A study of the effect of α-linolenic acid on the desaturation of dihomo-γ-linolenic acid using rat liver homogenates

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Summary. The effects of α-linolenic aцld on Δ5 desaturation of 20 : 3 n-6 dihomo-γ-linolenic acid) to 20 : 4 n-6 (arachidonic acid) and on Δ6 desaturation of 18 : 2 n-6 (linoleic acid) to 18 : 3 n-6 (γ-linolenic acid) were investigated in two different studies using rat liver homogenates.
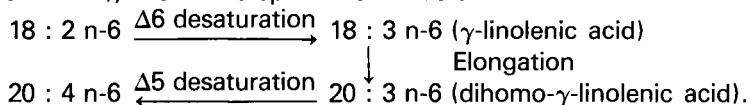
In the first study, rats were fed a semi-synthetic fat-free diet. When unlabelled 18 : 3 n-3 was added to the incubation of ^{14}C 18 : 2 or ^{14}C 20 : 3, the desaturation rates decreased. Δ6 Desaturation was more strongly inhibited than Δ5 desaturation. The position isomer, γ-linolenic acid, had no significant effect on Δ6 and Δ5 desaturation.

In the second study, the rats were fed a diet including α-linolenic acid in the form of rapeseed oil containing no erucic acid. The control animals were fed a peanut oil diet. When α-linolenic-fed rats were compared to control animals, we found that Δ6 desaturase activity was lower in the α-linolenic-fed animals. In contrast, there was no significant effect on the desaturation rate when ^{14}C 20 : 3 n-6 was used as a substrate.

Our results lead to the conclusion that α-linolenic acid, when given to rats as dietary rapeseed oil, partly inhibited Δ6 desaturation *in vitro,* but did not affect significantly subsequent Δ5 desaturation in the biosynthesis of arachidonic acid. The inhibition of the Δ5 desaturation step, when α-linolenic acid was added to the incubation, might be due to an unknown competitive mechanism. These observations have been discussed in relation with the current literature.

Introduction.

Dietary α-linolenic acid (18 : 3 n-3) tends to decrease the concentration of arachidonic acid (20 : 4 n-6) in animal tissue lipids (Hwang and Caroll, 1980 ; Mohrhauer and Holman, 1963 ; Rahm and Holman, 1964). Research *in vitro* on isolated rat liver microsomes (Brenner and Peluffo, 1966, 1969) has explained this process by showing that α-linolenic acid inhibits desaturation of linoleic acid (18 : 2 n-6), the first step of the conversion :

$$18 : 2 \text{ n-6} \xrightarrow{\Delta6 \text{ desaturation}} 18 : 3 \text{ n-6 (γ-linolenic acid)}$$
$$\downarrow \text{Elongation}$$
$$20 : 4 \text{ n-6} \xleftarrow{\Delta5 \text{ desaturation}} 20 : 3 \text{ n-6 (dihomo-γ-linolenic acid).}$$

However, the putative effect of α-linolenic acid on other steps of the bio-synthesis of arachidonic acid have been little studied and merit attention.

This paper describes the $\Delta5$ desaturation of dihomo-γ-linolenic acid *in vitro* using homogenates of rat liver. We have demonstrated that α-linolenic acid inhibits this desaturation when present in the incubation medium, but that it has no effect when only given to the rat in the feed.

Our results on $\Delta6$ desaturation have been compared to the present results and discussed.

Material and methods.

Animals and diets. — We used male Wistar rats (Iffa-Credo, l'Arbresle) weighing about 60 g when they arrived in the laboratory ; they were given one of the three following diets for 15 days :
— a fat-free diet (UAR, Villemoisson/Orge) ;
— a diet including a 10 % weight of rapeseed oil containing no erucic acid (22 : 1 n-9) ; this diet was prepared from the fat-free diet by replacing an equal weight of carbohydrates ;

TABLE 1

Composition of the diets used (in g/100 g).

