Kinetics of linoleic and arachidonic acid incorporation and eicosatrienoic depletion in the lipids of fat-deficient rats fed methyl linoleate and arachidonate

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SUMMARY Changes induced by dietary methyl linoleate and arachidonate in the fatty acid composition of liver phosphatidyl choline, phosphatidyl ethanolamine, cholesterol esters, and triglycerides were investigated in essential fatty acid-deficient rats. The esters were fed for 0, 24, 50, 96, 192, and 360 hr. The lipids were fractionated by thin-layer chromatography and the fatty acid composition was estimated by gas liquid chromatography. Acids bound to the α'- and β-carbons of phosphatides were separated by lipolysis with phospholipase A.

From the compositions found it was deduced that both dietary linoleate and arachidonate inhibited eicosatrienoate synthesis from oleate but that only arachidonate replaced eicosatrienoate quantitatively in the β-position of lecithin and cephalin. Both dietary acids displaced some of the β-positioned oleate. Monoenoic: saturated acid ratios were also decreased by both esters both in triglycerides and in α'-bound acids in phosphatides. In triglycerides this change preceded any significant incorporation of linoleate or arachidonate. Arachidonate effects seemed to be more rapid and more marked than those of linoleate and although final compositions were similar, because of the conversion of linoleate into arachidonate, a different pattern of reactions led to these results.

KEY WORDS kinetics · linoleic · arachidonic · incorporation · eicosatrienoic depletion · liver lipids · rat · choline glycerophosphatides · ethanolamine glycerophosphatides · phospholipase A · triglycerides

ALTHOUGH THE effect of linoleic and arachidonic acids on the fatty acid composition of different animal tissues has been studied for more than 10 years, only a few studies (1) of the kinetics of linoleic acid incorporation have been published. Holman's group in particular has carried out careful studies of the modification of fatty acid composition of the lipids of rats fed different levels of linoleic and arachidonic acid (2, 3), but no complete information is given by them or by others about the rates of their incorporation and of eicosatrienoic depletion.

A preliminary study[1] in this laboratory of the effects of dietary linoleate and arachidonate upon different tissues of essential fatty acid-deficient rats showed, as expected, a rapid incorporation of both acids into phospholipid-rich organs (liver and heart) and a very slow one into epididymal fat. The incorporation of either acid into liver or heart lipids followed an exponential curve and evoked an exponential decrease of eicosatrienoate. Only in the case of arachidonate, however, did the increasing curve correspond to the decrease of eicosatrienoate. Linoleate produced a much slower decrease of eicosatrienoate in heart than in liver; in the former organ its decreasing rate corresponded rather well to the increase of arachidonate synthesized by the animal from linoleate. In liver no correspondence between the slopes of eicosatrienoate and of arachidonate synthesized by the rats from linoleate could be shown; further, eicosatrienoate started decreasing before arachidonate increased.

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[1] Nervi, A. M., and R. R. Brenner, *Acta Physiol. Lat. Amer.* Submitted for publication.

All these results were for total lipids. To understand more properly the details of the changes induced, attention was focused in the present study on the progressive changes induced by dietary methyl linoleate and arachidonate in the fatty acid compositions of individual liver lipids of fat-deficient rats. The changes are compared with changes found in epididymal fat and heart lipids under the same experimental conditions.

MATERIALS AND METHODS

Methyl linoleate (86% pure) was obtained from sunflower seed oil by the formation of urea adducts and fractional distillation. Methyl arachidonate (73% pure) was obtained from hog liver by fractional crystallization at −20° and −60° (4) and vacuum distillation. It contained 4.2% linoleate, 2.8% eicosatrienoate, 2.9 and 9.0% of unknown acids with retention times near to that of arachidonate, 1.7% eicosapentaenoate, and minor amounts of palmitate, stearate, oleate, and eicosaenoate.

