Nagoya J. med. Sci. 33: 105-130, 1970

A POSSIBLE ROLE OF PYRIDOXINE IN LIPID METABOLISM

YUZO SATO

3rd Department of Internal Medicine, School of Medicine Nakoya University
(Director: Prof. Kozo Yamada)

ABSTRACT

The influence of pyridoxine upon the metabolic conversion of linoleic acid to arachidonic acid was studied in the livers of rats maintained with different kinds and conditions of dietary fats.

In the basal diet group, the liver phospholipid fraction of the pyridoxine deficient animals revealed significantly higher percentage of linoleic acid and significantly lower percentage of arachidohic acid, when compared with the control group, whereas in the liver phospholipid fraction, the proportion of arachidonic acid was higher and that of eicosatrienoic acid significantly lower in the pyridoxine deficient group fed the hardening oil diet. Recovery experiments were carried out. The time course of changes in the fatty acid composition of the liver lipids of rats fed cotton-seed oil, after 6 weeks on a hardening oil diet, revealed slower velocity of increase in arachidonate, slower velocity of decrease in eicosatrienoate and faster velocity of increase in linoleate in the pyridoxine deficient group. Changes in the fatty acid composition of the liver phospholipid after giving γ-linolenate to rats previously fed a fat free diet showed also slower velocity of increase in arachidonate and lower velocity of decrease in eicosatrienoate.

It is concluded that pyridoxine plays an important role in the essential fatty acid metabolism, chiefly in the conversion of γ-linolenate to arachidonate.

INTRODUCTION

The relationship between pyridoxine and essential fatty acid (EFA) metabolism is still to be clarified despite numerous investigations in this area during recent years[1]. Especially the possible role of pyridoxine in the metabolic conversion of EFA has been of interest since the report of Witten and Holman[2] that tissue concentrations of arachidonic acid were lower in rats maintained with a diet deficient in pyridoxine and essential fatty acids and given linoleate than in those maintained with a similar diet and given pyridoxine and linoleate. They emphasized that linoleate was a precursor of arachidonate in the rat, and that conversion was stimulated by pyridoxine. The importance of pyridoxine for utilization of linoleate was further docu-

佐 藤 祐 造
Received for publication February 14, 1970.

mented by the findings that in pyridoxine deficiency, with or without supplementation with linoleic acid, the content of linoleate particularly in the phospholipid fraction of the liver was increased while concomitantly arachidonate was decreased and the greatest effect was obtained during the first few days of supplementation[3)~5)]. These findings are supported by the experimentations in human beings[6)~7)]. The mechanism of the action of pyridoxine might possibly be explained by the *in vitro* experiments of Wakil[8)], in which pyridoxal- and pyridoxamine-phosphate appeared to be cofactors in the chain elongation of the fatty acid chain by incorporation of acetyl CoA. Kirschman and Coniglio[9)], on the other hand, indicated that pyridoxine was not concerned specifically with the conversion of linoleate to arachidonate, but "may effect in a general manner the metabolism of the saturated and monounsaturated as well as polyunsaturated fatty acid," since the greater increase in arachidonate in rats fed both pyridoxine and linoleate could have resulted from tissue growth. Greenberg and Moon[10)] observed that the presence or absence of pyridoxine in the diet did not influence the amount of arachidonic acid in the blood when fat deficient monkeys were fed corn oil. Other recent investigations[11)12)] also lent no support for a specific effect of pyridoxine supplementation on conversion of linoleate to arachidonate in liver. Thus up to the present time, no clear-cut conclusions have been drawn concerning a specific role of pyridoxine in the metabolism of the polyunsaturated fatty acids.

In the attempt to delineate a possible function of pyridoxine in relation to the metabolism of essential fatty acids, it seems pertinent to investigate the influence of dietary pyridoxine on the fatty acid distribution in the liver of rats maintained with different kinds of dietary fats and to study the time course of these changes.

EXPERIMENTAL PROCEDURE

Diet and care of animals

Male albino rats of Wistar strain weighing 50–70 g were used throughout the study, and were divided into five groups according to the diets. The rats were caged individually in raised wire bottom cages. Water was given *ad libitum*. The diets are described in Tables 1 and 2. Group A received a basal diet, whereas groups B and C were maintained on hardening oil diets. D group rats maintained with a hardening oil diet for 6 weeks, were transferred to the basal diet. The fatty acid compositions of liver lipids were determined at intervals for 6 months. E group rats were fed a fat free diet for 2 weeks. These dietary groups were subdivided into two sub-groups, pyridoxine deficient and pyridoxine supplemented. Group C only was sub-divided into 3 subgroups ···· (i) 1st fed a pyridoxine deficient diet for 10 weeks, (ii) 2nd a pyridoxine supplemented diet for 10 weeks, and (iii) 3rd a pyridoxine supplemented diet for 2 weeks after 8 weeks on a pyridoxine deficient diet.

TABLE 1

Composition of basal diet (rat/day)	
glucose	6.2 g
casein (vitamin free)	2.4 g
cotton seed oil	1.0 g
Mc Collum's salt	0.4 g
Vitamin mixture (rat/day)	
thiamine-HCl	40.0 μg
riboflavin	60.0 μg
pyridoxine-HCl	60.0 μg (when added)
vitamin B_{12}	0.1 μg
biotin	1.0 μg
folic acid	10.0 μg
niacinamide	0.2 mg
inositol	3.0 mg
Ca D-pantothenate	0.2 mg
choline-HCl	10.0 mg
PABA	0.5 mg
vitamin A	300 I.U.
vitamin D	30 I.U.

TABLE 2. Effect of dietary treatments on weight gain

Diet	pyridoxine (DOP)	No. of rats	body weight of rats initial (g)	body weight of rats final (g)	liver weight (g)	motality (%)	duration of experiments
(A) basal diet	(−)	7	71.4	124.3	3.7±0.3	0	7 wks
	(+)	7	69.4	160.0	4.4±0.4	0	7 〃
(B) hardening oil diet	(−)	8	46.8	67.5	3.0±0.3	20.0	6 wks
	(+)	7	48.4	104.5	4.4±0.4	0	6 〃
(C) hardening oil diet (i)	(−)	4	57.5	83.3	3.2±0.4	0	10 wks
(ii)	(+)	4	53.8	180.0	8.1±0.6	0	10 〃
(iii)	(−) 8 w → (+) 2 w	4	60.0	146.0	7.8±2.0	25.0	10 〃
(D) hardening oil diet → basal diet	(−)	27	60.1	112.8	5.2±2.3	7.4	from 6 wks
	(+)	22	59.9	194.8	7.6±1.0	4.5	to 6 months
(E) fat free diet	(−) DOP (+)	10	49.9	60.2	3.2±0.8	10.0	16 days
→ fed γ-linolenate	(+)	10	50.1	81.1	4.9±0.7	0	16 〃

Pyridoxine deficient sub-groups (in groups A, B, C and D) were produced by omission of pyridoxine from the vitamin mixture. In group E the pyridoxine deficient sub-group was intraperitoneally injected with desoxypyridoxine[13] 0.5 mg for one week daily, in addition to omission of pyridoxine from the vitamin mixture.

The fatty acid composition of cotton seed oil and hardening oil are of the

following weight percentages ···· cotton seed oil: 14 : 0 0.9, 16 : 0 21.7, 16 : 1 1.0, 18 : 0 2.7, 18 : 1 21.0, 18 : 2 52.7. hardening oil: 14.0 2.7, 16 : 0 25.3, 16 : 1 1.6, 18 : 0 44.0, 18 : 1 23.5.

The food intake of each pyridoxine supplemented sub-group was restricted to the average daily intake of the corresponding deficient sub-group. In this experiment the pyridoxine supplemented sub-groups were used as controls.

At the termination of experiments the rats which had received the pyridoxine deficient diet showed symptoms of severe pyridoxine deficiency characterized by loss of weight, acrodynia, capillary fragility etc. Food cups were removed from the cages 18 hr before the rats were killed by decapitation without anesthesia. The livers were removed quickly, weighed, and kept at −20° until analyzed.

Methods

Lipid extraction: Liver lipids were extracted with 20 volumes of chloroform-methanol (2 : 1, v/v) by the procedure of Folch *et al.*[14] Hydroquinone was added as an antioxidant.

Thin layer chromatography[15]: Liver lipids were fractionated on thin layer chromatography (TLC) into five fractions: I. cholesterol ester II. triglyceride III. free fatty acid IV. free cholesterol V. phospholipid.

The analyses were carried out on 20×20 cm chromatoplates coated with a thin layer (0.5 mm) of silicic acid containing 10% calcium sulfate as a binder. The chromatoplates for these studies were dried and activated by heating them for 2 hrs at 110°C in an oven and allowed to cool to room temperature. The solvent system used consisted of *n*-hexane-ethylether-acetic acid. 39 : 10 : 1 (v/v/v). 0.02% 2, 7-dichlorofluorescein[16] was applied in the adsorbent layer for visualizing spots in UV light after chromatography.

