# THE OXIDATIVE DESATURATION OF UNSATURATED FATTY ACIDS IN ANIMALS*

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(Received October 3, 1973)

**Summary**

The progress made in the knowledge of the microsomal fatty acid desaturation reaction is described:

1. Studies suggest that different enzymes produce 9, 6, and 5-desaturation.
2. The 6-desaturation of fatty acids would be the principal regulatory step in the biosynthesis of polyunsaturated fatty acids in rat microsomes.
3. The desaturation of fatty acids is quantitatively modified by competitive reactions among acids of the same or different families, and by competition with fatty acid incorporation in the lipids.
4. Dietary components, especially carbohydrates and protein modify quantitatively the fatty acid desaturation. Protein would induce the 6-desaturation reaction.
5. Insulin modifies the 6-desaturation reaction through a dual effect: one modifying glucose metabolism, and the other through enzymatic induction.
6. ATP increases the 6-desaturation of fatty acids.
7. By mild extraction of the microsomes with buffered KCl sucrose solution, a "soluble" fraction was separated that was necessary for a full desaturation activity of the microsomes. The nature of this factor is discussed.
8. The main features that characterize the 6-desaturation reaction are discussed and the possible structure of the enzyme outlined.

* An invited article.

** Member of the Carrera del Investigador of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

The importance of unsaturated fatty acids, especially linoleic and arachidonic acids in the normal physiological function of animals was firmly recognized after the work of BURR and BURR[1] in 1929 and the concept of essential fatty acids then appeared. However, a long time was still needed to arrive at the next step and to investigate the biosynthetic chains and relationships of unsaturated fatty acids as well as their biosynthetic mechanisms. Two reactions were recognized as essential: the building of the carbon chain and the introduction of the double bonds. The first one began to be known and rapidly developed, mainly after the works of WAKIL[2] and LYNEN[3]. The other developed more slowly after the pioneering work of BERNHARD et al. in 1958[4] who demonstrated for the first time that stearic acid could be desaturated aerobically to oleic acid by a mitochondria-free cellular supernatant. This result was expanded by STOFFEL in 1961[5], by proving that liver microsomes also convert eicosa-8,11,14-trienoyl-CoA into arachidonyl-CoA in the presence of NADPH and oxygen. Similarly, NUGTEREN in 1962[6] proved that microsomes desaturate labeled linoleic acid into γ-linolenic acid but in this case required ATP, CoA and $Mg^{++}$, besides NADPH or NADH and oxygen. HOLLOWAY et al.[7] showed in 1963 that oleyl-CoA could also be desaturated aerobically to octadeca-6,9-dienoic acid in the sole presence of NADH or NADPH. At that time we[8, 9] were also able to demonstrate that the rat could not elongate and desaturate *cis* 2-octenoic acid to linoleic acid. These results together with those discussed above showed that the main mechanism of unsaturated fatty acid biosynthesis in the rat is produced in the endoplasmic reticulum by

an aerobic mechanism that introduces new double bonds to the corresponding preformed long chain fatty acyl-CoA, and not by a mechanism similar to the anaerobic one found by SCHEUERBRANDT et al. in bacteria[10].

**Desaturation reactions involved in polyunsaturated fatty acids biosynthesis**

In 1966[11] we were able to confirm the work of NUGTEREN and of HOLLOWAY et al.[7] on linoleic and oleic acid desaturation. At the same time it was possible to show that rat liver microsomes also desaturate α-linolenic acid into octadeca-6,9,12,15-tetraenoic acid. It was remarked that all the acids produced from oleic, linoleic, and α-linolenic acids had the new double bond between the 6-7 carbons and in divynilic position with respect to the nearest double bond. From the kinetic data collected it was suggested that the same enzyme desaturated oleic, linoleic, and α-linolenic acids and that this enzyme was different from the enzyme or enzymes that desaturated stearic acid to oleic acid and palmitic acid to palmitoleic acid. The double bond in this last case was placed between carbons 9 and 10 and the enzymes are called 9-desaturases in contrast to the first enzyme that is termed correspondingly 6-desaturase. These results were confirmed afterwards in our laboratory[12] in a study of the different responses of 9 and 6-desaturation under different physiological stimuli and experimental conditions.

In the same publication[11] it was also demonstrated that the yield of the 6-desaturation was highly dependent on the nature of the substrate. It increased with the number of double bonds of the fatty acid and was small for oleic, higher for linoleic, and the highest for α-linolenic acid. Later on[12], it was also demonstrated that the stereochemical structure of the pre-existing double bonds was very important, and that the acids having a *trans* structure in the double bond nearest to the desaturating site were not desaturated in measurable amounts. Neither elaidic nor *trans-trans* linoleic acid were desaturated by rat liver microsomes. The increase of the affinity of the 6-desaturase from oleic to linoleic and α-linolenic acid, as well as the correlative increase of the rate of their desaturating reactions[12] explain the predominance of the polyunsaturated acids of the linoleic family or

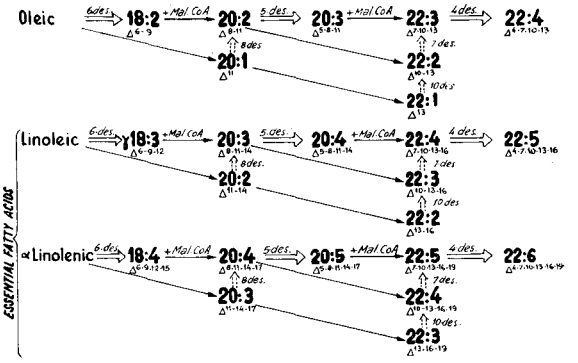


Fig. 1. Possible pathways in the biosynthesis of fatty acids of oleic, linoleic, and α-linolenic family.