Experimental Procedure

Weanling male albino rats from the strain of this Institute were raised on a semisynthetic fat-free diet described elsewhere (5). The constituents of the diet were: sucrose 77%, fat-free casein 19%, salt mixture 4%, and vitamins. After 90 days the rats were divided into 11 groups of six animals each. One group of animals was then sacrificed by ether anesthesia (group 0). Another five groups were fed on the fat-free diet supplemented with 110 mg of methyl linoleate per day. They were killed at 24 hr (group 1), 50 hr (group 2), 96 hr (group 3), 92 hr (group 4), and 360 hr (group 5) from the time they first received the ester. The other five groups of animals (groups 6–10) were fed on a fat-free diet supplemented with 110 mg of methyl arachidonate per day and were killed at the same periods of time. Whereas the basic diet was fed ad lib., the supplementary fatty esters were administered orally twice a day in 55-mg doses.

After the animals had been killed the livers were excised and weighed. They were pooled in the different groups and extracted immediately with chloroform–methanol 2:1 by the procedure of Folch, Lees, and Sloane Stanley (6). The lipid content was determined by vacuum evaporation of aliquots. Phosphorus was estimated by the procedure of Allen (7) and total cholesterol by the method of Abell, Levy, Brodie, and Kendall (8).

Separation of Triglycerides and Cholesterol Esters

Liver lipids were dissolved in chloroform and fractionated by adsorption on activated silicic acid (Baker, Analytical Reagent). Triglycerides and cholesterol esters were removed with chloroform and crude phospholipids were then obtained by repeated extraction with methanol. Pure triglycerides and cholesterol esters were obtained by thin-layer chromatography (TLC) on Silica Gel G (Merck, Darmstadt). Chromatoplates 20 × 20 cm and 0.5 mm in thickness were prepared by the procedure described by Stahl. The plates were developed (9) with petroleum ether (bp 60–80°)–ethyl ether–acetic acid 90:10:1 (v/v). Portions of the samples were run at the edges of the plate and components were located by means of iodine vapor; corresponding areas of the plate were scraped off and eluted.

Cholesterol was recognized by spraying with phosphotungstate (10). Cholesterol esters were extracted from the silica gel with methanol and saponified with methanolic KOH. Cholesterol was separated and the fatty acids were esterified with methanolic 3 N HCl at 60° for 20 min in stoppered tubes under nitrogen. The methyl esters were purified by sublimation (11), dissolved in petroleum ether (bp 30–40°), and kept in a freezer in stoppered conical tubes under nitrogen until they were analyzed by gas–liquid chromatography (GLC).

Triglycerides were methanolyzed without elution from the silica gel, using methanolic 3 N HCl for 3 hr at 60°. The methyl esters were purified and stored in the same way as those from cholesterol.

Phospholipids

Crude phospholipids were also fractionated by TLC, using chloroform–methanol–water 65:25:4 (v/v). The spots were located with iodine vapor. Ethanolamine glycerophosphatide (EGP), R_F 0.54, was recognized by comparison with a standard and by a positive ninhydrin reaction. Choline glycerophosphatide (CGP), R_F 0.32, was identified by means of a standard and its positive reaction with Dragendorff reagent (12); it gave no color with ninhydrin. EGP spots gave a weak positive reaction for aldehyde (13), showing that they contained some plasmalogen. Small amounts of these phosphatides were directly methanolyzed without elution, as described for triglycerides.

Phospholipid Lipolysis

The remaining EGP and CGP were eluted from the silica gel with chloroform–methanol 1:9 and hydrolyzed by snake-venom phospholipase A for 6 hr at room temperature. The incubation employed (14) ethereal emulsions of 10 mg of phospholipid in 1 ml of borate buffer (pH 7) containing 2.5 mM $CaCl_2$, with 2 mg of snake venom (*Crotalus adamantheus*, Ross Allen's Reptile Institute). The material was lyophilized, extracted with chloroform–methanol 1:9 and fractionated by TLC using chloroform–methanol–water 65:25:4. The spots were located with iodine vapor. Lysolecithin (R_F 0.08) and free fatty acids (R_F 0.82) produced from lecithin were identified by means of appropriate standards and by the

color reactions mentioned. No remaining CGP could be detected on the chromatoplates. Lysophosphatidyl ethanolamine (R_F 0.25) and free fatty acids (R_F 0.82) resulting from EGP were recognized similarly. Only very small amounts remained unhydrolyzed and appeared as a faint spot on the plates.