Interesterification: Interesterification of lipids was carried out by a slight modification of the method of Stoffel[17]. Lipid fractions separated by TLC were scraped off into test tubes equipped with glass cap; 5 ml of 5% H_2SO_4 in methanol and 0.2 ml of 2, 2-dimethoxypropane were added, and methylation was allowed to proceed for 12 hrs at 40°C in a water bath. The reaction mixture was diluted with an equal volume of water and extracted two times with 4 ml portions of *n*-hexane and evaporatcd to dryness.

Gas-liquid chromatography[18)19)]: A Yanagimoto model 5 DH with a 2000×3 mm stainless column and a flame ionization detector was used. Liquid phase was 15% diethylene glycol succinate polyester supported on 80–100 mesh Celite 545, nitrogen was used as carrier gas at a flow rate of 17.5 ml/min. All analyses were carried out at temperatures between 170 and 190°C. Identification of the peaks was made by comparison of retention time with known standards (Applied Science Lab. Inc.). The areas under the curves were computed by multiplying

their respective height by the width at half height. For statistical evaluation, the averages of the data from each group and the standard deviations of the mean were calculated. The significance of differences between the means was determined by students't test.

Determination:

Phospholipid[20]: Phosphlipid was assayed by the method of Hoeflmayr and Fried (PL Kit Haury). Namely the residue of Folch extract, after evaporation of solvent, was digested with 70% perchloric acid and nitric acid, and the liberated phosphate was determined by the molybdate colorimetric phosphorus of Fiske and Subbarow.

Triglyceride[21]: Triglyceride was determined by a slight modification of the method of Van Handel and Zilversmit. Namely, aliquots of the chloroform-methanol extract were saponified, the resultant glycerol was oxidized to form aldehyde, and the form aldehyde was estimated colorimetrically with chromotropic acid.

Separations of subcellular particles:

Mitochondria, microsomes, and supernatants were fractionated by the methods of Schneider[22]. Beckman Model L 4 Ultra centrifuge was used. One g of liver was homogenized in 9 ml of ice cold 0.25 M isotonic sucrose (8.5 g of sucrose per 100 ml of solution). Ten ml of homogenate were centrifuged for 10 minutes at 600 g to sediment the nuclei. The supernatant was centrifuged for 10 minutes at 10,000 g to sediment the mitochondria. The mitochondria were washed twice by resuspension in 2.5 ml of isotonic sucrose and recentrifugation at 10,000 g for 10 minutes. The washed mitochondria were labeled the mitochondrial fraction. The supernatant and washings from the mitochondrial fraction were combined and centrifuged for 60 minutes at 100,000 g to sediment the microsomes. The microsomes were washed once by resuspension in 2.5 ml of isotonic sucrose and recentrifugation at 100,000 g for 60 minutes. The washed microsomes were labeled the microsomal fraction. The supernatant and washings from the microsomes were combined and labeled the supernatant.

RESULTS

1. Growth

The average weights of rats and livers are listed in table 2.

The growth was retarded or nearly stopped in the pyridoxine deficient sub-groups and the growth curves for pyridoxine supplemented sub-groups showed steady weight gain during all experimental weeks. In the group C iii sub-groups previously depleted of pyridoxine and EFA, the growth curve came to show a steady weight gain after supplementation with pyridoxine.

2. Effect of pyridoxine deficiency on the fatty acid composition of liver lipids

(*i*) *Basal diet group*: Group A was fed a basal diet consisting of glucose 64%, vitamin free casein 24%, cotton seed oil 10%, Mc Collum's salt mixture 4% and adequate vitamin mixture. The average weight of the livers was 4.4 ±0.4 g for the control group and 3.7±0.3 g for the pyridoxine deficient sub-group. Liver phospholipid (lipoid-p) was lower in the deficient sub-group (669 μg/g) than in the corresponding pair-fed controls (907 μg/g). Liver triglyceride tended to be lower in the deficient sub-group (6.09 mg/g) than in the controls (7.30 mg/g). The fatty acid composition of the total liver lipids for both control and pyridoxine deficient rats is shown in Table 3. Pyridoxine deficient rats tended to show a higher proportion of linoleate and a lower proportion of arachidonate in comparison with the control, but these increase ($0.2<p<0.5$) and decrease ($0.1<p<0.2$) were statistically nonsignificant. The percentages of total saturated and mono-unsaturated fatty acids in the pyridoxine deficient sub-group were almost equal to those in the control group. The fatty acid compositions of liver phospholipid and triglyceride are shown in Table 4. In both control and pyridoxine deficient animals of phospholipid fraction, the poly-unsaturated fatty acids comprised 44 to 45% of the total fatty acids. Comparison of the phospholipid fraction in the two sub-groups of animals elucidated that the pyridoxine deficient animals indicated significantly higher proportion of linoleic acid ($p<0.05$) and significantly lower proportion of arachidonic acid ($p<0.05$). The percentages of saturated fatty acids were almost the same in both sub-groups of animals. The liver triglyceride fraction of the pyridoxine deficient sub-group had significantly higher proportion of ($p<0.01$) linoleic acid than the control group. Also, the deficient animals had lower proportions of ($p<0.05$) mono-unsaturated fatty acids (palmitoleic and oleic acids) in the phospholipid fraction. The percentages of arachidonic acid and saturated fatty acid showed the same figures in both sub-groups of animals.

TABLE 3. Effect of pyridoxine deficiency on the fatty acid spectrum of liver lipids (basal diet)

fatty acid	pyridoxine deficient (%)	control (%)
12 : 0	0.2±0.1	0.3±0.1
14 : 0	0.6±0.2	0.4±0.1
16 : 0	24.8±1.2	23.2±2.3
16 : 1	2.8±0.4	2.7±0.2
18 : 0	19.1±3.1	20.0±2.1
18 : 1	18.5±2.1	17.1±1.7
18 : 2	16.5±2.4	14.8±0.8
18 : 3	0.5+0.3	0.3±0.1
20 : 4	16.6±1.9	20.0±3.7
more longers	1.9±0.2	3.7±1.0
Saturated fatty acid	44.7	43.9
Mono-unsaturated fatty acid	21.3	19.8
Poly-unsaturated fatty acid	35.5	38.8

(mean±S.D.)

TABLE 4. Effect of pyridoxine deficiency (7 wks) on the fatty acid spectrum of the liver lipid fractions (basal diet)

Fatty acid	Triglyceride		Phospholipid	
	Pyridoxine deficient (%)	Control (%)	Pyridoxine deficient (%)	Control (%)
14 : 0	1.8±0.7	2.0±0.6	0.2±0.3	0.2±0.1
16 : 0	36.0±2.8	36.2±1.4	22.5±1.7	21.1±1.5
16 : 1	1.4±0.7	3.0±0.8	0.4±0.1	0.5±0.2
18 : 0	7.8±2.4	8.1±1.6	27.0±0.9	27.6±0.7
18 : 1	20.4±1.7	24.7±2.3	5.5±0.6	5.6±0.6
18 : 2	29.9±4.0	23.6±2.6	20.4±1.5	17.1±0.6
18 : 3	0.3±0.1	N.D.	N.D.	N.D.
20 : 4	2.6±1.1	2.3±1.0	24.0±2.3	27.9±2.2
Saturated	45.6	46.3	49.7	48.9
Mono-unsaturated	21.8	27.7	5.9	6.1
Poly-unsaturated	32.8	25.9	44.4	45.0

(mean±S.D.)

average liver weight (g)	control	4.4±0.4
	pyridoxine deficient	3.7±0.3
No. of rats	control	7
	pyridoxine deficient	7

(*ii*) *Hardening oil group*: Group B was fed a hardening oil diet containing 10% hardening oil instead of cotton seed oil. So the rats of this group B exhibited in EFA deficiency at the end of the experiment. The average weight of the livers for the control was 4.4±0.4 g and 3.0±0.3 g for the pyridoxine deficient sub-group. Liver phospholipid tended to be lower in the deficient sub-groups than in the corresponding pair-fed control. Liver triglyceride was significantly lower ($p<0.001$) in the deficient sub-group. The fatty acid composition of the total liver lipids for both control and pyridoxine deficient rats is shown in Table 5. Comparison of the total liver lipids in the two sub-group of animals indicated higher proportion of arachidonic acid. Also, the deficient

TABLE 5. Effect of pyridoxine deficiency (6 wks) on the total fatty acids of liver lipids (hardening oil diet)

Fatty acid	Pyridoxine-deficient rats (%)	Controls (%)
12 : 0	0.8±0.7	0.5±0.2
14 : 0	0.4±0.1	0.6±0.1
16 : 0	20.7±1.5	19.9±1.6
16 : 1	3.7±0.7	4.4±0.8
18 : 0	23.2±2.5	22.4±1.3
18 : 1	28.6±0.7	31.4±1.9
18 : 2	2.5±0.5	1.4±0.7
20 : 3	11.1±2.0	12.8±1.8
20 : 4	11.0±0.7	6.9±1.3
22 Carbons and longer	2.2±0.1	2.0±0.2
Saturated	45.1	43.4
Mono-unsaturated	32.3	35.8
Poly-unsaturated	26.8	23.1

(mean±S.D.)

