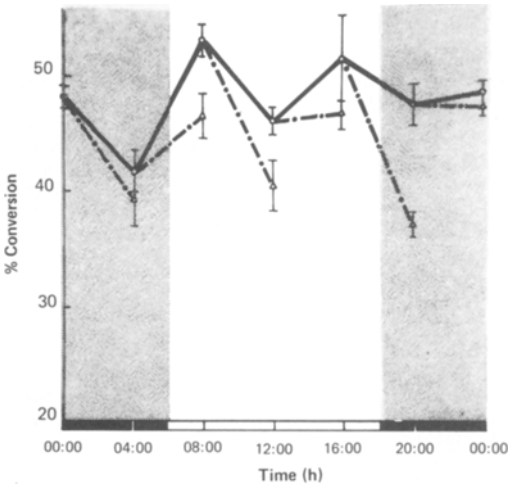

FIG. 6. Effect of cycloheximide on α-linolenic desaturation. o——o Normal animals; △—•—△ animals injected with cycloheximide.

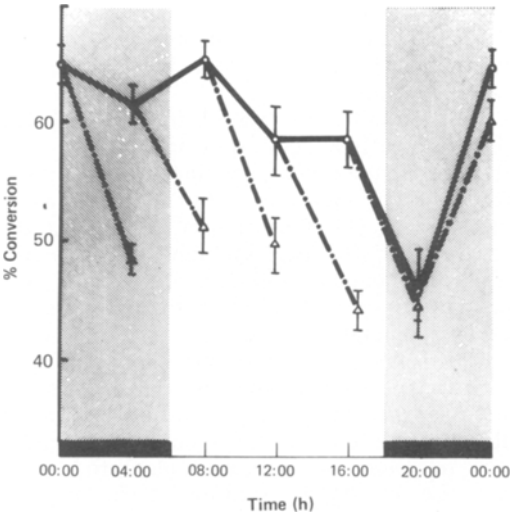

FIG. 7. Effect of cycloheximide on stearic acid desaturation. o——o Normal animals; △—•—△ animals injected with cycloheximide.

saturating capacity of the cellular endoplasmic reticulum was measured, but not necessarily the enzymatic activity. Besides, since the oxidative desaturation of linoleic acid and α-linolenic acid constitutes the first step in the synthesis of the fatty acids of the corresponding families (2,17) and is probably the critical step in the sequence of reactions (18-19), the results could be correlated with a circadian variation of the liver biosynthesis of the respective polyenoic fatty acids at the microsomal level.

As shown in Figures 4 and 5, the fatty acid compositions of whole liver and liver microsomes are also changed in the course of the day. However no apparent correlation was observed when comparing, for example, the oxidative desaturation of linoleic acid (Fig. 2) with the per cent of linoleic and arachidonic acids in liver (Figs. 4 and 5). Both curves of desaturation and fatty acid composition are apparently a consequence of the feeding rhythm of the animals that, besides the incorporation of dietary components, provokes a general rhythmic variation in hormonal secretion and enzymatic activity. They agree with those of Scott and Potter (15), who pointed out that the diurnal variations of lipogenesis depend mainly on the feeding period of the animals. Besides, the effect of different diets and fasting on fatty acid desaturation has already been proved (3,20,21).

The acceptable resemblance between the rhythms of linoleic and α-linolenic acid desaturation (Fig. 2) would also agree with the results that demonstrate that both acids are desaturated by the same enzyme, the 6 desaturase (2). Likewise, the remarkable difference between the curves of stearic and linoleic acid desaturation (Fig. 1) would confirm the existence of two different enzymes, 9 desaturase and 6 desaturase (2,3) regulated by at least partially different mechanisms.

The mechanism that evokes the diurnal change in the fatty acid oxidative desaturation is difficult to discern. The desaturation of fatty acids may be modified by competition with other fatty acids of the same or different series, by hormonal effects probably through enzymatic induction and by changes of related metabolites and cofactor concentrations (2). Therefore a possible cyclical change in the amount of endogenous microsomal FFA competing with the desaturation of the assayed substrate could produce an apparent circadian rhythm of the oxidative desaturation of this substrate.

In Figure 3 there is a clear evidence of the existence of circadian variations in the FFA content of liver microsomes. They may be correlated to the feeding of the animals, since the greatest increase is produced during the first part of the dark period, in which they feed actively. Besides, the peak observed at 16:00 hr is probably related to the nonesterified fatty acids released by the adipose tissue during the light period of rest and fasting (22). However the curve of Figure 3 is apparently not correlated with any of the desaturation curves. Consequently the microsomal FFA concentration does not seem to be a direct cause of the circadian changes of the desaturation. Nevertheless, if the activity of the desaturating enzyme is really related in some way to the concentra-

tion of liver FFA, the results obtained indicate that its effect would be complemented by other factors that make both cruves (FFA and desaturation) noncorrelative.

According to the results in Figure 3, the protein synthesis does not follow the same rhythm as either stearic or linoleic acid desaturation. This indicates that, if the changes observed in the desaturation depend on the synthesis of the corresponding enzymes, the latter may have a particular rhythm that does not exactly correspond to that of the circadian changes of total proteins.

The experiment with cycloheximide provides more information on the relationship between protein synthesis and desaturation. In the desaturation of α-linolenic acid, the previous injection of cycloheximide produces a decrease in the desaturation levels (Fig. 6). Considering that cycloheximide inhibits protein synthesis, these results would indicate that the changes of desaturation are somehow dependent on the synthesis of the related enzymes. Besides, the fact that cycloheximide does not prevent the decay in the desaturation, and even accelerates it, is interpreted, by Shapiro and Rodwell (13) and other authors (23,24) in similar situations, to mean that the variations of the desaturation are not affected by the synthesis of a degradative or inhibitory protein of the enzymes. For stearic acid the interpretation may be similar. However the increase produced at 00:00 hr is not inhibited by cyclohexamide and consequently is probably not related to enzyme synthesis.

The enzymes responsible for the observed changes in the fatty acid desaturation may be specifically the desaturase, acyl-CoA synthetase or even other enzymes that may compete with the desaturase. However the contribution of the acyl-CoA synthetase may be discarded, since Lippel (25) has shown that acyl-CoA synthetases are unaffected by fasting and has suggested that they are not adaptive enzymes.

Therefore the present experiments suggest that the fatty acid desaturation follows circadian changes related to the food intake and governed by protein synthesis and other factors. Moreover circadian changes are also found in the liver fatty acid composition, microsomal FFA content and protein synthesis. These circadian changes must be taken into consideration when comparative studies are made on fatty acid metabolism.

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