Defatted casein	23
Starch and saccharose	63 or 53
Salt mixture (ref. 205 f UAR)	7
Cellulose	6
Vitamins (ref. 200 UAR)	1
Lipids (vegetable oil)	0 or 10

TABLE 2

Fatty acid composition of the dietary oils (in % of total fatty acid).

Fatty acids	Rapeseed oil	Peanut oil
16 : 0	6.4	12.0
16 : 1	0.6	0.5
18 : 0	1.5	3.6
18 : 1 n-9	58.5	57.0
18 : 2 n-6	21.5	21.8
18 : 3 n-3	9.5	—
20 : 0	0.7	2.0
20 : 1	1.2	1.2
22 : 0	0.1	1.7
others	—	0.2
$\dfrac{18 : 3 \text{ n-3}}{18 : 2 \text{ n-6}}$	0.44	—

— a diet including a 10 % weight of peanut oil and prepared in the same way using the fat-free diet.

The composition of the diets and the fatty acid composition of the oils used are given on tables 1 and 2.

Radioactive substrates and other reagents. — [1 ^{14}C] 18 : 2 n-6 and [2 ^{14}C] 20 : 3 n-6 acids (The Radiochemical Centre, Amersham), having a specific activity of 51 mCi/mmol (*), were diluted with the corresponding radioactive fatty acids (Nu Chek Prép., Elysian) to obtain ethanol solutions having a specific activity of about 5 mCi/mmol (200 nmol/100 µl of solution).

Non-radioactive α-linolenic and γ-linolenic acids (Nu Chek Prép., Elysian) were used in ethanol solutions at a concentration of 30 nmol/100 µl.

The coenzymes and other reagents were pure products from Sigma or Merck.

Preparation of the homogenates. — Immediately after the rats were killed, 2 g of the liver were homogenized and ground in a Potter with 18 ml of a 0.25 M saccharose solution and a 0.05 M phosphate buffer, pH 7.4, at 4 °C. The ground liver was centrifuged at a minimum of 8 000 × g for 20 min. The supernatant, containing microsomes and cytosol and used without further preparation, will be referred to as the homogenate. We determined its protein content by the biuret reaction (Layne, 1957).

Measurement of desaturase activity. — Incubations were carried out in open recipients with constant stirring at 37 °C. Each incubation medium included in µmol and for a total volume of 6 ml : phosphate buffer at pH 7.4 : 600 ; MgCl$_2$: 30 ; ATP : 20 ; coenzyme A : 1 ; NADPH : 2.5 ; [1 ^{14}C] linoleic acid or [2 ^{14}C] dihomo-γ-linolenic acid : 0.010 to 0.040. The amount of α-linolenic acid added varied with the experiment. The homogenate, corresponding to between 4 and 20 mg of protein, was added last and determined time 0 of the reaction.

Incubation was stopped by adding 12 % alcoholic potassium hydroxyde, and the solution was refluxed for 1 h. The fatty acids released by the addition of hydrochloric acid were extracted by ethyl ether. After evaporation of the ether and alcohol drying, the fatty acids were methylated (Stoffel *et al.,* 1959). After the methyl esters had been extracted by pentane and purified on a silicic acid column, they were separated by thin-layer chromatography on Merck silica gel H impregnated with 12.5 % of silver nitrate (eluent : ethyl ether/petroleum ether 50/50 V/V) or by gas-liquid radiochromatography with radioactive recovery (Bézard *et al.,* 1964). The radioactivity of each fraction was measured by liquid scintillation in a Packard counter (Prias PLD Tricarb) to obtain the conversion percentage, *i.e.* the ratio of the radioactivity of the desaturation product to initial substrate radioactivity. From this ratio, we deduced specific desaturase activity, expressed in pmol of substrate converted per min and per mg of homogenate protein.

(*) Abbreviations used : mCi = millicurie ; mmol = millimole ; nmol = nanomole ; pmol = pico-mole ; ml = milliliter ; µl = microliter.