TABLE 6. Effect of Pyridoxine deficiency (6 wks) on the fatty acids of liver phospholipids (hardening oil diet)

Fatty acid	Pyridoxine-deficient rats (%)	Controls (%)
12 : 0	0.3±0.2	0.1±0.2
14 : 0	0.4±0.1	0.2±0.1
16 : 0	18.9±1.7	16.6±1.3
16 : 1	3.9±0.4	3.5±0.8
18 : 0	25.6±2.0	26.2±2.3
18 : 1	25.2±1.7	24.3±1.7
18 : 2	2.3±0.5	1.9±1.1
20 : 3	10.9±1.3	17.7±3.1
20 : 4	12.2±1.8	8.6±0.6
22 Carbons and longer	1.8±0.4	2.0±0.1
Saturated	45.2	43.1
Mono-unsaturated	29.1	27.8
Poly-unsaturated	27.2	30.2
(mean±S.D.)		
Tissue lipoid-p (Hoeflmayr and Fried)	618 (μg/g)	700 (μg/g)

animals had higher proportion of ($p<0.01$) linoleic and lower proportion of ($p<0.01$) oleic acid than the control group. Eicosatrienoic acid (20 : 3 ω 9), characteristic of EFA deficiency[23], tended to be lower, but the decrease was statistically not significant in the deficient group. The percentage of saturated fatty acid was almost the same in both sub-groups of animals. The phospholipid fraction (Table 6) of the pyridoxine deficient group showed higher percentage of arachidonic acid and lower percentage of ($p<0.001$) eicosatrienoic acid when compared with the control group. Also the proportion of palmitic acid was significantly higher ($p<0.02$) in the deficient group. The percentage of mono-unsaturated fatty acid was the same in both sub-groups of animals.

TABLE 7. Effect of pyridoxine deficiency (6 wks) on the fatty acids of liver triglycerides (hardening oil diet)

Fatty acid	Pyridoxine-deficient rats (%)	Controls (%)
12 : 0	0.2±0.1	0.3±0.1
14 : 0	1.0±0.2	0.9±0.3
16 : 0	28.9±1.3	28.8±2.0
16 : 1	4.5±0.9	5.3±0.9
18 : 0	6.8±2.2	5.9±1.6
18 : 1	53.9±3.3	56.2±3.6
18 : 2	1.2±0.6	0.7±0.4
20 : 3	2.2±0.5	1.2±0.4
20 : 4	1.2±0.9	0.3±0.2
22 Carbons and longer	trace	trace
Saturated	36.9	35.9
Mono-unsaturated	58.4	61.5
Poly-unsaturated	4.6	2.2
(mean±S.D.)		
Tissue triglyceride (Van Handel and Zilversmit)	2.44 mg/g	5.38 mg/g

The proportion of poly-unsaturated fatty acids remained relatively unchanged since the higher arachidonate values were counteracted by the lower eicosatrienoate.

The fatty acid composition of liver triglyceride is shown in Table 7. Oleate tended to be lower, but the decrease was nonsignificant ($0.2<p<0.5$) in the deficient sub-group.

In cholesterol esters (Table 8), the proportion of oleate was significantly lower ($p<0.05$) in the deficient sub-group.

TABLE 8. Effect of pyridoxine deficiency (6 wks) on the fatty acids of the liver cholesterol esters (hardening oil diet)

Fatty acids	Pyridoxine-deficient rats (%)	Controls (%)
12 : 0	1.4±0	1.9±0.6
14 : 0	2.7±1.2	1.9±0.9
16 : 0	41.4±7.3	39.8±7.5
16 : 1	5.8±1.5	7.2±1.5
18 : 0	14.1±3.1	11.2±2.7
18 : 1	29.3±5.8	35.6±4.8
18 : 2	2.0±1.7	1.0±0.2
20 : 3	1.8±1.0	2.0±0.8
20 : 4	2.5±1.5	1.0±0.4
Saturated	59.6	54.8
Mono-unsaturated	35.1	42.8
Poly-unsaturated	6.3	4.0

(mean±S.D.)

(*iii*) *Recovery experiment*: This group C was also fed a hardening oil diet. After given the deficient diet for 8 weeks, in the (iii) sub-group, the diet was switched from the deficient to the supplemented control. Average weight of the livers was 3.2 g for the pyridoxine deficient sub-group (i), 7.8 g for the recovery sub-group (iii) and 8.1 g for the control group (ii).

The fatty acid compositions of liver total lipid and phospholipid are shown in Table 9. Comparison of the total liver lipid indicated that the proportion of fatty acids in the recovery sub-group (iii) became similar to that in the control group (ii). In the recovery sub-group (iii) the percentage of arachidonic acid decreased to 3.3% while the percentage of oleate increased to 35.8%. Comparison of the phospholipid fraction also revealed that the proportion of fatty acids in the recovery sub-group became nearly the same as that in the control group. Therefore in the recovery sub-group (iii), the decrease in the percentage of arachidonate was accompanied by an equivalent increase in that of eicosatrienoate. The proportion of oleate increased too.

Comparison of the total fat in the deficient and the control sub-groups for 10 weeks indicated higher concentration of arachidonate and lower concentration of eicosatrienoate in the pyridoxine deficient sub-groups. The phospholipid

TABLE 9. Changes in the fatty acid composition of liver total fat and phospholipid of rats fed hardening oil

	total fat (%)			phospholipid (%)		
	B_6 (−) 10 w	B_6 (−) 8 w →B_6 (+) 2 w	control	B_6 (−) 10 w	B_6 (−) 8 w →B_6 (+) 2 w	control
12 : 0	N D.	N.D.	0.6	0.1	1.1	0.4
14 : 0	0.9	0.4	0.8	0.3	0.3	0.4
16 : 0	20.9	25.4	23.1	17.6	20.4	17.8
16 : 1	4.5	5.0	5.4	3.9	4.5	3.7
18 : 0	21.6	16.8	17.3	24.6	23.7	23.9
18 : 1	29.4	35.8	37.0	22.3	27.0	26.2
18 : 2	1.2	2.7	1.2	1.6	1.6	1.3
18 : 3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20 : 3	12.7	10.5	11.2	14.9	16.6	18.4
20 : 4	8.9	3.3	3.1	9.5	4.5	5.7
longers	N.D.	0.9	0.7	5.5	2.1	1.9

fraction of the pyridoxine deficient sub-group for 10 weeks showed higher percentage of arachidonate and lower percentage of eicosatrienoate when compared with the control group. These changes showed patterns similar to those of pyridoxine deficiency for 7 weeks.

Comparison of the total fat and phospholipid fraction in the pyridoxine deficient sub-groups for 6 weeks (group B) and 10 weeks (group C) indicated that proportion of arachidonate decreased and equivalently proportion of eicosatrienoate increase in the last 4 weeks of depletion.

3. Sequential changes in liver lipids after giving cotton-seed oil diets to rats previously fed hardening oil diets for 6 weeks

Tables 10 and 11 and Fig. 1 show the time course of changes in the fatty acid composition of liver total fat of rats fed cotton-seed oil after 6 weeks on hardening oil (group D). Zero time values are for rats fed a hardening oil diet before transfer to cotton-seed oil diet. Lipids from the rats fed the hardening oil diet were low in the concentrations of linoleic and arachidonic acids, and high in the concentrations of palmitoleic, oleic and eicosatrienoic acids in

TABLE 10. Changes in the fatty acid composition of liver total fat of rats fed cotton seed oil after 6 weeks on hardening oil (Control)

	0	1/2 day	1 day	2 day	3 day	6 day	6 month
12 : 0	0.5%	0.5%	0.4%	0.2%	0.2%	0.5%	0.6%
14 : 0	0.6	0.5	0.7	0.3	0.4	0.4	1.0
16 : 0	19.9	23.3	24.1	19.2	20.5	22.4	33.5
16 : 1	4.4	3.7	3.9	1.8	1.9	1.3	2.2
18 : 0	22.4	19.0	19.2	27.9	22.6	24.5	31.3
18 : 1	31.4	25.3	26.6	16.9	18.2	14.2	12.2
18 : 2	1.4	7.9	7.1	9.1	9.1	13.8	18.7
18 : 3	N.D.	0.2	N.D.	N.D.	N.D.	0.5	0.2
20 : 3	12.8	9.7	3.7	2.9	2.2	0.3	N.D.
20 : 4	6.9	8.1	11.1	20.7	19.0	18.9	33.0
longer	N.D.	3.5	5.6	1.3	10.3	3.3	2.2

both control and pyridoxine deficient rats. Comparison of the total fat in the two sub-groups of animals indicated that the concentration of linoleate increase more rapidly in the pyridoxine deficient sub-group than in the control

