The Activating Effect of Dietary Protein on Linoleic Acid Desaturation

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ABSTRACT

The desaturation of ^{14}C-1-linoleic acid to γ-linolenic acid and their incorporation into the microsomal lipids of rats fed on a balanced diet and a protein diet were measured in vitro. It was shown that a protein diet does not change significantly the distribution of the radioactivity among the different lipidic fractions compared to the animals fed on a balanced diet. However the microsomal desaturation of linoleic acid to γ-linolenic acid increased in the rats maintained on a protein diet. Besides, the amount and composition of the free fatty acids present in the microsomes of the animals fed on both diets were similar enough to discard the hypothesis that they may modify the desaturation of linoleic acid produced by the diet. The enzymic activity of the linoleyl desaturase of liver microsomes of animals fed on a protein diet, measured in substrate saturating conditions, is greater than in animals with balanced diet. Consequently the results support the hypothesis that a protein diet increases specifically the desaturating activity of the microsomes.

INTRODUCTION

Studies on the biosynthesis of polyenoic fatty acids in liver microsomes from normal rats have demonstrated that a protein diet produces an increase in the desaturation of linoleic acid to γ-linolenic acid (1). Considering that this increase produced by the diet is maintained during 24 hr but is not evoked when Actinomycin D, Puromycin or Cycloheximide are simultaneously injected, it was thought that the protein diet probably increased the linoleic acid desaturation by induction of the 6-desaturase (1). Nevertheless, such an effect could be produced by another mechanism.

One of the characteristics of liver microsomes is that they contain a large amount of phospholipids, especially phosphatidylcholine, and a smaller amount of triglycerides, cholesterol, cholesterol esters and free fatty acids (2). Besides, the enzymes that intervene in the synthesis and degradation of the different lipidic fractions are present in the microsomes. Brenner et al. (3) have demonstrated that liver microsomes not only convert linoleic acid into linoleyl-CoA and desaturate it into γ-linolenyl-CoA, but also incorporate both acids into phospholipids. The same authors have found evidence of the competition of the synthesis of phospholipids with the desaturation reaction for the fatty acids in the microsomes (4). Therefore the effect of the proteins could be produced through a modification of phospholipid synthesis and also by a change in the distribution of the microsomal lipidic fractions. This change would produce a variation in the total proportion of linoleic acid and γ-linolenic acid. For this reason the incorporation of linoleic acid in the microsomal lipids and the desaturation to γ-linolenic acid were studied in animals fed on a balanced diet and a protein diet.

On the other hand, the desaturation of linoleic acid to γ-linolenic acid measured in vitro depends on the presence and concentration of other free fatty acids in the medium, as it has been demonstrated before (5). Therefore we also considered it of importance to investigate the amount and composition of the free fatty acids of the microsomes before and after the incubation, of animals fed on a balanced or protein diet.

MATERIAL AND METHODS

Animals

Adult female Wistar rats weighing 250-300 g and maintained on standard Purina chow were used.

Treatment of Animals

The animals were divided into two groups of five animals each. One group was fed a Purina chow diet and water ad libitum, and was used as a control. The other group was force-fed a 20% suspension of casein (Casenolin, Glaxo-Argentina) for 48 hr. These animals received isocaloric diet (25 kcal/100 g body wt), and the total daily food intake was administered accordingly at 4 hr intervals. Water was given ad libitum.

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TABLE I

Effect of Dietary Protein on the Per Cent Distribution of Labeled Fatty Acids in the Microsomal Lipids After Incubation[a]

Thin layer chromatography zones	Incubation time			
	5 min		20 min	
	Balanced diet	Protein diet	Balanced diet	Protein diet
Origin[b]	0.8[c]± 0.13	0.7 ± 0.1	1.5 ± 0.3	1.5 ± 0.3
Lisophosphatides	0.7 ± 0.07	0.7 ± 0.1	1.5 ± 0.2	1.6 ± 0.2
Choline phosphatides	12.0 ± 2.7	11.1 ± 2.3	29.8 ± 6.3	32.3 ± 3.7
Ethanolamine phosphatides	13.3 ± 2.0	9.1 ± 1.9	8.4 ± 0.5	6.4 ± 0.8
Free fatty acids	25.4 ± 4.8	31.1 ± 4.9	13.1 ± 0.8	11.8 ± 2.0
Neutral lipids	47.8 ± 3.7	47.3 ± 4.6	45.7 ± 6.4	46.4 ± 4.2

[a]10 nmoles of ^{14}C-1-linoleic acid were incubated with 2 mg microsomal protein at 25 C with the cofactors detailed in the Materials and Methods.