The fatty acid compositions found for the lyso derivatives corresponded to acids initially bound to the α'-carbon while the free fatty acids were from the β-position. Phospholipase A specificity for fatty acids bound to the β-carbon has been demonstrated by Tattrie (15) and corroborated by many others.

Calculated compositions of acids bound to α'- and β-carbons of CGP and EGP corresponded only approximately to the total fatty acid composition.

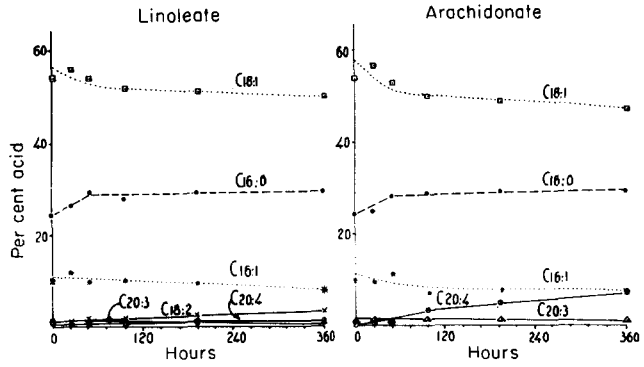

FIG. 1. Changes in the fatty acid composition of liver triglycerides of rats fed methyl linoleate or arachidonate after 3 months on a fat-free diet. Percentages plotted against hours after beginning the diet.

Gas–Liquid Chromatography

Fatty acid composition were determined by GLC in a Pye apparatus with argon ionization detector. The samples were run in 4-ft columns packed with 10% polyethylene glycol adipate on Celite (80–100 mesh) at 195°. Certain samples were also chromatographed on 10% Apiezon columns; other samples were hydrogenated and reanalyzed by GLC. The peaks of palmitate, palmitoleate, stearate, oleate, linoleate, eicosatrienoate, arachidonate, eicosapentaenoate, and docosahexaenoate were identified by comparison with the corresponding standards while the other acids were tentatively recognized by the procedure of Ackman (16, 17). GLC data were calculated as area per cent. Quantitative results with a sunflower seed oil standard[2] agreed with the stated composition data with a relative error of less than 8% for major components (>10% of total mixture) and less than 33% for minor components (<10% of total mixture). To check the statistical error of the estimated fatty acid compositions, the livers from three groups of animals fed linoleate and arachidonate were not pooled but individually analyzed, and standard errors were calculated. Standard errors of 0.05, 0.3, and 0.12 were found for octadecadienoate; 0.14, 0.85, and 0.75 for eicosatetraenoate; and 0.63, 0.26, and 0.17 for eicosatrienoate for rats of groups 0 (fat-free), 3 (fed linoleate 96 hr), and 8 (fed arachidonate 96 hr) respectively (6 rats in each group).

RESULTS

In the liver of fat-deficient rats, dietary linoleate or arachidonate caused a decrease in the total lipid content, which reached a nearly constant level after 4 days of supplementation. This decrease was attributable to loss of triglycerides and cholesterol esters, for phospholipids actually increased. Simultaneously with this mobilization of nonpolar lipids, a general modification in the fatty acid pattern of all the lipids took place. These changes were shown by plotting the composition against time and calculating the curves by the method of least squares (18).