α-linolenic family over the acids of the oleic family in animals fed a complete diet.

Oleic, linoleic, and α-linolenic acid are starting points of three different series of polyunsaturated fatty acids and the possible sequences of reactions that build the acids are outlined in Fig. 1. Besides the already discussed 6-desaturation, the existence of a 5-desaturation has also been demonstrated. It has been proved that rat liver microsomes convert eicosa-8,11-dienoic acid and eicosa-8,11,14-trienoic acid to eicosa-5,8,11-trienoic and araquidonic acid respectively[5, 13, 14, 15]. These acids belong to the oleic and linoleic series respectively. The 5-desaturation of acids of 20 carbons in rat liver microsomes is higher than the 6-desaturation of the corresponding acids of 18 carbons[13, 14, 15].

The 4-desaturation of acids of 22 carbons has been demonstrated *in vitro* by incubating microsomes with labeled linoleic under elongating and desaturating conditions[16]. However, we have not been able yet to find measurable conversion of tritiated docosa-7,10, 13,16-tetraenoic acid to docosa-4,7,11,13,16-pentaenoic acid either in rat liver or testicle when the acid is directly incubated with the microsomes of the organs in the presence of the necessary cofactors.

The ability of liver microsomes to elongate unsaturated fatty acids by the addition of two carbon units has been well demonstrated. This reaction completes the biosynthetic chain of unsaturated fatty acid families. However, the three acids, oleic, linoleic, and α-linolenic, when incubated in strictly elongating conditions are converted by liver microsomes, in the presence of malonyl-CoA to eicosa-11-enoic, eicosa-11,14-dienoic, and eicosa-11,14,17-trienoic acid, and

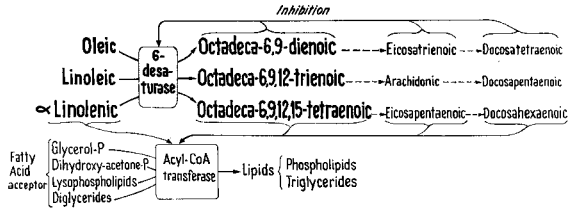


Fig. 2. Links between fatty acid desaturation and incorporation into lipids.

to higher homologs respectively[16, 17, 18, 19]. These acids could then be desaturated giving place to an alternative route of polyunsaturated fatty acid biosynthesis (Fig. 2). If these alternative routes proceed normally, an 8-desaturation should be necessary.

The 8-desaturation of acids of 20 carbons has been studied specially in Sprecher's laboratory[20]. His results showed that although rat liver microsomes are able to desaturate both eicosa-11-enoic and eicosa-11, 14-dienoic acid, the acids synthesized are eicosa-5, 11-dienoic and eicosa-5,11,14-trienoic acid respectively. The double bond is opened in both acids in the 5–6 position by the 5-desaturase. 8-desaturase showed little if any activity. The same authors also demonstrated that either eicosa-5,11-dienoic acid or eicosa-5, 11,14-trienoic acid would not be further desaturated. Therefore, these represent real dead ends in the biosynthetic chains.

All these results, and similar ones obtained by MARCEL et al.[21], indicate that the main route of polyunsaturated acids follows an alternating sequence of desaturations and elongations represented by the horizontal lines of Fig. 1. These sequences of reactions begin with a 6-desaturation, followed by an elongation, a 5-desaturation, a new elongation and a 4-desaturation.

## Factors that modify the desaturation of fatty acids

*Incorporation of fatty acids in the lipids*

The incubation of liver microsomes with labeled linoleic acid in the presence of ATP, CoA, $Mg^{++}$, NADH or NADPH in air was shown not only to activate the acid to the acyl-CoA and desaturate it to γ-linolenic acid, but also to incorporate both acids simultaneously in lipids[11]. The incorporation was mainly performed in phosphatidylcholine, phosphatidylethanolamine and triglycerides. The incorporation of substrate fatty acid in the lipids was shown to compete with the desaturation reaction[11, 22]. The addition of lysolecithin or α-glycerol phosphate decreased the conversion of linoleic acid into γ-linolenic due to the increased incorporation of the substrate in the lipids. This effect was mainly evident when the ratio of substrate fatty acid to microsomes was low. Considering that this relationship *in vivo* may be generally expected to be low, the yield of the desaturation may be highly dependent on the amount of lysolecithin, diglycerides, and other lipids capable of incorporating fatty acids (Fig. 2).

The fact that the microsomal oxidative desaturation of fatty acids is carried out together with the incorporation of the fatty acids in the lipids has led some investigators[23] to suggest that the former reaction could take place not on the acyl-CoA but on the fatty acids bound to the lipids. At least this mechanism appears to be operative in some reactions in vegetables[24]. However, this suggestion at least in the case of the microsomal oxidative desaturation studied in the animals would not be supported by the results just mentioned. Besides while studying simultaneously the incorporation and desaturation of linoleic acid, we were able to demonstrate[25] that the preincubation of liver microsomes with ATP, CoA, $Mg^{++}$ and labeled linoleic acid under nitrogen incorporates the label into the aforementioned lipids, but that the desaturation produced by subsequent incubation in the presence of NADH and $O_2$ was less than the one produced without preincubation.