TABLE 11. Changes in the fatty acid composition of liver total fat of rats fed cotton seed oil after 6 weeks on hardening oil (Pyridoxine deficient)

	0	1/2 day	1 day	2 day	3 day	6 day	6 month
12 : 0	0.8%	0.8%	N.D.	0.4%	0.5%	0.6%	0.6%
14 : 0	0.4	0.6	0.5%	0.5	0.4	0.5	0.7
16 : 0	20.7	23.6	21.9	21.6	21.0	20.2	32.2
16 : 1	3.7	3.7	3.9	1.6	1.5	0.8	2.7
18 : 0	23.2	16.5	20.4	28.4	23.7	27.0	24.6
18 : 1	28.6	24.9	22.2	13.3	14.4	8.3	18.7
18 : 2	2.5	11.4	9.8	11.7	14.1	15.1	28.4
18 : 3	N.D.	0.5	N.D.	N.D.	0.4	0.3	0.7
20 : 3	11.1	4.3	4.0	2.0	1.9	0.2	N.D.
20 : 4	11.0	8.8	11.7	17.8	20.5	23.0	15.2
longer	2.2	5.3	8.9	0.9	4.0	3.4	4.1

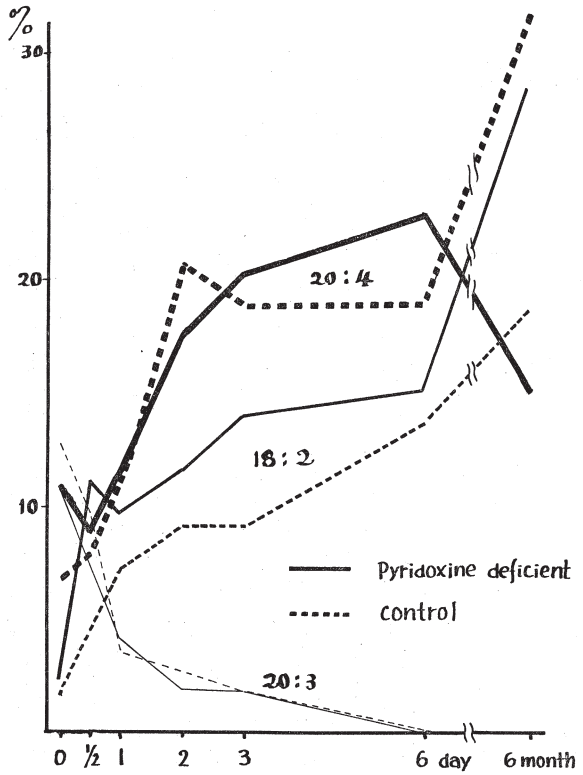

FIG. 1. Changes in the fatty acid composition of liver total fat of rats fed cotton seed oil after 6 weeks on hardening oil.

group. The rates of increase of arachidonate and decrease of linoleate in the control and the pyridoxine deficient sub-group showed no significant difference during this experiment.

Tables 12 and 13 and Fig. 2 show the time course of changes in the fatty acid composition of liver phospholipid of rats fed cotton-seed oil after 6 weeks on a hardening oil diet. Zero time values are for rats fed hardening oil diets before transfer to cotton-seed oil diets. During the supplementation, we found that arachidonate level increased gradually, to values of 26–30%, while eicosatrienoate decreased to 0% in both control and pyridoxine deficient subgroups. Also the proportion of linoleate increased and the proportions of palmitoleate and oleate decreased. In both sub-groups little changes occurred in the proportions of other fatty acids. With the pyridoxine deficient rats, the proportion of arachidonate increased to 11.7% after 2 days, a significantly smaller rise than in the control rats (17.6%). In the pyridoxine deficient sub-group the percentage of linoleate increased more rapidly (12.3% after 2 days) than in the control group (8.3% after 2 days). The percentage of eicosatrienoate declined more rapidly in the control group than in the deficient sub-group. Thus, we

TABLE 12. Changes in the fatty acid composition of liver phospholipid of rats fed cotton seed oil after 6 weeks on hardening oil (Control)

	0	1/2 day	1 day	2 day	3 day	6 day	6 month
12 : 0	0.1%	0.2%	0.1%	0.1%	0.1%	0.1%	0.1%
14 : 0	0.2	0.3	0.2	0.1	0.2	0.1	0.1
16 : 0	16.6	18.5	17.0	14.7	18.9	17.0	20.4
16 : 1	3.5	3.2	2.2	1.4	1.3	1.0	0.7
18 : 0	26.2	26.7	28.6	28.1	28.1	29.7	30.3
18 : 1	24.3	16.6	13.3	12.4	13.2	6.9	5.4
18 : 2	1.9	7.6	7.9	8.3	9.6	10.8	13.1
18 : 3	N.D.	0.3	0.4	0.5	0.4	0.2	0.3
20 : 3	17.7	12.2	6.1	3.4	1.8	0.4	N.D.
20 : 4	8.6	11.2	16.3	26.2	26.7	28.9	29.8
longer	2.0	3.9	6.0	5.2	6.5	7.3	2.2

TABLE 13. Changes in the fatty acid composition of liver phospholipid of rats fed cotton seed oil after 6 weeks on hardening oil (Pyridoxine deficient)

	0	1/2 day	1 day	2 day	3 day	6 day	6 month
12 : 0	0.3%	N.D.	0.3%	0.1%	0.3%	0.1%	0.2%
14 : 0	0.4	0.3%	0.2	0.2	0.4	0.2	0.3
16 : 0	18.9	19.0	17.8	15.6	18.7	16.7	19.5
16 : 1	3.9	2.3	3.1	1.6	1.5	0.7	0.6
18 : 0	25.6	26.1	24.6	31.4	26.7	31.4	28.7
18 : 1	25.2	17.1	15.7	10.3	10.7	6.4	6.0
18 : 2	2.3	9.7	12.8	12.3	12.3	14.5	17.7
18 : 3	N.D.	N.D.	1.2	0.6	0.5	N.D.	N.D.
20 : 3	10.9	8.0	5.1	2.7	1.9	0.4	N.D.
20 : 4	12.2	13.4	15.5	23.9	24.3	27.0	25.7
longer	1.8	4.4	7.5	2.6	4.0	3.5	4.2

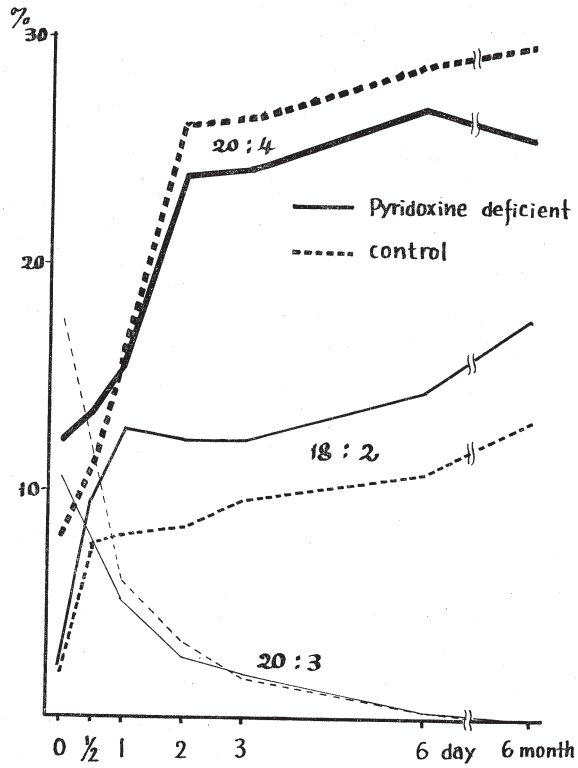

FIG. 2. Changes in the fatty acid composition of liver phospholipid of rats fed cotton seed oil after 6 weeks on hardening oil.

found significant differences between the pyridoxine deficient sub-group and the control group in the time course of changes of the three poly-unsaturated fatty acids contents of rat livers.

In the triglyceride and cholesterol ester fractions no definite tendency was seen.

4. Changes in the fatty acid composition of liver phospholipid of rats fed γ-linolenate after 2 weeks on a fat free diet

A fat free diet with and without pyridoxine (60 μg/day/rat) was fed to rats for 2 weeks before feeding 100 mg γ-linolenate (77% pure by gas liquid chromatography) daily for two days (group E). As was mentioned in previous experiments with pyridoxine deficient and control groups, the most notable differences appeared in the phospholipid fatty acids, quantitatively the most important fraction[5] (50–80% of total liver lipid). Accordingly, only the fatty acid composition of liver lipids was analysed this time in the phospholipid fraction.