[b]Lipids were separated in choloroform-methanol-water 65:25:3 v/v/v.

[c]Mean values of five observations ± one standard error of the mean (SEM).

Isolation of Microsomes and Assay Procedure

The rats were killed by decapitation without anesthesia. The liver was rapidly excised, and placed immediately in ice cold homogenizing medium. The liver was then homogenized in a cold solution (3:1 v/w) consisting of 0.15 M KCl, 0.005 M $MgCl_2$, 0.004 M EDTA, 0.004 M N-acetyl-cysteine, 0.05 M phosphate buffer (pH 7), and 0.25 M sucrose. Cell debris and mitochondria were removed by sedimentation at 12,000 x g for 10 min at 0 C. The microsomes were isolated in the cold by differential centrifugation at 140,000 xg for 60 min in a Spinco Model L2 centrifuge as described previously (3,6).

The desaturation of linoleic acid to γ-linolenic acid by liver microsomal preparation was measured by estimation of the conversion per cent of ^{14}C-1-linoleic acid (53.0 mci/mmole; 98% Radiochemically pure, Radiochemical Centre, Amersham, England) to γ-linolenic acid. Ten nmoles of labeled linoleic acid were incubated with 2 mg of microsomal protein in a Dubnoff Shaker at 25 C for 5 or 20 min in a total volume of 3 ml of a 0.15 M KCl, 0.25 M sucrose solution containing 4 μmoles ATP, 0.2 μmoles CoA, 2.5 μmoles NADH, 15 μmoles $MgCl_2$, 4.5 μmoles glutathione, 125 μmoles NaF, 1 μmoles nicotinamide and 125 μmoles phosphate buffer (pH 7). The distribution of radioactivity between substrate and product was measured by gas liquid radiochromatography of the methyl esters in a Pye apparatus with a proportional counter under the conditions described in a previous work (1).

TABLE II

Specific Radioactivity of Thin Layer Chromatography Fractions, cpm/μmole[a]

Fraction	Balanced diet	Protein diet
Choline phosphatides	7130[b]± 1700	13150 ±5200
Ethanolamine phosphatides	13720 ± 3400	17790 ± 4400
Neutral lipids	9555 ± 2300	13140 ± 3200

[a]Experimental conditions as in Table I. Microsomes incubated for 20 min.

[b]Mean values ± 1 SEM.

To measure the specific activity of linoleic acid desaturase, the effect of time, substrate concentration and amount of enzyme were specially tested and fixed. The incubation medium contained: 4 μmoles ATP, 0.2 μmoles CoA, 2.5 μmoles NADH, 7.5 μmoles $MgCl_2$, 2.25 μmoles glutathione, 62.5 μmoles NaF, 0.5 μmoles nicotinamide and 62.5 μmoles phosphate buffer (pH 7) in a total volume of 1.5 ml 0.15 M KCl and 0.25 M sucrose solution. The incubation procedure was the same as before.

The effect of time of incubation on the linoleic acid converted to γ-linolenic acid is shown in Figure 1. Up to 30 min the conversion increased linearly with time. Therefore 30 min of incubation were chosen to get the highest possible yield and increase the accuracy of the measurement. Besides, the linoleic acid desaturation was proportional to the amount of enzyme up to 5 mg of microsomal protein. For this reason the desaturating specific activity of the microsomes was measured in the presence of 5 mg microsomal protein during 30 min and with increasing concentrations of ^{14}C-1-linoleic acid from 3.3 x 10^{-2} mM to 13.2 x 10^{-2} mM (diluted with unlabeled fatty acid to maintain 200,000 cpm in each tube).

Lipid Fractionation

The lipids of the incubation system were

TABLE III

Desaturation Per Cent of Linoleic to γ-Linolenic Acid in Lipidic Fractions[a]

Thin layer chromatography zones	Incubation time			
	5 min		20 min	
	Balanced diet	Protein diet	Balanced diet	Protein diet
Origin	9.4	14.1	30.1	42.0
Lisophosphatides	9.1	11.3	28.6	33.5
Choline phosphatides	3.9	7.4	12.1	17.6
Ethanolamine phosphatides	4.1	11.0	9.4	24.0
Free fatty acids	3.0	3.5	18.8	32.9
Neutral lipids	5.0	10.3	10.1	16.7
Total lipids	6.4	9.2	18.7	26.1

[a]Experimental conditions as in Table II.