*Competition between acids*

In spite of having worked with microsomal preparations and under experimental conditions that did not assure a saturation of the enzyme with the substrate, it was possible to show[11] that the three acids, oleic, linoleic and α-linolenic, that are desaturated by 6-desaturase showed a competitive effect. However, the competition increased with the number of double bonds and α-linolenic acid was a stronger competitor than oleic acid. These results parallel the higher affinity (smaller approximate Km) of α-linolenic acid for the desaturating enzyme than that of oleic acid. At the same time, it was shown that the saturated acids palmitic and stearic in amounts equivalent to the substrate did not inhibit linoleic acid desaturation. These results were subsequently confirmed by ULLMAN and SPRECHER[15]. The inhibition produced by different

concentrations of isomeric monoenoic acids of 18 carbons: oleic, vaccenic, and petroselinic was small, and of the same order[26]. Much higher inhibition was produced by the polyunsaturated acids. In this respect, it was shown that the following acids or acyl-CoA belonging to the linoleic acid family: γ-linolenic[26] or γ-linolenyl-CoA[27], eicosa-8,11,14-trienoic acid[14], arachidonyl-CoA[27], docosa-7,10,13,16-pentaenoic acid or docosa-7,10,13,16-pentaenoyl-CoA[28] inhibited linoleic acid desaturation to γ-linolenic acid *in vitro*. The inhibition was effected by γ-linolenic acid, the product of the reaction; by arachidonic acid, the intermediate member of the series, which by virtue of its structure does not allow the opening of new double bonds, and by eicosa-4,7,10,13,16-pentaenoic acid that also cannot be further desaturated and is the last member of the series at the level of acids of 22 carbons. Therefore, the amount of fatty acids of linoleic acid family in the cell controls the biosynthesis of polyunsaturated fatty acids of the same family. Polyunsaturated fatty acids of α-linolenic family also compete with or inhibit *in vitro* linoleic acid oxidative desaturation[11, 27, 28], showing an important mechanism of control over the biosynthesis among fatty acids of different families (Fig. 2). These results confirm similar ones obtained *in vivo* some years before[29] involving oleic and linoleic acid families. The study related to the kinetics of linoleic and arachidonic acid incorporation to eicosa-5,8,11-trienoic acid depletion in the lipids of fat deficient rats fed methyl linoleate and arachidonate. It was shown that either linoleic acid or arachidonic acid inhibited oleic acid conversion to eicosa-5,8,11-trienoic acid. The data collected in that article were analyzed statistically by LINDSTROM and TINSLEY[30], who confirmed the existence of competitive reactions among the synthesis of acids of linoleic and oleic families.

Competition of unsaturated fatty acids in the 5-desaturation of eicosa-8,11-dienoic acid and eicosa-8,11,14-trienoic acid has been shown by BRENNER[14], ULLMAN and SPRECHER[15] and CASTUMA et al.[31]. It has been found that some unsaturated fatty acids of a shorter chain length and placed earlier in the series, such as linoleic and α-linolenic acids, may compete with the 5-desaturation of fatty acids of 20 carbons. Polyunsaturated acids of 20 and 22 carbons also inhibit or compete with the 5-desaturation of fatty acids, and this competition is generally increased by the increase in the number of double bonds. The most powerful competitor and inhibitor of all the acids tested for eicosa-8,11-dienoic acid desaturation was found to be eicosa-5,8,11,14,17-pentaenoic acid. Therefore, a regulation in the biosynthesis of polyunsaturated fatty acids may be produced by competition with other fatty acids of the same or different series, not only at the level of the first step represented by the 6-desaturation, but also at the intermediate step at the 5-desaturation.

*Competition of fatty acids and incorporation in the lipids*

The effect produced by polyunsaturated acids (e.g. arachidonic acid or eicosa-5,8,11,14,17-pentaenoic acid) on the oxidative desaturation of linoleic acid may not be always inhibitory[11]. When low concentrations of substrate (labeled linoleic acid) were incubated with high concentrations of microsomal protein and increasing concentrations of either arachidonic or eicosapentaenoic acid, the conversion of linoleic into γ-linolenic acid was actually enhanced by the addition of the polyunsaturated acids. However, the activating effect of arachidonic acid disappeared by the addition of increasing amounts of heated microsomes or lysolecithin to the incubation mixture, but not by lecithin or Tween 20[22]. Similar results were found for γ-linolenic acid[26].

To explain these results it is necessary to consider that polyunsaturated acids are preferentially bound to the β-position of phospholipids. NERVI et al.[22] also showed that the incubation of liver microsomes with increasing amounts of $^{3}H$ arachidonic acid in the presence of a fixed amount of 1-$^{14}C$ linoleic acid, under desaturating conditions, increased the amount of arachidonic acid incorporated in all the lipids. This increase was correlative with a decrease of the linoleic acid bound to phospholipids, and it provoked an increase in the amount of the free $^{14}C$ acid, the acid bound to the CoA, and the 6-desaturation. These results led to the conclusion that with low substrate concentration arachidonic acid competed with linoleic acid for the esterification of microsomal lysolecithin and other fatty acid binders, leaving increasing amounts of substrate (linoleic acid) available for desaturation. With saturated and *trans* acids that are preferentially bound to the α-carbon of phospholipids, the activating effect on linoleic acid desaturation was not seen[26] and a similar situation was also found

with monoenoic acids that are not preferentially bound to the same carbon. Therefore, the inhibitory effect of different fatty acids on the desaturation of other fatty acids depends on the relative proportion of substrate fatty acid to the microsomal enzymes available, type of binding lipid, and position of reaction in the lipid as well as relative amount and affinities of the fatty acids for the desaturase and the accepting lipid.