TABLE 14. Changes in the fatty acid composition of liver phospholipid of rats fed γ-linolenate after fat free diet

	Control (%)		Pyridoxine deficient (%)	
	fat free diet	fed γ-linolenate	fat free diet	fed γ-linolenate
12 : 0	0.1±0.05	0.1±0.04	0.3±0.05	0.2±0.04
14 : 0	0.3±0.1	0.2±0.1	0.3±0.1	0.2±0.1
16 : 0	19.0±0.8	19.3±1.2	20.2±0.8	20.9±2.2
16 : 1	4.6±0.5	3.9±0.5	4.5±0.4	4.1±1.1
18 : 0	32.0±1.4	32.4±1.7	29.0±0.6	32.7±3.4
18 : 1	21.5±2.2	17.9±1.2	20.4±2.1	18.2±1.9
18 : 2	3.0±0.5	1.4±0.3	3.1±0.4	2.2±0.5
18 : 3 (Δ 6.9.12)	N.D.	0.2±0.1	0.1	0.2±0.1
20 : 3	11.1±1.6	3.1±0.7	5.6±0.4	3.6±1.7
20 : 4	8.3±0.7	20.0±2.6	12.9±0.5	18.3±3.4
longer	0.2±0.1	3.1	3.7±2.7	1.9
liver weight	4.5±0.6 g	5.1±0.6 g	2.7±0.9 g	3.5±0.7 g

(mean±S.D.)

Data obtained on the fatty acid composition of liver phospholipid of the control and the pyridoxine deficient rats fed γ-linolenate are shown in Table 14. Before treatment with γ-linolenate, the pyridoxine deficient group showed a higher proportion of arachidonate and significantly lower proportion of eicosatrienoate ($p<0.02$), when compared with the control group. So the decrease in the concentration of arachidonate was accompanied by an equivalent increase in the concentration of eicosatrienoate, typical of EFA deficiency[23]. The same pattern of changes in fatty acid composition has been seen when EFA deficiency

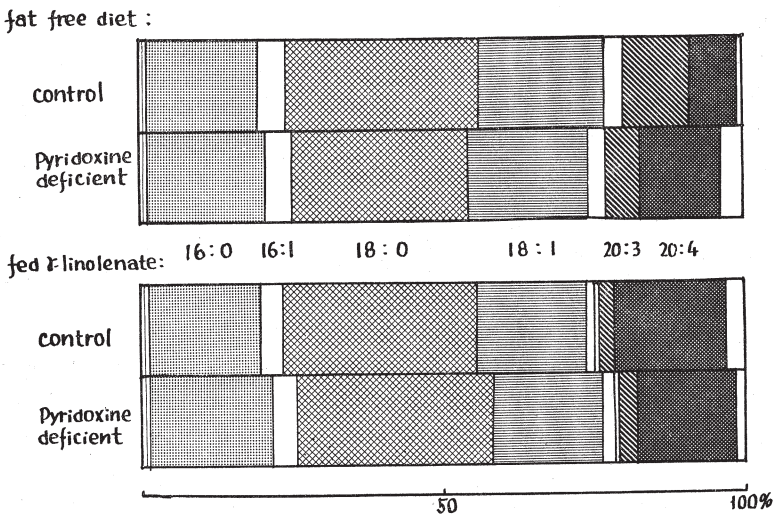

FIG. 3. Changes in the fatty acid composition of liver phospholipid of rats fed γ-linolenate after fat free diet.

was produced by feeding a hardening oil diet (see group B and C). After 2 days of γ-linolenate treatment, the percentage of eicosatrienoate decreased in both pyridoxine deficient and control sub-groups. In livers of the two sub-groups, comparison of the phospholipid fractions indicated that there were no significant differences in the proportions of the fatty acids (Fig. 3). As shown in Fig. 4, the proportion of arachidonate increased from 155% to 220% after two days—a smaller rise in the pyridoxine deficient sub-groups than in the control group with a rise from 100% to 240% (in this figure the proportion of individual fatty acid is given in %). In the pyridoxine deficient sub-group the proportion of eicosatrienoate decreased from 50.5% to 32.4%—a diminution smaller than in the control group, from 100% to 27.9%. Therefore, the increase in ratio of the percentage of arachidonate and the decrease in ratio of the percentage of eicosatrienoate were slower in the pyridoxine deficient sub-group than in the control group.

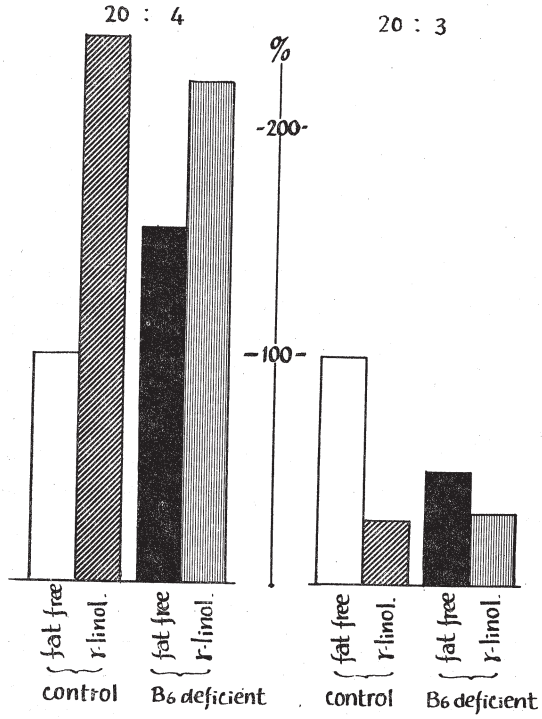

FIG. 4. Changes in the fatty acid composition of liver phospholipid of rats fed γ-linolenate.

5. Effect of pyridoxine deficiency on the fatty acid composition of liver subcellular particles

The fatty acid composition of liver subcellular particles for both pyridoxine deficient and control rats is shown in Table 15.

TABLE 15. Effect of pyridoxine deficiency on the fatty acid spectrum of liver cellular particles

	mitochondria		microsome		supernatant	
	Pyridoxine deficient	Control	Pyridoxine deficient	Control	Pyridoxine deficient	Control
12 : 0	0.5±0.3%	0.8±0.4%	0.4±0.1%	0.4±0.1%	2.1±1.2%	3.1±2.3%
14 : 0	1.7±1.2	0.5±0.3	0.4±0.1	0.3±0.2	1.5±0.7	1.6±0.9
16 : 0	25.8±3.9	23.4±1.7	21.6±1.3	20.5±0.9	35.8±5.3	36.4±6.5
16 : 1	2.9±1.2	2.8±0.5	2.2±0.5	1.9±0.3	5.2±0.7	4.1±1.3
18 : 0	20.9±1.9	21.3±1.2	23.5±1.7	24.9±1.5	6.5±1.6	6.0±1.7
18 : 1	15.9±3.0	14.4±2.0	13.3±1.9	13.7±1.7	24.0±3.6	25.9±6.3
18 : 2	14.0±1.8	14.6±1.8	11.7±1.8	10.8±0.9	17.5±3.7	17.2±3.9
18 : 3	0.4±0.1	0.3±0.1	0.3±0.1	0.4±0.3	1.0±0.6	0.7±0.2
20 : 4	17.6±2.6	22.0±1.3	25.4±2.3	25.9±1.8	5.4±1.6	4.0±1.7
longer	3.0±0.9	4.5±1.0	2.7±0.6	2.7±0.1	1.5±1.0	1.6±0.4
saturated fatty acid	48.9	46.0	45.9	46.1	45.9	47.1
monounsaturated fatty acid	18.8	17.2	15.5	15.6	29.2	30.0
polyunsaturated fatty acid	35.0	41.4	40.1	39.8	25.4	23.5

(*i*) *Mitochondria*: Composition of the mitochondrial fraction in the two subgroups of animals indicated a significant lower concentration of arachidonic acid ($p<0.05$) in the pyridoxine deficient animals. The proportions of polyunsaturated fatty acids were lower and the proportions of saturated fatty acids higher in the pyridoxine deficient group when compared with the control group.

(*ii*) *Microsomes*: The percentage of linoleate tended to be higher but the increase was statistically not significant ($0.2<p<0.5$) in the pyridoxine deficient animals. The percentages of saturated, monounsaturated and polyunsaturated fatty acids in the pyridoxine deficient group were almost equal to those in the control group.

(*iii*) *Supernatant*: In the supernatant fraction no significant difference was found between the proportions of fatty acids in the pyridoxine deficient and the control animals.