extracted with chloroform-methanol 2:1 v/v. The lipid extract obtained was freed of nonlipid impurities by the procedure of Folch et al. (7), concentrated in a rotary evaporator at room temperature, made up in chloroform-methanol 2:1 v/v, and stored at 4 C. Aliquots of this stock solution were evaporated under nitrogen and analyzed by thin layer chromatography (TLC) on plates of Silica Gel G (Merck). The lipids were separated with chloroform-methanol-water 65:25:3 v/v/v. The spots were compared to standards run at the same time. They were developed with iodine vapor. The corresponding fractions of the samples were scraped off and either phosphorous determined according to Doizaki and Zieve (8) or fatty acid ester groups following Rapport and Alonzo (9). Aliquots were counted in a Mark 1, Nuclear Chicago scintillation counter with scintillation solution prepared according to Bray (10). Other aliquots were heated with methanolic 3 M HCl for 3 hr at 68 C and label distribution in fatty acid methyl esters analyzed by gas liquid radiochromatography as formerly described (1).

Free Fatty Acids

The free fatty acid content of the microsomes was determined by TLC on plates of Silica Gel G (Merck). They were separated with redistilled petroleum ether-ethyl ether-glacial acetic acid 70:30:1.2 v/v/v. Standards of pure linoleic acid (10, 20 and 40 nmoles) were run at the same time. Lipid components were located by aqueous sulfuric acid (1:1 v/v) spray and the plates were heated for 30 min at 180 C. The amount of fatty acids was measured with a Zeiss PMQ II spectrophotometer for chromatograms. The light absorbed by the spots of the fatty acids was measured and compared with the standards.

The composition of the free fatty acids separated by TLC was determined by analyzing their methyl esters by gas liquid chromatography in a Pye apparatus with an argon ionization detector. Six foot columns heated to 180 C and packed with 10% polyethyleneglycol succinate chromosorb W (80-100 mesh) were used. The main peaks were identified by comparison of the adjusted retention times relative to stearate with the corresponding standards. Fatty acid composition was calculated measuring the surface of the peaks by triangulation.

TABLE IV

Free Fatty Acids in Microsomes, nmoles/mg protein

Diet	Before incubation	After incubation
Balanced	34.8[a]± 3.3	20.5 ± 1.3
	P>0.05	
Protein	48.1 ± 7.0	19.5 ± 3.4
	P<0.001	

[a]Mean values ± 1 SEM.

RESULTS AND DISCUSSION

In our experimental conditions, the desaturation of linoleic acid to γ-linolenic acid is performed in the microsomes through a series of reactions. The linoleic acid is first converted

TABLE V

Composition of Microsomal Free Fatty Acids of Animals Fed on Balanced Diet and Protein Diet

Fatty acids	Balanced	Protein
C 16	36.0	37.8
C 16:1	4.2	6.7
C 18	24.3	25.3
C 18:1	13.4	13.4
C 18:2	2.5	2.2
C 20:4	10.7	10.4
Others	8.9	4.3

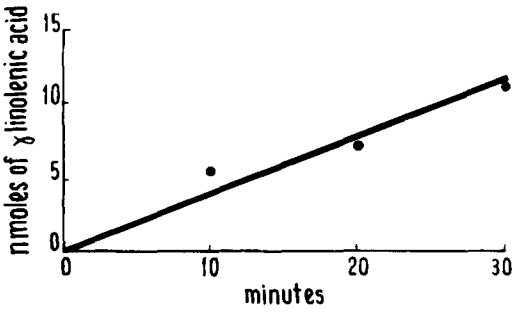

FIG. 1. Effect of time on the speed of linoleic acid desaturation to γ-linolenic acid. Five milligrams microsomal protein incubated with 100 nmoles of labeled linoleic acid.

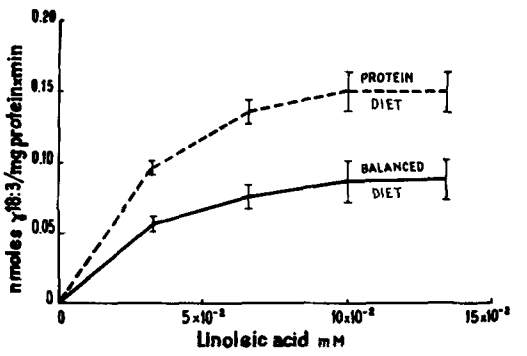

FIG. 2. Effect of a protein diet on the substrate saturation curve of linoleic acid desaturation.

into linoleyl-CoA and then at least two types of reactions are performed: the desaturation into γ-linolenyl-CoA on one hand, and the esterification to synthesize principally phospholipids and triglycerides on the other. The esterification of linoleyl-CoA must be considered as a reaction parallel to the desaturation, and if it decreases under certain conditions, it may consequently favor the desaturation. Because of these reasons, liver microsomes were incubated with ^{14}C-1-linoleic acid in desaturating conditions, studying the distribution of radioactivity among the different microsomal lipidic fractions (Table I).