*Effect of ATP*

Preincubation of rat liver microsomes with ATP alone in $N_2$ or in the presence of CoA and $Mg^{++}$ followed by subsequent incubation with 1-$^{14}$C linoleic acid together with NADH and $O_2$ enhances linoleic acid desaturation to γ-linolenic acid, as well as the 6-desaturation of oleic or α-linolenic acids[32]. The increase depended on ATP concentration following a sigmoidal curve and was not shown by GTP, CTP, ADP or AMP. It could not be attributed to the simple increase of the acylation of the CoA and was also shown in the direct conversion of linoleyl-CoA to γ-linolenate. It is interesting to remark that CATALÁ and BRENNER* also found that the injection of ATP to normal rats also increased the desaturating activity of the isolated microsomes towards linoleic acid conversion to γ-linolenic acid. This result also suggests a possible physiological significance of the cellular concentration of ATP upon the desaturation of fatty acids and as a consequence upon the general synthesis of polyunsaturated acids.

It was also shown[33] that preincubation of rat liver microsomes with 0.25 M sucrose, 0.15 M KCl solution in the presence or absence of ATP followed by ultracentrifugation at 100,000 × g for 1 hr allows the separation of a "soluble" factor that is necessary for full desaturating activity of the washed microsomes. However, the activation produced by ATP remains bound to the particles.

*Hormones and diets*

IMAI in 1961[34] and GELLHORN and BENJAMIN in 1964[35] demonstrated respectively that the oxidative desaturation of palmitic to palmitoleic and stearic to oleic acid was depressed in the diabetic rats. In 1966 MERCURI et al.[36] also found a defect in the desaturation of the essential fatty acid: linoleic acid to γ-linolenic by the liver microsomes of the alloxan diabetic rats. In 1967[37] the same authors also found a similar defect in the other two 6-desaturations of oleic and α-linolenic acids. These defects were corrected by insulin injection in 38 hr or less. However, the simultaneous injection of Actinomycin D or Puromycin which abolish protein synthesis, impaired the reparative effect of insulin. The possibility that these effects could be attributed specifically to the alloxan or to the high blood glucose level of the diabetic is not supported by similar experiments performed with animals made diabetic by intravenous injection of streptozotocin[38] and with starved animals and animals refed with glucose[39]. Starvation for 48 hr significantly decreased the microsomal 9- and 6-desaturation activity that was largely recovered by refeeding the animals for 12 hr with 10% glucose in the drinking water. In this experiment again the injection of Actinomycin D prevented the recovery. On the basis that starvation lowers blood insulin levels and that glucose refeeding stimulates insulin secretion, both results were considered to indicate that fatty acid 9- or 6-desaturation activity depended on insulin level in the animal and that insulin appeared to be an inducer of the enzymes. However, the effect could also be produced by insulin mediated via the utilization of glucose at the cellular level. For this reason, a study was made on the effect of different diets on the microsomal oxidative desaturation of linoleic acid and on the activities of liver enzymes involved in glycolysis[40]. Whereas fasting produced in liver a correlative decrease on linoleic acid desaturation, "glucokinase" and piruvate kinase activity; glucose refeeding, in spite of increasing the three parameters including blood insulin no correlative changes were evoked. Specifically, the 6-desaturation decayed after a transient increase, whereas the glycolytic enzymes still went on increasing. These results would indicate that the effect of insulin is not mediated via the glycolysis. This was confirmed when the same parameters were studied in rats fed a balanced diet, a carbohydrate-free diet, a lipid-free diet, and a protein free diet. It was shown that whereas the lipid free diet produced no important changes in the 6-desaturation and the other parameters related to the glycolysis, the carbohydrate free diet increased the desaturation and decreased the glycolytic enzyme. Therefore, from these results the real effects of carbohydrate and protein metabolism seem to be

* CATALÁ, A. and BRENNER, R. R., unpublished results.

Table 1

Responses of different liver microsomal desaturases involved in unsaturated fatty acid synthesis to dietary changes.

| Enzyme | Reaction | Fasting | EFA deficiency | Glucose | Fructose | Glycerol | Protein |
|---|---|---|---|---|---|---|---|
| 9-desaturase | 18:0→18:1(n−9) | ⇓ | ⇑ | ⇑ | ⇑ | ⇑ | → |
| 6-desaturase | 18:2→18:3(n−6) | ⇓ | ⇑ | ⇓ | ⇓ | ⇓ | ⇑ |
| 5-desaturase | 20:2→20:3(n−9) | ⇓ | → | | | | ⇑ |

antagonistic. Carbohydrates appeared to lower the oxidative desaturation of linoleic acid in liver microsomes, whereas the proteins appeared to increase it. The inhibitory effect of a carbohydrate diet on linoleic acid desaturation was further reproduced in our laboratories by feeding normal rats a carbohydrate diet[41]. A similar inhibitory effect of carbohydrate diet on the 6-desaturation was also found in our laboratory[42] using a high glycerol diet. (Table 1). Considering that carbohydrate and glycerol metabolism are intimately connected through glycerol-phosphate and dihydroxyacetone phosphate, both effects are very probably produced by the same mechanisms. It is important to remark that the high glycerol diet had no inhibitory effect on the diabetic rat. Since glycerol phosphate *in vitro* decreased the microsomal conversion of linoleic acid to γ-linolenic[11], it is tempting to propose that carbohydrate and glycerol metabolism may evoke their inhibitory effect through glycerol phosphate formation. All these results lead to the conclusion that the products of carbohydrate metabolism appear to inhibit the 6-desaturation of fatty acids. However, when glucose is administered to fasting rats, it provokes the secretion of insulin that promotes an increase of the 6-desaturation activity. This increase may be due to an induction of the enzyme or enzymes related to the 6-desaturation of fatty acids and is only transient due to the immediate enhancement of the glycolysis. (Fig. 3).