Triglycerides

Oleic, palmitic, and palmitoleic acids were the main components both of liver triglycerides (Fig. 1) and of epididymal fat (not shown); minor amounts of polyunsaturated and stearic acids were present. The triglycerides slowly incorporated dietary linoleate and arachidonate during the 15 days of the experiment; higher percentages were reached in liver triglycerides than in epididymal fat in the same period of time. The most significant changes induced by both acids were a rapid depression of oleate levels with a corresponding increase in palmitate, shown both by liver triglycerides and by epididymal fat 2–3 days after supplementing the diet and before any important change in linoleate or arachidonate percentage was detected. After this early modification the proportion of monoenoic and saturated acids remained practically constant. This rapid change in the proportions of palmitic and oleic acids in epididymal fat is difficult to reconcile with the long average lives of fatty acids found in the same tissue by Stein and Stein (19), and the change is too large to be accounted for as a change in composition of the rapidly turning over compartment whose existence has been suggested by the same authors.

Cholesterol Esters

Oleic, palmitic, and palmitoleic acids were also the main fatty acids in liver cholesterol esters (Fig. 2), which incorporated dietary linoleate and arachidonate at about the same rate as did liver triglycerides, but more slowly than phospholipids. In rats fed linoleate, part of the 18:2 was converted into 20:4; this acid was also incorporated

[2] 1963 program of the "Division de Matières Graises," Union International de Chimie Pure et Appliqué.

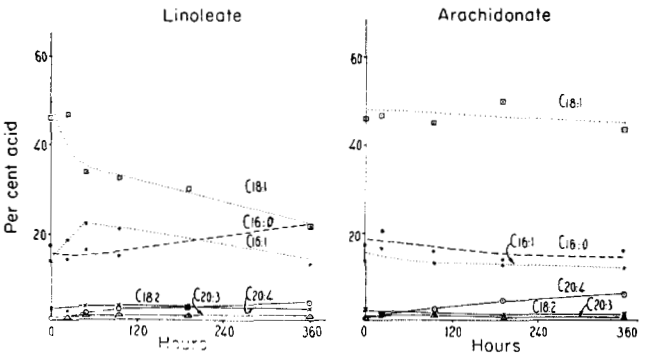

FIG. 2. Changes in the fatty acid composition of liver cholesterol esters of fats fed methyl linoleate or arachidonate after 3 months on a fat-free diet. Percentages plotted against hours after beginning the diet.

into cholesterol esters, but at a faster rate than linoleate. Eicosatrienoate was a minor component which decreased slowly. Slowly decreasing percentages of 18:1, 16:0, and 16:1 were found in rats fed arachidonate, whereas different changes were observed in animals fed linoleate.

Choline and Ethanolamine Glycerophosphatides

The main fatty acid components of CGP and EGP were stearic, palmitic, and oleic acids, and either eicosatrienoic or arachidonic acid. The relative proportions of all these acids underwent similar changes in both phosphatides of rats fed on linoleate or arachidonate, but minor differences were also found which have important implications.

Effect of Arachidonate. Both arachidonate and linoleate were incorporated very quickly into both phospholipids and attained a constant percentage in a short time (Figs. 3 and 4). The plateau for arachidonate was attained in approximately 8 days and was considerably higher than that for linoleate. While arachidonate was being incorporated into CGP and EGP decreases were observed in the proportions of 20:3, 18:1, and 16:1, whereas the percentage of palmitate increased. All these acids nearly reached the steady state between the first 2 and 4 days but it was attained somewhat faster in EGP than in CGP. Arachidonate tended to displace the residual amounts of linoleate (20) that remained in the phospholipids in spite of the essential fatty acid-deficient diet fed to the rats, as well as other octadecadienoic acids synthesized by the animals under these conditions (20,21). This displacement was complete in somewhat more than 4 days. A similar but much slower effect was found in triglycerides and cholesterol esters. Whereas all the proportions of the acids recently mentioned changed in the same direction both in CGP and in EGP, stearate