The agreement between the values obtained by thin layer or gas chromatography was satisfactory (Blond, 1981).

Results.

Effect of incubation time and protein concentration in the medium. — Figure 1 shows that the kinetics of the reaction with both substrates was linear in time when incubation lasted less than 30 min. Moreover, enzyme activities were proportional to the protein concentration up to values ranging between 10 and 15 mg per incubation (fig. 2).
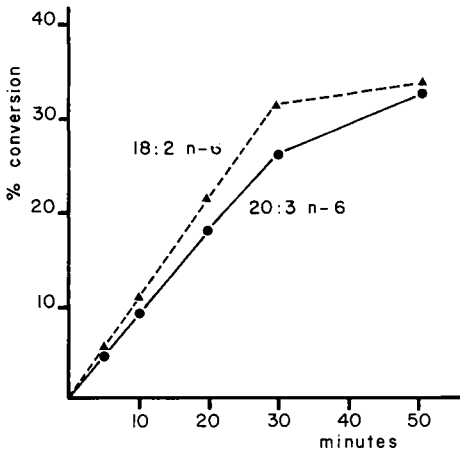

FIG. 1. — *Effect of incubation time on the desaturation of ^{14}C substrates using rat liver homogenates.* Means of two experiments per substrate carried out on rats fed the fat-free diet ; 10 mg of homogenate protein and 40 nmol of ^{14}C substrate per incubation. The other incubation conditions are described in Material and methods.
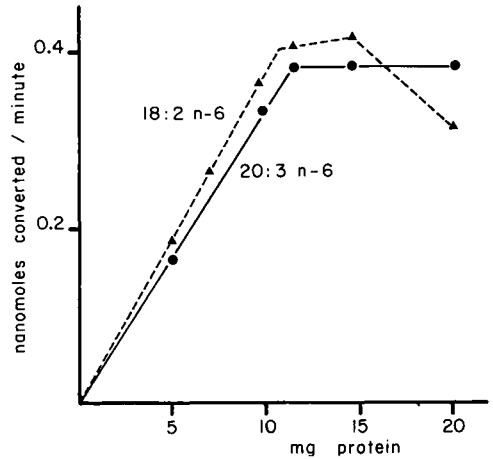
FIG. 2. — *Effect of the amount of homogenate proteins in the medium on the desaturation activity with each ^{14}C-substrate.* Means of two experiments per substrate carried out on rats fed the fat-free diet ; 40 nmol of ^{14}C substrate per 30 min incubation. The other incubation conditions are described in Material and methods.

These preliminary results showed that the optimal incubation conditions for determining desaturase activity were 10 mg of homogenate protein and 10 or 30 min of incubation. We therefore used these criteria during the rest of the study.

Effect of adding α-linolenic acid to the incubation medium

— In the first set of experiments, we incubated increasing amounts of each substrate and compared desaturase activity with or without α-linolenic acid. The results on table 3 show that this fatty acid inhibited both the desaturation of linoleic acid and that of dihomo-γ-linolenic acid. When inhibitor concentration

was equal to that of the substrate (30 nmol/incubation), Δ6 desaturase activity was reduced by 55 % and Δ5 desaturase activity by less than 30 %.

TABLE 3

Desaturase activity of rat liver homogenates with (+) or without (0) 30 nmol of α-linolenic acid (in pmol of substrate converted/min/mg of protein ± SD).

Substrate	Addition of 18 : 3 n-3	Nanomoles of substrate per incubation			
		10	20	30	40
18 : 2 n-6 [14C]	+		16.1 ± 0.3 (3)	17.7 ± 3.0 (3)	18.6 ± 0.6 (2)
	0		30.0 ± 1.2 (2)	39.0 ± 1.0 (2)	47.6 ± 0.7 (2)
20 : 3 n-6 [14C]	+	15.9 ± 1.3 (2)	24.0 ± 12 (3)	31.2 ± 2.4 (2)	42.8 (1)
	0	25.8 ± 0.4 (2)	34.6 ± 1.4 (3)	42.9 ± 4.8 (3)	48.4 (1)

The rats were fed the fat-free diet. Results were obtained after 10 min incubation with 10 mg of homogenate protein. The numbers in parenthesis are the numbers of experiments carried out.