DISCUSSION

The liver has been regarded as the important site of lipogenesis, though proof of the synthesis of fatty acids by adipose tissue, mammary gland, intestine, lung and other organs has been presented[24]. It is now generally assumed that two different enzyme systems play a role during fatty acid synthesis in the rat. A distinction can be made between synthesis *de novo* whereby mainly palmitic acid is formed from low-molecular-weight precursors and enzymic chain elongation during which fatty acids already present are converted, *e.g.* palmitic acid to stearic acid and linoleic acid to arachidonic acid. During synthesis *de novo* 1 mole of palmitic acid is formed from 1 mole of acetyl CoA

and 7 moles of malonyl-CoA[25].

The mechanism of fatty acid synthesis *de novo* is as follows: $ATP+HCO_3^-+Acetyl-CoA \rightleftarrows ADP+Po+Malonyl\ CoA$ (Biotin enzyme)

$$CH_3-(CH_2-CH_2)_n-CO-SCoA+\overset{\overset{COOH}{|}}{CH_2}-CoSCoA+2TPNH+2H^+ \rightarrow CH_3-(CH_2-CH_2)_{n+1}-COSCoA+CO_2+HSCoA+2TPN^++H_2O$$

The latter reaction is separated to 6 steps and fatty acid synthesis is accomplished through the repetition of a cycle of 6 consecutive reactions[26]. Fatty acid synthetase catalyzes an orderly and progressive sequence of reactions. This fatty acid synthetase is localized in the supernatant and is distributed in the liver, brain, adipose tissue and lactating mammary gland[26].

On the other hand, the enzymic chain elongation of fatty acid has been shown to take place in the microsomal fraction of the liver[27]. The fatty acid chain is elongated starting from the corresponding acyl-CoA with the aid of malonyl-CoA and NADPH. During chain elongation β-ketoacyl-CoA, β-hydroxy acyl-CoA and trans-α, β-enoyl-CoA occur succesively as intermediates. This enzymic chain elongation can clearly be distinguished from synthesis *de novo* in the supernatant and from a proposed reversal of the mitochondorial β-oxidation.

The synthesis of the monounsaturated fatty acid is also an oxygenase reaction requiring molecular oxygen and DPNH and TPNH. The site of the reaction has been localized to the microsomes[28].

Nutritional experiments with rats and chicks have established the overall pattern of reactions in the formation of polyunsaturated fatty acids. Linoleate (18 : 2ω6), linolenate (18 : 3ω3), oleate (18 : 1ω9) and palmitoleate (16 : 1ω7), each serve as the initial unsaturated precursor for an independent family of polyunsaturated fatty acids and these fatty acids can undergo either chain lengthening or dehydrogenation, and either reaction can proceed first[29]. Linoleic, for example, is converted to arachidonate (20 : 4ω6) through either of two pathways, (a) an initial dehydrogenation to 18 : 3ω6, followed by elongation to 20 : 3ω6[30], or (b) an initial elongation to 20 : 2ω6, followed by dehydrogenation[31]. Recent studies by Davis and Coniglio[32] indicated that 22 : 4ω6 and 22 : 5ω6 were continueing members of this biosynthetic sequence. Although there is no direct cross-over in the metabolism between the four families of fatty acids[33], both feeding (*in vivo*)[34] and enzymatic[35)~37] (*in vitro*) studies suggest that competitive relationship, at the enzyme level, is important in regulating the type of polyunsaturated fatty acid found in tissue lipids. Nutritional experiments by Holman[34] showed that linoleate inhibited the conversion of oleate to 20 : 3ω9 and likewise, that conversion of linoleate to arachidonate was inhibited by feeding oleate or linolenate and that in addition to the metabolic competitions between unsaturated fatty acids, dietary saturated fatty acid likewise exerted influences upon the polyunsaturated acids of tissues. Con-

sequently, it was postulated that the metabolic conversions of polyunsaturated acids were dependent not only upon the concentrations of the various precursors, but also upon the concentrations of other families of polyunsaturated acids which acted as competitive inhibitors, so the concept of balanced diet must include a consideration of balanced concentrations of several polyunsaturated, monounsaturated and saturated fatty acids. On the other hand, Majima[38] from his nutritional experiments using a spectrophotometer stated that: 1) Even if rats were fed on a fat deficier t diet or starved, there hardly occured decreases of linolenic and arachidonic acids, despite the decreases of linoleic acid in phospholipid; 2) Under the existence of pyridoxine and linoleate, linoleic and arachidonic acids changed into each other in phospholipid. In pyridoxine deficiency, linoleic acid failed to change into arachidonic acid, but arachidonic acid was changed into linoleic acid; 3) From these results, he proposed that arachidonic acid was metabolized to linoleic acid in phospholipid of albino rats, and suggested a possibility of a cycle formation between these two acids. However, in Sprecher's experiments[29] on the metabolism of arachidonate and 7, 10, 13, 16 docosatetraenoate ($22:4\omega6$) in rats, the 18 : 2 content was greater in the fat deficient controls than in rats receiving arachidonate. This failure to increase linoleate after feeding arachidonate or 22 : 4 confirms previous observations that there is little catabolism of arachidonte to linoleate. Stoffel and Schiefer[39] also stated that there was no gross measurable conversion of arachidonate to linoleate.

Brenner and Peluffo[37] investigated the desaturation of ^{14}C-labeled linoleate, linolenate, oleate, palmitate and stearate by liver microsomal preparation, under conditions where chain elongation or fatty acid oxidation was inhibited. The rates of conversion of oleate into octa-deca-6, 9-dienoate ($18:2\omega9$), linoleate into γ-linolenate ($18:3\omega6$) and linolenate into octadeca-6, 9, 12, 15-tetraenoate ($18:4\omega3$) were measured, and Lineweaver-Burk plots showed the existence of competitive reactions among the three acids in agreement with the suggestions made from nutritional experiments with rats. Saturated acids were ineffective in altering the percentage of desaturation of the acids, whereas polyunsaturated acids of 20 and 22 carbon atoms generally activated the conversion of the three acids. Docosahexaenoate ($22:6\omega3$) decreased the percentage of desaturation of oleate, linoleate and linolenate. The unsaturated acids of 18 carbons apparently competed for the same oxygenase. Mohrhauer *et al.*[40] studied the chain elongation of linoleate ($18:2\omega6$ to $20:2\omega6$) and found that this reaction also was inhibited *in vitro* by other fatty accids. This reaction was studied again with liver microsomal preparations from rats fed fat free diets. Microsomes, malonyl CoA, NADPH, ATP, $MgCl_2$ and linoleate-$1^{14}C$, with or without inhibitor fatty acids, were incubated in buffer in an anaerobic atmosphere. The anaerobic combinations prevented the oxidative desaturation of the fatty acids. Oxidation of fatty acids was minimized also

by adding KCN and KF to the incubation system. When the liver microsomes were incubated with ^{14}C-linoleate, appreciable radioactivity was found only in linoleate itself and 20 : 2ω6, the first product of chain elongation. When the incubation was made in oxygen, dehydrogenation as well as chain lengthening occurred. The products isolated showed that the following reactions all occurred: 18 : 2ω6 to 18 : 3ω6; 18 : 2ω6 to 20 : 2ω6, 20 : 2ω6 to 20 : 3ω6 and to 20 : 4ω6. Approximately 50 percent of the overall conversion was represented by chain lengthening. Mitochondria also catalyzed some elongation of ^{14}C-linoleate, but this was only about 25 percent of that catalyzed by the microsomal preparations. Thus the endoplasmic reticulum (microsomes) appears to be the major site of chain elongation. As mentioned above the elongation reaction was inhibited by a number of other saturated or unsaturated fatty acids. All the unsaturated fatty acids tested (carbon chains of 18, 20 or 22 carbons) were inhibitory. Though elongation of linoleate was inhibited by a large number of fatty acids, dehydrogenation was inhibited only by oleate, linolenate, and 22 : 6ω3, which fact suggests that the substrate specificity of the enzyme system involved in dehydrogenation is greater than that of the elongation system. In addition, the amount of linolenate needed to inhibit the elongation system is much less than the amount required to inhibit the desaturation reaction. Consequently in the presence of fatty acids, such as occur in a living system, conversion of linoleate to arachidonate may proceed to a greater extent by dehydrogenation followed by chain elongation, than by an initial chain elongation, a reaction which appears much more sensitive toward inhibition.