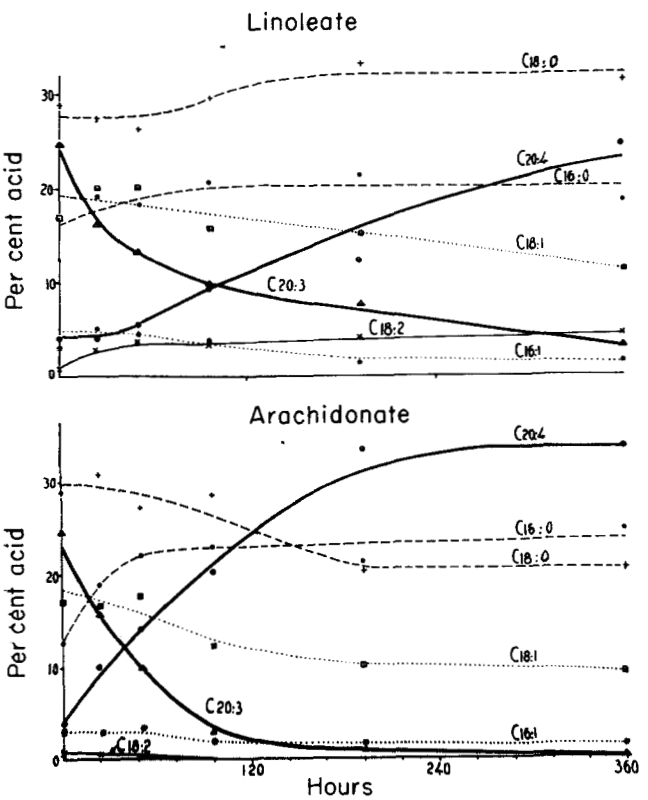

FIG. 3. Changes in the fatty acid composition of liver choline glycerophosphatides (CGP) of rats fed methyl linoleate or arachidonate after 3 months on a fat-free diet.

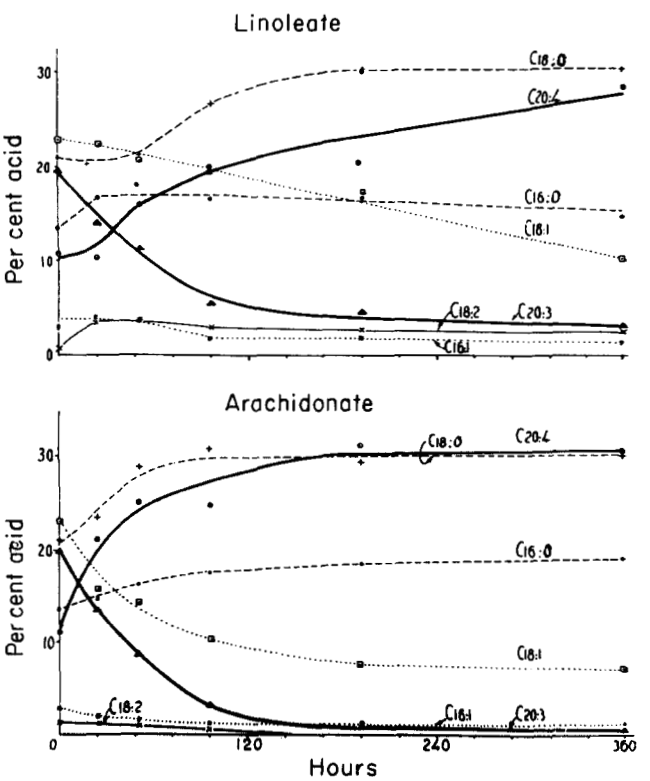

FIG. 4. Changes in the fatty acid composition of liver ethanolamine glycerophosphatides (EGP) of rats fed methyl linoleate or methyl arachidonate after 3 months on a fat-free diet.

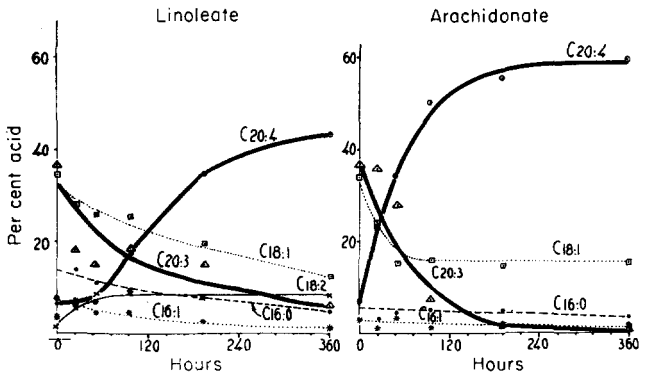

FIG. 5. Changes in the composition of fatty acids bound to the β-carbon of liver CGP of rats fed methyl linoleate or arachidonate after 3 months on a fat-free diet.