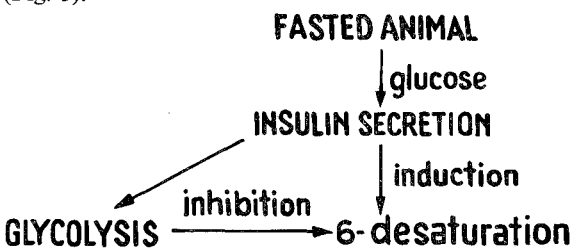


Fig. 3. Effect of glucose metabolism on fatty acid 6-desaturation.

On the other hand, MERCURI, PELUFFO and DE TOMAS[43] have confirmed that the effects of glucose and glycerol on the 9-desaturation of stearic acid are completely different. The administration of glycerol, glucose, fructose, or stearic acid to normal rats increased the microsomal specific activity of the 9-desaturase (Table 1). The same effect was shown on the diabetic rat except for glucose. Considering that in the diabetic rat the metabolism of both fructose and glycerol circumvent the glucokinase step that is insulin dependent and are converted to acetyl-CoA, a precursor of palmitic and stearic acid, the authors propose that the enhancing effect of these substances on the 9-desaturase activity is produced through an increase of palmitic or stearic acid synthesis.

The activating effect of a protein diet on linoleic acid desaturation was also further confirmed using casein or a mixture of aminoacids*. It was also shown to be not correlative with the activity of the glycolytic enzyme piruvate kinase[41]. The activating effect of the proteins was also inhibited by Actinomycin D[41]. This result was consistent with the effect of a protein diet on the 6-desaturation of α-linolenic acid found by INKPEN et al.[44]. However, proteins evoked a completely different effect on the 9-desaturation of stearic acid, showing again that both enzymes respond in a different way under the same stimuli (Table 1).

It was of interest to inquire if the effect of protein could be related with the activating effect of insulin. It was found that the simultaneous effect of dietary protein and insulin on linoleic acid desaturation in the normal rat could be antagonistic and whereas protein increased the desaturation, insulin decreased it[41]. However, in the alloxan diabetic rat the effect of insulin showed the following characteristics: a) Insulin restored linoleic acid desaturation of diabetic rats to normal, but the restoration was not proportional

* PELUFFO, R. O. and BRENNER, R. R., paper in preparation.

to the insulin dose. Very low doses of 0.2 or 0.05 IU per animal produced a higher effect. b) Both a protein diet and insulin administration restored linoleic acid desaturation to normal, but no significant additive effect was demonstrated. In view of the results reported by WOOL[45] showing that very low doses of insulin increase protein synthesis without modifying blood glucose level, it was deduced that insulin apparently had two effects in vivo: enhancement of 6-desaturation of fatty acids, probably through an increase of protein synthesis, and a decrease of the desaturation rate through stimulation of glycolysis (Fig. 3). The increase of oxidative desaturation of linoleic acid by casein or a mixture of aminoacids of similar composition was further investigated and found not to be related to the amount of free fatty acids in the microsomes[46]. Protein diet did not either modify the relative incorporation of different fatty acids in the lipids. Therefore, the enhancement of the 6-desaturation of fatty acids produced by protein cannot be attributed to any of these factors, but very probably to an induction of the enzyme.

Further information was obtained studying the circadian changes in the oxidative desaturation of linoleic, α-linolenic and stearic acids in mouse liver microsomes[47]. These oscillations were shown to be related to the food intake periods but were not correlative with oscillations in total protein synthesis and free fatty acid concentration in the microsomes. Linoleic and α-linolenic desaturation showed similar responses during the day-cycle that were different from stearic acid desaturation. The injection of cycloheximide (an inhibitor of protein synthesis) 4 hr before measuring the desaturation modified the circadian variation of the 9 and 6 desaturations. It decreased the desaturation levels, but it did not prevent the decay of the desaturation activity during the negative slopes of the cycle. Therefore, it was assumed that the cycles of the oxidative desaturation of fatty acids were related to the synthesis of specific proteins but not to a degradative inhibitory protein. These cycles are very probably related to the food and hormonal effects already discussed.

A comparative study of the effect of diabetes and protein diet on the activity of 5-desaturation was also performed[13]. The acid chosen was the 1-$^{14}$C eicosa-8,11-dienoic. It was shown that alloxan diabetes decreased the microsomal desaturation of eicosa-8, 11-dienoic acid to eicosa-5,8,11-trienoic acid, but the decrease was less pronounced than with the 6-desaturation of linoleic acid. Protein diet increased both the 5- and 6-desaturations but again the 5-desaturation was less responsive.