— In the second set of experiments, we incubated 40 nmol of each substrate with increasing amounts of α-linolenic acid (fig. 3). Δ6 Desaturation decreased rapidly as inhibitor concentration increased ; Δ5 desaturation first decreased, then tended to stabilize at about 60 % of the desaturase activity measured without the inhibitor.

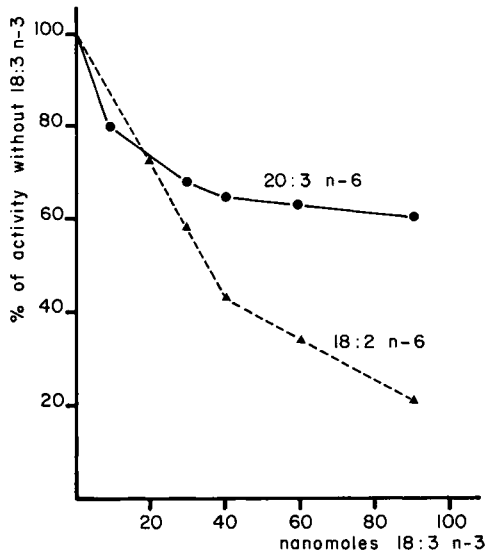FIG. 3. — *Effect of increasing amount of α-linolenic acid on the desaturation of the 14C substrates, expressed as the % of specific activity without α-linolenic acid in the medium.* The values correspond to the mean of results obtained from two experiments with 40 nmol of substrate and increasing amounts of 18 : 3 n-3 acid. Incubation time : 10 min ; fat-free diet.

Effect of adding γ-linolenic acid to the incubation medium. — To determine if α-linolenic inhibition was specific, we carried out a series of incubations with its position isomer, γ-linolenic acid ; the results on table 4 show that γ-linolenic acid inhibited neither of the two desaturations in our experimental conditions.

TABLE 4

Desaturase activity of rat liver homogenates using increasing amount of γ-linolenic acid
(in pmol of substrate converted/min/mg of protein).

Nanomoles of 18 : 3 n-6 per incubation		0	10	20	30
Substrate	18 : 2 n-6 [14C]	37.7	40.7	39.3	40.1
	20 : 3 n-6 [14C]	32.8	32.4	34.7	39.2

The rats were fed the fat-free diet. 10 mg of homogenate protein and 40 nmol of [14C] substrate were incubated for 30 min.

Effect of dietary α-linolenic acid. — Table 5 shows the Δ5 and Δ6 desaturase activities of homogenates from rats given rapeseed or peanut oil. The main difference between these two dietary oils concerns α-linolenic acid which is not found in peanut oil (table 2). Δ6 Desaturation of linoleic acid was clearly lower in rats given the rapeseed oil than in those given peanut oil or the fat-free diet (table 3). On the contrary, Δ5 desaturation was not significantly different in the two types of rats but was higher than in those fed the fat-free diet (table 3).

TABLE 5

Effect of dietary α-linolenic acid on the desaturation of [14C] substrates. Desaturase activity
(pmol of substrate converted/min/mg of homogenate protein).