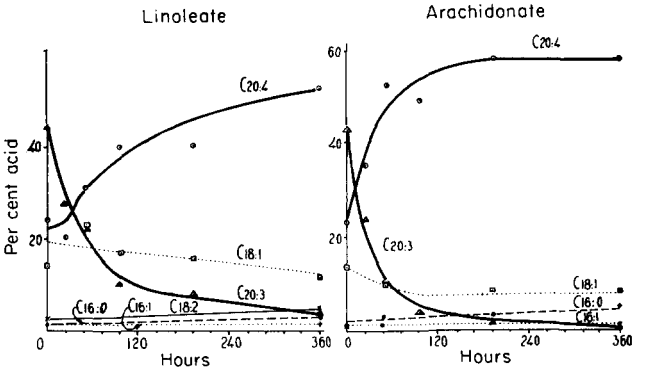

FIG. 7. Changes in the composition of fatty acids bound to the β-carbon of liver EGP of rats fed methyl linoleate or arachidonate after 3 months on a fat-free diet.

percentages were modified in completely different directions in the two phosphatides. During the first 4 days they decreased in CGP whereas they increased rapidly and sharply in EGP.

Fatty acid distributions between β- and α'-positions were similar in CGP and EGP (Fig. 5–8). Polyunsaturated acids were mainly confined to the β-position whereas saturated acids were preferentially bound to α'-carbons, although palmitic acid was also found in significant amounts in the β-position. Monoenoic acids were found in both positions. In the β-position, arachidonate increased to a steady value of about 60% and reciprocal changes were shown by eicosatrienoate and oleate. These changes can be related to those in the α'-acids. In CGP, palmitate increased whereas stearate and oleate decreased, while in EGP both saturated acids increased.

Effect of Linoleate. The linoleate rapidly incorporated into the phosphatides was shown to reach a constant percentage (approximately 4%) earlier in EGP than CGP (Figs. 3 and 4). Linoleate was converted into 20:4 and 22:5 and the synthesized arachidonate began to be incorporated after a delay of more than 24 hr, after which it attained very high percentages. This delay was more marked in CGP than EGP. Both linoleate and synthesized arachidonate were incorporated into the β-position (Figs. 5 and 7) of phosphatides. Besides this, dietary linoleate displaced eicosatrienoic acid rapidly and the other β-positioned acids, 18:1, and 16:1, more slowly. The changes in 20:4, 20:3, and monoenoic acids were slower than those produced in animals fed arachidonate and they did not reach the steady state even in a 15 day period.

Of the acids bound to α'-carbon (Figs. 6 and 8) oleic was equally and exponentially decreased in both phosphatides, whereas saturated acid curves changed differently in CGP and EGP. Palmitic and stearic acids followed a similar pattern of changes in animals fed either linoleate or arachidonate, but in CGP palmitic acid increased the most while it was stearic acid that increased in EGP.

DISCUSSION

The data presented here demonstrate that the effects of dietary methyl linoleate and arachidonate on liver and adipose tissue of fat-deficient rats are sometimes similar and sometimes distinct. The two esters induced similar

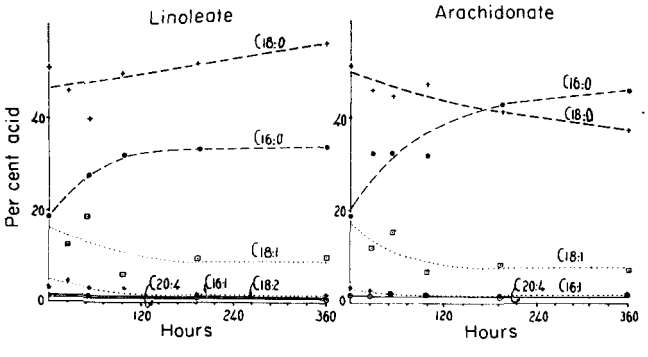

FIG. 6. Changes in the composition of fatty acids bound to the α'-carbon of liver CGP of rats fed methyl linoleate or arachidonate after 3 months on a fat-free diet.