An essential fatty acid free diet was also shown[13] to provoke different effects on the 6- and 5-desaturation activity of liver microsomes (Table 1). It evoked equal increases of the 6-desaturation of oleic and linoleic acid, whereas the 5-desaturation of eicosa-8, 11-dienoic acid was not changed. These results could be easily explained if the existence of two different desaturating enzymes for the 6- and 5-positions is postulated. Further, it could be also postulated that in the general scheme of polyunsaturated fatty acid synthesis (Fig. 1), the 6-desaturation (that is the first desaturation reaction) would be the key controlling step in the synthesis, whereas the 5-desaturation would play a secondary controlling role. This postulates that the elongation reaction of the first members of the series, oleic, linoleic, and α-linolenic acid (Fig. 1) that lead towards parallel routes of synthesis, are not quantitatively important or lead to dead ends. The results of MARCEL et al.[21] and of ULLMAN and SPRECHER[20], already discussed, support this postulate. This is further supported by the findings that the elongation was not modified by the protein diet, whereas the desaturation was quantitatively increased[48].

*Distribution and effect of age on the oxidative desaturation activity*

The oxidative desaturation of fatty acids to polyunsaturated fatty acids in mammals is restricted to the endoplasmic reticulum. Therefore, the activity of an organ is dependent primarily on the amount of endoplasmic reticulum. Consequently, it would be expected to be high in the liver and low in the heart. High 6-desaturation activity was found in the liver, testis, and adrenals, and much lower in the brain, heart, and kidneys[12].

The activity of the testis may be very high in the immature rate, but it falls quickly with age to very low values[16]. On the other hand, in the liver there is no change in the 6-desaturation activity with age during the first three months of life, but it is significantly decreased after one year[49]. This difference between liver and testis may be related to the constant and central function of the liver for the synthesis of lipoproteins and for special hormonal regulation of the testis. As has been described above, there are also

circadian variations in the 6-desaturation of fatty acids[47], and PELUFFO has even suggested the possibility of seasonal changes of the same reaction.*

**Components of the fatty acid oxidative desaturation in animals**

The components that interact in the microsomal oxidative desaturation of stearic acid to oleic acid has been partially resolved, principally by SATO's group[50, 51] and HOLLOWAY[52, 53]. This reaction needs molecular oxygen and the reduced cofactors NADH or NADPH. Electrons are transported from the reduced cofactor by a non-phosphorylating microsomal electron transport chain that includes a flavoprotein, cytochrome $b_5$, and a $CN^-$ sensitive factor[50]. This last factor has been considered for some time to contain iron[54], but the recent results of SATO's group[55] suggest that it is iron-free. Furthermore, OSHINO and SATO[56] showed that the amount of cyanide sensitive factor is modified by the diet and they even suggest that this factor may be the fatty acid desaturase itself.

The 5- and 6-desaturations of fatty acids also require the same reduced cofactors, NADH or NADPH and oxygen, and the conversion of linoleic to γ-linolenic acid has also been shown to be inhibited by cyanide[26]. Therefore, it is reasonable to consider that a similar electron transport system functions with these enzymes (Fig. 4). The activities of the 9-desaturation of stearic acid and the 6-desaturation of linoleic and α-linolenic acid have both been shown to be significantly reduced by simple suspension and centrifugation of the microsomes at 100,000 × g for 1 h with buffered 0.15 M KCl 0.25 M sucrose. However, the readdition of the supernatant solution reactivates the extracted microsomes to their original activity (Table 2). The deactivation of the whole microsomes produced by this mild treatment

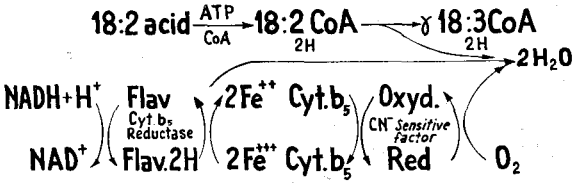


Fig. 4. Hypothetical scheme of linoleic acid oxidative desaturation reactions.

* PELUFFO R. O. and BRENNER, R. R., paper in preparation.

Table 2

Dissociation of the rat liver microsomal system that desaturates linoleic acid to γ-linolenic acid by centrifugation in buffered 0.15 M KCl 0.25 M sucrose.

| Fractions | Desaturation |
|---|---|
| | % |
| Original microsomes (1 mg) | 16.5 |
| Extracted microsomes (1 mg) | 1.1 |
| Supernatant (1 ml) | 0.0 |
| Extracted microsomes (1 mg) + Supernatant (1 ml) | 13.5 |

5 mg microsomal protein were extracted with 3 ml of a solution composed of 0.15 M KCl, 0.25 M sucrose, 0.004 M buffer phosphate (pH 7.0) and 1.5 mM glutathione, and centrifugated at 100,000 × g during 1 h in the cold.

8 nmol {1-$^{14}$C} linoleyl-CoA were incubated with 1 mg microsomal protein or the fraction specified and NADH, ATP, and $MgCl_2$ for 20 min at 35 °C.

Table 3

Effect of tripsyn on the activating properties of the microsomal supernatant upon linoleic acid desaturation activity of the microsomes.

| Fractions | Desaturation activity |
|---|---|
| | % |
| Whole microsomes | 100 |
| Extracted microsomes (with 0.15 M KCl 0.25 M sucrose) | 7.7 |
| Extracted + 3 ml supernatant | 98.0 |
| microsomes + 3 ml tripsynized supernatant | 7.7 |

Extraction of microsomes as in Table 2.

6 ml microsomal extracted supernatant were incubated with 6 mg trypsin for 1 h at 37 °C, then 12 mg trypsin inhibitor was added.