All of these *in vitro* studies provide comfirmation for the concept of metabolic competition among different families of unsaturated fatty acids, which was proposed from the results of the nutritional studies. In our hardening oil diet groups (Groups B, C and D) the conversion of linoleate to arachidonate (ω6) was inhibited by different families of unsaturated fatty acids. Comparison of the fatty acid composition of liver phospholipid fraction between hardening oil diet and fat free diet groups indicated that the level of oleate is elevated with a corresponding low level of stearate in hardening oil groups. Therefore, there is almost an identical meaning between the hardening oil diet groups and fat free diet groups in regard to EFA deficiency. Gibson[41] emphasized the variations in the activity of enzymes catalyzing the synthesis of long chain fatty acids from acetate or malonyl CoA in liver during periods of alloxan diabetes, starvation and lipid free alimentation. He indicated that in livers of animals maintained on a fat free diet, the levels of the enzymes directly catalyzing the synthesis of saturated fatty acids exceeded normal values 10-fold, but in starvation and diabetes the synthetic activities were greatly depressed[42]. His later studies showed that the capacity of the liver to synthesize long chain fatty acids was greatly enhanced during early linoleate deficiency in mice. The period of dietary restriction of linoleate was also accompanied

by a rapid fall in the relative linoleate content of the liver; by marked changes in the composition of the fatty acids in liver; and by an accumulation of liver triglyceride.

Walker[43] indicated that the fatty acid composition of liver lipids from the fat deficient rats was low in linoleate and arachidonate and high in palmitoleic and eicosatrienoic acids. In our observations (Tables 5–14 Figs. 1–3), fatty acid composition of those animals maintained on the hardening oil diets (Groups B, C and D) and the fat free diet (Group E) agreeded with that reported by earlier investigators[23]. The level[44)45] of eicosatrienoate was elevated with a corresponding low level of linoleate and arachidonate.

The trien to tetraene ratios, an index which has been used by Holman[44] to indicate to what extent animals are deficient in essential fatty acids, are shown in Table 16. Mohrhauer and Holman[23] suggested that a trien to tetraene ratio of 0.4 indicated that rats were normal in regard to essential fatty acid intake. In both hardening oil and fat free diets, the pyridoxine deficient groups had smaller trien to tetraene ratio than the control groups. The recovery group showed almost the same tendency as that of the control group.

Two monoenes, the proportions of palmitoleate and oleate were elevated in the hardening oil groups and the fat free groups than in the basal diet groups. These changes are perhaps due to the fact that these two monoenes are the initial unsaturated precursors of all polyunsaturated fatty acid biosynthesis in animals receiving no essential fatty acids[30]. Supplemental feeding with cotton-seed oil or γ-linolenate resulted in a rapid increase in the concentrations of linoleate and arachidonate, and decrease in the concentrations of

TABLE 16. Trien to tetraen ratio of fatty acids

a) hardening oil diet

	Pyridoxine deficient	Control
total fat	1.01	1.86
phospholipid	0.89	2.06
triglyceride	1.83	4.00
cholesterol ester	2.00	0.72

b) recovery exporiment fed hardening oil diet

	Pyridoxine deficient	Control	Recovery
total fat	1.43	3.61	3.18
phospholipid	1.57	3.23	3.68

c) fat free diet

	Pyridoxine deficient		Control	
	fat free diet	fed γ-linolenate	fat free diet	fed γ-linolenate
phospholipid	0.43	0.20	1.34	0.16

palmitoleate, oleate and eicosatrienoate in the liver phospholipids in both control and pyridoxine deficient rats (Tables 10–14, Figs. 1–4). In our basal diet experiments, the fatty acid compositions of the liver lipids of both control and pyridoxine deficient rats are given in Table 4. Comparison of the liver phospholipids in the two sub-groups animals indicated that there were significantly higher percentage of linoleic acid ($p<0.05$) and significantly lower percentage of arachidonic acid ($p<0.05$) in the pyridoxine deficient sub-groups, wheras in the hardening oil diet groups the phospholipid had higher proportion of arachidonic acid ($p<0.001$) and lower proportion of ($p<0.001$) eicosatrienoic acid in the pyridoxine deficient sub-group than in the control group. These data are in agreement with previous observations[2)5)9)11)]. The higher proportion of arachidonate in the pyridoxine deficient group in the hardening oil diets (Table 8) cannot be assumed to indicate a net gain of arachidonate[45)]. Ostwald *et al.*[46)] indicated that the amount (mg per liver) of arachidonate remained essentially constant in both long-term pyridoxine and EFA deficiencies. The proportion, however, decreases because the amount (mg) of total phospholipid fatty acids increases as liver weight increases. The eicosatrienoate, typical of EFA deficiency, appears when the amount of arachidonate can no longer increase because of the lack of available linoleate. The velocity of gain of linoleate and arachidonate and the loss of eicosatrienoate are more rapid in the control groups than in the pyridoxine deficient groups (Figs. 1, 2, 4). These data are also identical with previous investigations[4)5)], and these changes occur in both supplementation with cotton-seed oil to hardening oil groups and γ-linolenate to fat free diet groups. In Group E also the velocity of incorporation of γ-linolenate to arachidonate and loss of eicosatrienoate are more rapid in the control groups than in the deficient groups. In group E the percentage of the 18 : 2 was greater in the fat-deficient rats than in those rats receiving γ-linolenate. The 18 : 2 in the rats receiving no essential fatty acids was probably a mixture of linoleate (18 : 2 ω6), 18 : 2 ω9 formed by desaturation of oleate and 18 : 2 ω7 formed from cis-vaccenate in a similar manner[29)].

Wise and Elwyn[47)] showed with the use of Serine 3 ^{14}C, that turn-over times for liver lipid serine, ethanolamine, and choline were respectively, 442, 127 and 72 minutes; in small intestine they were 69 minutes for lipid serine and 25 minutes for lipid ethanolamine. These results showed that rates of synthesis via the cytidine diphosphate pathway[48)] were an order of magnitude greater than the rates of other reactions involved in phosphatide synthesis. Our studies showed that feeding cotton-seed oil to the pyridoxine deficient animals resulted in rapid changes in the fatty acid composition of liver phospholipids. In the first 6 days, the concentrations of palmitoleate, oleate and eicosatrienoate decreased rapidly while the concentrations of linoleate and arachidonate increased rapidly in both control and pyridoxine deficient group, but the velocity of incorporation of linoleate and arachidonate into the liver phospholipid of

rats maintained with pyridoxine deficient diets was slower than that with pyridoxine supplemented diets. Walker[43] investigated the ability of various tissues to incorporate essential fatty acids. Rats maintained with a diet deficient in essential fatty acids for 25 weeks were transferred to a diet containing 10% corn oil. Result of feeding corn oil to the EFA deficient animals pointed out rapid changes in the fatty acid composition of liver lipids. During the first 6 days, the concentrations of oleate and eicosatrienoate decreased rapidly while the concentrations of linoleic and arachidonic acids increased rapidly. Oleic, arachidonic, palmitic, and palmitoleic acids approached the corn oil control levels by day of 15 of supplemental feeding. However, the level of linoleic acids remained slightly below the level in the control animals for a longer period. Similarly eicosatrienoate remained slightly elevated until the 29 day of supplemental feeding. Quite similar results were obtained in our present study except that the plateau concentrations reached somewhat sooner. Our results agreed with the faster phosphatide turn-over rates reported by Wise *et al.*[47]. A difference in the velocity of incorporation of linoleate and arachidonate into the liver phospholipid between the pyridoxine deficient and the control groups indicates that pyridoxine may play specific roles in phospholipid synthesis. The report of Haskell and Snell[49] of decreased concentration of phytosphingosine in yeast grown in a pyridoxine deficient medium is pertinent. Goswami and Coniglio[11] pointed out that the structure of phytosphingosine suggested that it, like sphingosine, might be formed through a pyridoxal-phosphate-dependent condensation of L-serine with a fatty acyl derivative.

Takahashi and Fukazawa[50] investigated the fatty acid changes in liver injury and concluded that arachidonate synthesis in liver phospholipid was reflected by the changes in the proportion of arachidonate in liver phospholipid fractions. It has been established in our experiments that pyridoxine deficiency produces a significant depression of arachidonate synthesis, from the significantly higher percentage of arachidonic acid in the liver phospholipid of the control rats than of the pyridoxine deficient rats, and faster increase of arachidonate percentage in the control rats than in the pyridoxine deficient rats after supplementation with cotton-seed oil (linoleate) and γ-linolenate. It is suggested from these results that pyridoxine plays an important role in the essential fatty acid metabolism, especially in the conversion of γ-linolenate to arachidonate, but this does not eliminate the possibility that these changes may be mediated through an essential but presently undefined intermediate role in the fatty acid metabolism of pyridoxine sensitive phospholipid. Further investigations should be performed at subcellular levels.

SUMMARY AND CONCLUSION

In an attempt to clarify a possible role of pyridoxine in the metabolism

of essential fatty acids, the influence of dietary pyridoxine on the fatty acid distribution in the livers of rats maintained with different kinds and conditions of dietary fats were investigated and the following results were obtained.