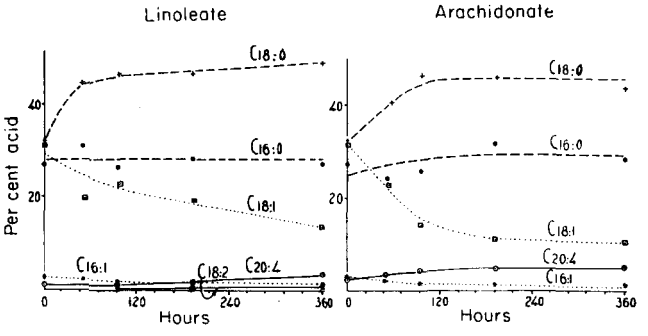

FIG. 8. Changes in the composition of fatty acids bound to the α'-carbon of liver EGP on rats fed methyl linoleate or arachidonate after 3 months on a fat-free diet.

changes in the lipid class composition, especially the proportion of polar and nonpolar lipids. Both acids were very rapidly incorporated and esterified to the β-carbon of CGP and EGP, specifically decreasing, not only the amount of eicosatrienoate and oleate bound to the β-carbon, but also the relative proportions of the acids bound to the α′-carbon. New synthesis via Kennedy's pathway (22) of at least 20–30% of the CGP and EGP molecules (Figs. 6 and 8) may be calculated to have occurred, from the variation in the percentages of α′-fatty acids. The remaining molecules could be produced either through this mechanism or the β-transacylase one. Specific β-fatty acyl transferase has been found (23, 24) for PC and PE in rat liver and this short cut for phospholipid synthesis may explain quite easily the reciprocity existing between the slopes of arachidonate incorporation and the depletion of eicosatrienoate and oleate (Figs. 5 and 7). Similar reciprocity was also found in heart lipids,[1] which are rich in phospholipids.

From these results dietary arachidonate appeared not only to prevent the synthesis of eicosatrienoic from oleic acid but also to replace it in its main store in the phospholipids. All octadecadienoates are also displaced from CGP and EGP.

The effect of dietary linoleate is somewhat different. Although it prevented the desaturation and elongation of oleic into eicosatrienoic acid (Figs. 3 and 4) and was incorporated into the β-position of phospholipids, the shape of the curves does not indicate that it replaces eicosatrienoate directly. Linoleate is converted into arachidonic acid by the rat. Although this acid was found in the β-position of both phospholipids it did not seem to be incorporated in this case through a direct replacement of eicosatrienoate; the curves of arachidonate and eicosatrienoate (Figs. 5 and 7) were different in shape and not reciprocals.

Although linoleate is rapidly incorporated into heart lipids under the same experimental conditions,[1] synthesized arachidonate seemed to be available in this organ only after some delay (4 days after linoleate incorporation). The decreasing curve for eicosatrienoate followed exactly the increase of arachidonate. In consequence, these results agree with our previous ones (5) showing that linoleate reduces the percentage of eicosatrienoate when it is actually converted to arachidonate. They also seem to agree with our proposal (20) that arachidonate may produce a decrease of 20:3 and octadecadienoates by reason of its specific incorporation into the β-position of phospholipids.

However, when arachidonate is synthesized from linoleate in the same tissue, it does not seem to provoke a rapid reduction of eicosatrienoate percentage through phospholipid synthesis (20)—or at least it is produced with much delay compared with an earlier one directly due to linoleate. According to the observations of Dhopeshwarkar and Mead (25) and Holman and Mohrhauer (26), this direct linoleate effect seems to be due to a competition with oleate for the enzymes involved in their transformation into polyunsaturated fatty acids.

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