Linoleic acid desaturation to γ-linolenic acid was measured on 5 mg microsomal protein under the conditions described in Table 4.

shows that fatty acid oxidative desaturation of the microsomes requires a complex structure and some components are held together by very weak forces. The nature of the activating factor extracted from the microsomes has not yet been identified. However, it is known to be deactivated by trypsin (Table 3), lipid extraction (Table 4), or heating at 100 °C for 10 min,

Table 4

Effect of lipid separation on the activating properties of the microsomal supernatant upon microsomal linoleic acid desaturation activity.

| Fractions | Desaturating activity |
|---|---|
| | % |
| Whole microsomes | 100 |
| Extracted microsomes (with 0.15 M KCl 0.25 M sucrose) | 34.5 |
| Extracted microsomes + 3 ml whole supernatant | 99.5 |
| Extracted microsomes + 3 ml supernatant delipidized with 10% acetone-water | 68.8 |

5 mg microsomes were extracted with 3 ml of reaction mixture composed of 0.15 M KCl, 0.25 M sucrose, 0.04 M phosphate buffer pH 7.0, and centrifuged at 100,000 × g. The supernatant was delipidized with 10% acetone-water. Linoleic acid desaturation to γ-linolenic was measured using 5 mg microsomal protein plus the specified supernatant when stipulated. 20 nmoles of {1-$^{14}$C} linoleic acid were incubated with NADH, ATP, CoA, and $MgCl_2$ for 20 min at 35 °C.

but to resist 10 min. heating at 60 °C[57]. It is excluded from Sephadex G100 and is partially included but deactivated in Sephadex G200. It has very low cytochrome $b_5$ reductase activity and this activity is not inducible by a proteical diet*. These results suggest that the extracted factor is a protein and very probably has a lipoproteic structure. The importance of lipids for maximal activity of the microsomal stearic acid 9-desaturase has been shown by JONES et al.[58] and HOLLOWAY[59]. Similarly, ROGERS and STRITMATTER[60] demonstrated that the microsomal cytochrome $b_5$ reductase requires lipids to have a maximal activity. The results discussed earlier, in conjunction with this information, would suggest that in addition to the lipids needed to build the membrane structure of the endoplasmic reticulum, a special lipoprotein which is easily detachable, plays a specific role in the oxidative desaturation of both 9- and 6-desaturases. This lipoprotein would be common or similar for both enzymes and is very probably required in a reaction also common or similar to both desaturations.

* NERVI, A. M., PELUFFO, R. O., CATALÁ, A., and BRENNER, R. R., paper in preparation.

**Specificity and structure of unsaturated fatty acid desaturases**

It has been impossible up to now to get pure fatty acid desaturases and for this reason there is not only a lack of exact kinetic constants, but also it has been very difficult to determine the specificity of the enzymes. Exact kinetic data cannot be obtained because of the particular physicochemical properties of acyl-CoA that form miscelles at relatively low concentrations.

Several indirect methods have been used to determine the specificity of the desaturating enzymes. As has been described above, indirect evidence has been gathered that show that the 6-desaturations of oleic, linoleic, and α-linolenic acids are produced by the same enzyme, and that this enzyme is different from the 9-desaturase and the 5-desaturase[11, 12, 13]. Other supporting evidence is: 1) Similar relative responses of the 6-desaturations of oleic, linoleic, and α-linolenic acid to activating factors or inducers of enzyme synthesis as are insulin, dieldryn*, protein, and environmental temperature**. However, these differ for the 9- or 5-desaturation. 2) Similar relative effect of inhibitors or deactivators of fatty acid desaturation such as temperature*, NaCN, etc., on the 6-desaturation. Some of these results are shown in Tables 1, 5 and 6.

Table 5

Different inactivation effect of heating on the microsomal 9-, 6- and 5-desaturating activity.

| Reaction | | Inactivation |
|---|---|---|
| | | % |
| 9-desaturation | 16:0→16:1 | 38.9 |
| | 18:0→18:1 | 63.4 |
| 6-desaturation | 18:2→γ18:3(n−6) | 84.0 |
| | α18:3→ 18:4(n−3) | 86.0 |
| 5-desaturation | 20:2→20:3(n−9) | 61.9 |
| | 20:3→20:4(n−6) | 52.3 |

Rat liver microsomes were heated 15 min at 40 °C and the fatty acid desaturating activity was compared to microsomes maintained at 0–4 °C.

* NINNO, R., CASTUMA, J. C. and BRENNER, R. R., paper in preparation.

** POLERO DE TORRENGO, M. and BRENNER, R. R., paper in preparation.

Table 6

Effect of environmental temperature on the activity of 6- and 9-desaturases in fish (Pimelodus maculatus).

| Reaction | | Relative desaturating activity $\frac{15.7}{29.2}$ °C |
|---|---|---|
| 9-desaturation | 16:0→16:1 | 2.43 |
| | 18:0→18:1 | 1.43 |
| | 18:1→ 18:2 | 2.04 |
| 6-desaturation | 18:2→γ18:3 | 2.03 |
| | α18:4→ 18:4 | 2.03 |

Microsomal desaturating activity was measured in groups of 35 animals kept in a water bath at 15.7 °C and 29.2 °C during 3 weeks.