The results in Table I show the per cent of labeling of the different lipidic fractions of the microsomes after 5 and 20 min of incubation. They show a rapid labeling in all the lipids, including a remarkable incorporation in the neutral lipids. The radioactivity in the free acids decreases with the increase of the time of incubation. From 5 to 20 min this decrease is correlated with an increase of the per cent of radioactivity in the phosphatidylcholine fraction. After 20 min of incubation it is twice as high as that corresponding to the 5 min. During this interval the incorporation into neutral lipids remains constant. Consequently the labeled acids would be incorporated principally by the lecithin during this period. It may be pointed out that the fact that incorporation of the labeled acid among the fractions of neutral lipids remains constant for 15 min, after a rapid incorporation during the first 5 min, by no means implies a static process, but the existence of a turnover, as is demonstrated by the increase in the levels of γ-linolenic acid between the 5th and 20th min of incubation (Table III).

The data in Table I also show that a protein diet does not change significantly the distribution of the radioactivity among the free acids and the different lipidic fractions when compared to the animals fed on a balanced diet.

The measuring of the specific activity of the phosphatidylcholine, phosphatidylethanolamine and triglyceride fractions (Table II) also shows similar values for the normal rats and the rats maintained on a protein diet.

Table III shows the per cent of distribution of radioactivity among linoleic acid and its conversion product, γ-linolenic acid, in each of the lipidic fractions. The microsomes were incubated 5 and 20 min. The data indicate that there is an increase in the conversion per cent of linoleic acid to γ-linolenic acid along with the time of incubation, and that this increase is shown in all the lipidic fractions when the rats are maintained either on a normal or a protein diet. Besides, the protein diet increases the microsomal desaturation of linoleic acid to γ-linolenic acid, and this increase is distributed in a similar way among all the lipidic fractions.

Therefore, and considering that in our experimental conditions the incorporation of labeled acids into the different lipidic fractions of the microsomes is not substantially modified when a protein diet is administered, and that on the other hand the desaturation is increased in all the same fractions, we may suppose that the increase of desaturation cannot be attributed to a modification of lipid synthesis or to a change in the incorporation of substrate or product, or both, in the lipids. Therefore the protein diet apparently produces the increase of linoleic acid desaturation by means of a different mechanism.

In previous experiments we have demonstrated that the microsomal conversion of linoleic acid to γ-linolenic acid, measured in vitro, depends on the presence and concentration of other fatty acids in the incubation medium (3,5,11,12). Considering that the increase of desaturation observed in the liver microsomes caused by a protein diet could depend on a modification in the concentration of free fatty acids in the microsomes, we

measured the amount of free fatty acids present in them, before and after the incubation period, and for animals fed on both diets. The results are summarized in Table IV. As can be observed although there are significant differences in the fatty acid content of the microsomes before and after incubation ($P<0.001$), there are not significant differences between the control group and the group with protein diet either before or after the incubation. So we can apparently reject the possibility of a modification in the microsomal free fatty acid content being responsible for the increase in the desaturation produced by the protein diet in our experimental conditions.

Nevertheless, considering that the microsomal desaturation of linoleic acid to γ-linolenic acid is modified in different ways by different fatty acids (3,5), it was necessary to check the composition of the free acids of the liver microsomes. The results appear in Table V and they show, as expected, that the composition does not have comparatively important variations between the control animals and the ones maintained on a protein diet.

Therefore the increase of the desaturation of linoleic acid produced by the protein diet cannot be attributed to a change of the composition of the microsomal free fatty acids induced by the protein diet.

Finally, Figure 2 compares the effect of a protein diet and a balanced one in the microsomal desaturation velocity of linoleic acid to γ-linolenic acid for different concentrations of substrate. In these experimental conditions, substrate saturation curves are found, and the effect of collateral reactions on linoleic acid desaturation are undoubtedly minimized when rising the high concentrations of substrate. Therefore, in these conditions and at the plateau, they may measure with rather good reliability the specific desaturating activity of the microsomes for linoleic acid. It can be observed that in these experimental conditions the enzymic activity of liver microsomes is greater in the rats fed a protein diet than in the animals with normal balanced diet. Consequently the data collected in this series of experiments would add considerable evidence to the hypothesis that a protein diet increases specifically the desaturating activity of the microsomes. Its importance and its mechanism of activation are still to be further investigated.

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