Substrate [14C]	18 : 2 n-6			20 : 3 n-6		
Nanomoles of substrate	20	30	40	20	30	40
Rapeseed diet (a)	16.5 (14.9-18.1)	19.8 (16.9-22.8)	17.8 (16.4-19.2)	39.3 (36.8-41.8)	49.6 (47.2-52.0)	55.1 (52.2-58.0)
Peanut diet (b)	27.3 (23.8-30.8)	30.6 (27.4-33.8)	29.0 (27.0-31.0)	35.7 (34.6-36.8)	54.0 (51.4-56.6)	52.6 (52.0-53.2)
Ratio $\frac{a}{b}$ × 100	60.4	64.7	61.4	110	91.9	104.8

The results are the means of two experiments per diet ; individuals values are indicated in parenthesis. Incubation conditions were the same as for rats fed the fat-free diet (table 3).

Discussion and conclusions.

It is well known that desaturases are situated in the endoplasmic reticulum of the cell (Brenner, 1974), and that linolenyl-coenzyme A is the substrate of the desaturation (Brenner, 1971). However, although it was shown that dihomo-γ-linolenyl-coenzyme A was a putative substrate in Δ5 desaturation (Stoffel, 1961), more recent research has demonstrated direct desaturation of the rest of the dihomo-γ-linolenyl esterified at the inner position of lecithins by rat liver microsomes (Pugh and Kates, 1977). In any case, thioester is formed from the fatty acid before desaturation. For this reason, we preferred to use the supernatants of centrifugation at 8 000 × g, which includes microsomes and hepatic cytosol that increase acyl--coenzyme A synthetase activity (Lippel, 1971) and favor thioester desaturation (Jeffcoat et al., 1978).

Our results concerning the inhibition of linoleic acid desaturation by α-linolenic acid agree with those of other authors using microsomes (Brenner and Peluffo, 1966, 1969). According to these authors, linoleic acid and α-linolenic acid compete for the same enzyme, Δ6 desaturase, that has a higher affinity for the triene than for the diene. Moreover, our results demonstrate that α-linolenic acid in the incubation medium also inhibits Δ5 desaturation of dihomo-γ-linolenic acid (table 3, fig.3). This effect was specific as long as its isomer, γ-linolenic acid, had no inhibitory effect in the same conditions (table 4).

Considering the complexity of the reactions obtained (including the activation of dihomo-γ-linolenic acid, Δ5 desaturation of thioester and possibly incorporation into microsomal phospholipids), it is difficult to draw any conclusions as to the exact nature of the reaction inhibited by α-linolenic acid. The latter itself is a substrate for acyl-coenzyme A synthetase but this reaction does not appear to be limiting in the homogenate system we used since γ-linolenic acid, also a substrate of the enzyme, had no inhibitory effect (table 4).

It is interesting to note that, in spite of relatively high concentrations of α-linolenic acid in the incubation medium, Δ5 desaturation tended to stay at a residual level of about 60 % of the initial activity (fig. 3). This suggests that this residual activity might be that of an enzyme not inhibited by α-linoleic acid. If the two substrates proposed in the literature (thioester on the one hand and 1-acyl-2-dihomo-γ-linonelyl phosphorylcholine on the other). were desaturated by two specific enzymes, only one of them might be affected by α-linolenic acid in the incubation medium.

In the second part of this study, we showed that α-linolenic acid from dietary rapeseed oil did not affect Δ5 desaturation in vitro in rat liver homogenates, but lowered significantly the Δ6 desaturation of linoleic acid (table 5). These results confirm those obtained in vivo in our laboratory (Lemarchal et al., 1976 ; Blond et al., 1978 ; Blond, 1981). We know that dietary α-linolenic acid is rapidly converted into higher polyenoic acids in n-3 (Klenk and Oette, 1960), the first step being Δ6 desaturation as for linoleic acid. As compared to homogenates from control animals, those from the rapeseed oil-fed rats showed a lower value for arachidonic acid, and a higher one for polyenoic fatty acids of the n-3 family,

particularly α-linolenic acid (table 6). These n-3 polyenes might have been liberated through lipid hydrolysis during the incubations, thus affecting the $\Delta 6$ desaturation of linoleic acid directly. However, it is difficult to conclude whether the inhibition shown is due to linolenic acid or to its longer chain metabolites.