A careful study of the fatty acid specificity of the 9-desaturation in relation to chain length has been done by BRETT et al.[61]. They measured the oxidative desaturation of fatty acids from 10 to 19 carbons for different organisms. From these results it was deduced that in hen liver and goat mammary gland there seems to exist two enzymes, one for long chain fatty acids (with a maximum for stearic acid), and the other for shorter chain length (with a maximum for myristic acid). In Torulopsis bombicola and rat[62], the last enzyme is absent. BRETT et al.[61] studied the microsomal desaturation of a whole series of isomeric methyl-stearic acids and showed that stearic acid with methyl substituents at positions 2, 3, 4, 16, 17, or 18 were desaturated by the enzyme, but methyl side chains at carbons 5, 6, 8, 9, 10, 11, 12, 14, and 15 eliminated the activity. These results led the authors to propose an enzymatic structure that would bind the substrate, holding the carboxyl group fixed and locating the 9–10 carbons at the active center, and an enfolding by the enzyme of the substrate acyl-chain between positions 5 and 15. Enzyme and substrate interactions would also lead to a rotation of about 9, 10 C-C that would bring the two hydrogens together in an eclipsed conformation, allowing a simultaneous concerted removal of both hydrogens[63].

Some of the necessary features of the 6-desaturase were outlined by us in 1971[12]. The criteria used were various: one was to compare the reactivities of the *cis* acids: oleic, linoleic, and α-linolenic, and the similar *trans* acids: elaidic and all *trans* linoleic. Another criteria was to compare the inhibitions produced by saturated and unsaturated fatty acids with different chain length, the extent of unsaturation, and the position of double bonds.

The main difference with the 9-desaturation that was pointed out was that the substrate fatty acid in the 6-desaturation requires preexisting double bonds. Moreover, an important fact that was also taken into account was that oleic, linoleic, and α-linolenic acids were desaturated at carbons 6–7 in divinylic position from double bond 9–10, leaving the rest of the molecule unmodified.

The only difference between oleic and linoleic or α-linolenic acids is the existence of additional double bonds in 12–13, 12–13 and 16–17 respectively. Therefore, it was deduced that the structure of the enzyme not only must recognize the hydrocarbon tail of the fatty acids but also the 9–10 double bonds and the additional 12–13 and 16–17 bonds. An hypothetical structure was suggested which could fulfill these requirements. It would be one in which the configuration and binding forces of the enzyme would mimic the hydrophobic tail of the α-linolenic acid from the 9–10 double bond and so on. The principal binding forces would be weak polar attractions of the double bonds and London-Van der Waals dispersion forces. The latter would increase with the number of $CH_2$ groups attached to the enzyme[64]. The kink of the molecule produced by the double bonds would increase the number of $-CH_2-$ groups near the enzyme, going from oleic to α-linolenic acid with a corresponding increase of this attraction.

The hydrocarbon chain, where the 6–7 double bond is to be produced, was considered to be at the desaturation site of the enzyme and perfectly fixed in relation to the site of binding of the 9–10 double bond. Therefore, the tail of the acid from the 9–10 carbon is not only recognized and bound, but also preserved by the 6-desaturase during the oxidative desaturation. However, new results obtained by BRENNER and CATALÁ* suggest that the enzyme must also recognize the $-C^{=O}_{-S}$ CoA group or similar functional groups of the molecule. This conclusion was suggested by a study of the inhibition produced by unsaturated acyl pantetheines and alcohols on linoleic acid desaturation (Tables 7 and 8). Although both groups of substances have structures similar to the hydrocarbon tail of α-linolenyl-CoA, and therefore could be bound to the specific binding site of the enzyme, no inhibition was found.

Table 7

Effect of different fatty alcohols on the microsomal desaturation of linoleic acid to γ-linolenic acid.

| Additions | | Conversion |
|---|---|---|
| | nmoles | % |
| No additions | | 22.1 |
| + linoleyl alcohol | 5 | 22.6 |
| | 10 | 22.0 |
| + αlinolenyl alcohol | 5 | 20.5 |
| | 10 | 20.8 |
| + octanol | 5 | 21.8 |
| | 10 | 20.8 |
| + αlinolenic acid | 5 | 14.7 |
| | 10 | 10.8 |

5 nmoles of {1-$^{14}$C} linoleic acid were incubated with ATP, CoA, $MgCl_2$, NADH and 5 mg microsomal protein of rat liver at 35 °C and pH 7.0 for 20 min.

Table 8

Effect of unsaturated fatty acyl pantetheines on the microsomal desaturation of linoleic acid to γ-linolenic acid.

| Additions | Conversion |
|---|---|
| | % |
| No addition | 10.1 |
| + linoleyl pantetheine | 9.8 |
| + αlinolenyl pantetheine | 10.9 |
| + arachidonyl pantetheine | 10.6 |
| + αlinolenic acid | 2.0 |
| + arachidonic acid | 3.1 |

100 nmoles of {1-$^{14}$C} linoleic acid and equivalent amounts of acyl pantetheines were incubated with ATP, CoA, $MgCl_2$, NADH, and 5 mg microsomal protein of rat liver for 20 min at 35 °C pH 7.0.

The experimental results discussed above suggest that the 5-desaturase is different from the 6-desaturase. Therefore, the enzyme must recognize the $-CH_2-$ difference in the fatty acid molecule. These desaturases probably bind the acid through the carboxylic end of the molecule and also recognize the distance between the $-C^{=O}_{-S}$ CoA or similar functional group of the acid and the C-C bond to be desaturated, which would be properly located in relation to the other preexisting double bonds.

Enzyme and substrate interactions very probably lead to the rotation of the 6,7-C-C, similar to the one proposed by BRETT et al.[62]. This rotation would bring the two hydrogens together in an eclipsed conformation that would not only allow the concerted removal of the two hydrogens[63], but also produce an additional kink in the molecule, very similar to the one produced by a double bond. Although additional data are needed to resolve the problem, these new results would favour the existence of a cavity at the active site of the enzyme in which the unsaturated fatty acids could be easily embeded and accommodate the shape of the molecule.

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