TABLE 6

Fatty acid composition of the lipids extracted from rat liver homogenates (% of total fatty acids).

Fatty acids	Rapeseed diet	Peanut diet
14 : 0	3.1	6
16 : 0	23.6	20.5
16 : 1	3.7	3.2
18 : 0	11.7	11.3
18 : 1	28.8	27.4
18 : 2 n-6	12.4	11.1
18 : 3 n-3	2.0	–
20 : 3 n-6	1.0	0.7
20 : 4 n-6	8.1	12.3
22 : 4 / 22 : 5 n-6	1.6	4.0
22 : 5 / 22 : 6 n-3	2.7	1.8

The homogenates were those used for incubation experiments and were prepared as described in Material and methods. Lipids were extracted and their fatty acids further analysed by gas-chromatography. Means of 4 determinations.

Rather recent studies (Hassam, 1977) have demonstrated that an elevated supply of dietary α-linolenic acid can partly inhibit the conversion of γ-linolenic acid into arachidonic acid, which involves the $\Delta 5$ desaturase. The observations of Hassam were made during nutritional experiments using dietary ratios of 18 : 3 n-3/18 : 3 n-6 that were very much higher than 1, while the ratio n-3/n-6 in rapeseed oil is about 0.44 (table 2). This fact supports the conclusions that the inhibitory effect of dietary α-linolenic acid on the biosynthesis of arachidonic is related essentially, if not solely, with linoleic acid desaturation.

Résumé. *Influence de l'acide α-linolénique sur la désaturation de l'acide dihomo-γ-linolénique par des homogénats de foie de rat.*

Les effets de l'acide α-linolénique (18 : 3 n-3) sur la $\Delta 5$ désaturation de 20 : 3 n-6 (acide dihomo-γ-linolénique $\rightarrow$ linolénique) $\rightarrow$ 20 : 4 n-6 (acide arachidonique) et la $\Delta 6$ désaturation de 18 : 2 n-6 (acide linoléique) $\rightarrow$ 18 : 3 n-6 (acide γ-linolénique) sont étudiés chez le rat. Deux études différentes sont conduites en utilisant des homogénats de foie.
Dans la première étude, des rats reçoivent un régime semi-synthétique lipidoprive. Si 18 : 3 n-3 non radioactif est additionné aux incubations de 18 : 2 ^{14}C ou de 20 : 3 ^{14}C, les vitesses de désaturation sont abaissées. La $\Delta 6$ désaturation est plus fortement inhibée que

la Δ5 désaturation. Pour un rapport inhibiteur/substrat de 1 : 1 (30 nanomoles), 18 : 3 n-3 réduit l'activité de Δ6 désaturation à 55 %, tandis que l'activité de Δ5 désaturation est abaissée à moins de 30 %, dans ces conditions. 18 : 3 n-6, l'isomère de position, n'a pas d'effet significatif sur les désaturations (Δ6 et Δ5).

Dans la seconde étude, quelques animaux reçoivent un régime contenant de l'acide α-linolénique, sous forme d'huile de colza (sans acide érucique). Des animaux témoins reçoivent un régime à l'huile d'arachide. En comparant les résultats des deux lots de rats, on trouve que l'activité de Δ6 désaturation a été abaissée par l'α-linolénate alimentaire. Au contraire, on n'observe pas d'effet significatif pour la Δ5 désaturation.

Les résultats montrent que l'acide α-linolénique, qui est présent dans quelques huiles (telles que huile de colza et huile de soja) inhibe partiellement la désaturation *in vitro* de l'acide linoléique, mais ne doit pas affecter les réactions suivantes intervenant dans la biosynthèse de l'acide arachidonique. L'inhibition de la Δ5 désaturation, si 18 : 3 n-3 est présent dans le milieu d'incubation, peut être due à un mécanisme compétitif, non connu jusqu'ici. Les observations sont discutées en liaison avec la littérature.

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