1) In the liver phospholipid fraction of rats fed a basal diet, the proportion of linoleate was significantly higher, and the proportion of arachidonate significantly lower in the pyridoxine deficient group than in the pair fed control. In this diet group the fatty acid compositions of subcellular particles of the livers were also studied. The total fat of the mitochondrial fraction in the pyridoxine deficient group revealed significantly lower proportion of arachidonate than in the control group.

2) In the groups fed a hardening oil diet, the liver phospholipid fraction of the pyridoxine deficient group showed higher proportion of arachidonic acid and significantly lower proportion of eicosatrienoic acid when compared with the control group. Recovery experiments were carried out.

3) Sequential changes in the liver lipids after giving cotton-seed oil diets to rats previously fed a hardening oil diet showed slower velocity of increase in arachidonate, slower velocity of decrease in eicosatrienoate and faster velocity of increase in linoleate in the pyridoxine deficient groups.

4) After supplementation with γ-linolenate to rats previously fed a fat free diet, there were also seen slower velocity of increase in arachidonate and slower velocity of decrease in eicosatrienoate.

From these results it becomes evident that pyridoxine plays an important role in the essential fatty acid metabolism, especially in the conversion of γ-linolenate to arachidonate.

ACKNOWLEDGEMENT

The author wishes to express his deep gratitude to Prof. K. Yamada, director of the 3rd Department of Internal Medicine, School of Medicine, Nagoya University, for his constant interest and helpful guidance in this study. Grateful acknowledgement is also made to Inst. N. Sakamoto and Dr. K. Yasuda for their careful criticisms and useful comments. The author is deeply indebted to Dr. N. Yamanaka, Institute of Biochemistry, School of Medicine, Nagoya University, for the preparation of subcellular particles by Beckman Model L 4 Ultracentrifuge.

This work was supported in part by a Grant in Aid for Scientific Research from the Education Ministry of Japan.

REFERENCE

1) Holts, P. and Palm, D., Pharmacological aspects of vitamin B_6, *Pharmacol. Rev.*, **16**, 113, 1964.

2) Witten, P. W. and Holman, R. T., Polyethenoid fatty acid metabolism VI. Effect of pyridoxine on essential fatty acid conversion, *Arch. Biochem. Biophys.*, **41**, 266, 1952.

3) Anonymous, The effect of pyridoxine on the conversion of linoleic acid to arachidonic acid, *Nutr. Rev.*, **20**, 109, 1962.
4) Swell, L., Law, M. D., Schools, P. E. and Treadwell, C. R., Tissue lipid fatty acid composition in pyridoxine deficient rats, *J. Nutr.*, **74**, 148, 1961.
5) Scheier, G. E. and Williams, M. A., Sequential changes in liver and heart lipids after giving linoleate or linoleate plus pyridoxine to rats depleted of fat and pyridoxine, *Biochem. J.*, **92**, 422, 1964.
6) Mueller, J. F. and Iacono, J. M., Effect of desoxypyridoxine induced vitamin B_6 deficiency on polyunsaturated fatty acid metabolism in human beings, *Am. J. Clin. Nutr.*, **12**, 358, 1963.
7) Watanabe, N., Gimbel, N. S. and Johnston, C. G., Effect of polyunsaturated and saturated fatty acids on the cholesterol holding capacity of human bile, *Archiv. Surg.*, **85**, 136, 1962.
8) Wakil, S. J., Mechanism of fatty acid synthesis, *J. Lipid Res.*, **2**, 1, 1961.
9) Kirschman, J. C. and Coniglio, J. G., The role of pyridoxine in the metabolism of polyunsaturated fatty acids in rats, *J. Biol. Chem.*, **236**, 2200, 1961.
10) Greenberg, L. D. and Moon, H. D., Alterations in the blood fatty acids in single and combined deficiencies of essential fatty acids and vitamin B_6 in Monkeys, *Acta. Biochem. Biophys.*, **94**, 405, 1961.
11) Goswami, A. and Coniglio, J. G., Effect of pyridoxine deficiency on the metabolism of linoleic acid in the rat, *J. Nutr.*, **89**, 210, 1966.
12) Williams, M. A., Mclntosh, D. J., Hincenbergs, I. and Tamai, K. T., Comparative effects of pyridoxine, riboflavin and thiamine of linoleate utilization in rats, *Biochem. Biophys. Acta.*, **137**, 388, 1967.
13) Umbreit, W. W. and Waddell, J. G., Mode of action of desoxypyridoxine, *Proc. Soc. Exp. Biol. Med.*, **70**, 293, 1949.
14) Folch, J., Lees, M. and Sloanstamley, G. H., A simple method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem.*, **226**, 497, 1957.
15) Mangold, H. K., Thin layer chromatography of lipids, *J. Am. Oil Chemists' Soc.*, **41**, 762, 1964.
16) Renkonen, O. and Varo, P., Thin-layer chromatography of phosphatides and glycolipids, *In* Lipid chromatographic analysis Vol. I, Edited by G. V. Marinetti, Marcel Dekker, Inc, New York, 1967, p. 41.
17) Stoffel, W. Chu, F. and Ahrens, Jr, E. H., Analysis of long-chain fatty acids by gas liquid chromatography, *Anal. Chem.*, **31**, 307, 1959.
18) Stein, R. A., Slawson, V. and Mead, J. F., Gas liquid chromatography of fatty acids and derivatives, *In* Lipid chromatographic analysis Vol. I, Edited by G. V. Marinetti, Marcel Dekker, Inc., New York, 1967, p. 361.
19) Tachisawa, N., The lipid and fatty acid compositions of brain tumor, *Brain and Nerve*, **18**, 234, 1966 (in Japanese).
20) Hoeflmayr, J. und Fried, R., Eine Methode zur routinemässigen Bestimmung des Lipidphosphors und der Phosphatide, *Med. und Ern.*, **7**, 9, 1966.
21) Van Handel, E. and Zilversmit, D. B., Micromethod for the direct determination of serum triglycerides, *J. Lab. and Clin. Med.*, **50**, 152, 1957.
22) Schneider, W. C., Intracellular distribution of enzymes. III The oxidation of octanoic acid by rat liver fractions, *J. Biol. Chem.*, **176**, 259, 1948.
23) Mohrhauer, H. and Holman, R. T., The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver, *J. Lipid Res.*, **4**, 151, 1963.
24) Favarger, P., Relative importance of different tissues in the synthesis of fatty acids, *In* Handbook of physiology section 5: Adipose Tissue, Edited by A. E. Renold and G. F. Cahill Jr, Williams and Wilkins Comp., Washington, D. C., 1965, p. 19,

25) Bressler, R. and Wakil, S. J., Studies on the mechanism of fatty acid synthesis, *J. Biol. Chem.*, **236**, 1643, 1961.
26) Lynen, F., Biosynthesis of saturated fatty acids, *Fed. Proc.*, **20**, 941, 1961.
27) Nugteren, D. H., The enzymic chain elongation of fatty acids by rat liver microsomes, *Biochem. Biophys. Acta.*, **106**, 280, 1965.
28) Gellhorn, A. and Benjamin, W., The intracellular localization of an enzymatic defect of lipid metabolism in diabetic rats, *Biochem. Biophys. Acta.*, **84**, 167, 1964.
29) Sprecher, H., The total synthesis and metabolism of 7, 10, 13, 16-docosatetraenoate in the rat, *Biochim. Biophys. Acta.*, **144**, 296, 1967.
30) Mead, J. F., Lipid metabolism, *Ann. Rev. Biochem.*, **320**, 241, 1963.
31) Stoffel, W. and Ach, K. L., Der Stoffwechsel der ungesättigen Fettsäuren II. Eigenschaft des kettenverlangenden Enzyms. Zur Frage der Biohydrogenierung der ungesättigen Fettsäuren, *Z. Physiol. Chem.*, **337**, 123, 1964.
32) Davis, J. T. and Coniglio, J. G., The biosynthesis of docosapentaenoic and other fatty acids by rat testes, *J. Biol. Chem.*, **241**, 610. 1966.
33) Klenk, E., The metabolism of polyenoic fatty acids, *J. Am. Oil Chemists' Soc.*, **42**, 580, 1965.
34) Holman, R. T., Nutritional and metabolic interrelationships between fatty acid, *Fed. Proc.*, **23**, 1062, 1964.
35) Brenner, R. R. and Peluffo, R. O., Effect of saturated and unsaturated fatty acids on the desaturation *in vitro* of palmitic, stearic, oleic, linoleic and linolenic acids, *J. Biol. Chem.*, **241**, 5213, 1966.
36) Anonymous, Synthesis of polyunsaturated fatty acids *in vitro*, *Nutr. Rer.*, **26**, 154, 1968.
37) Brenner, R. R. and Peluffo, R. O., Regulation of unsaturated fatty acids biosynthesis I., *Biochim. Biophys. Acta.*, **176**, 471, 1969.
38) Majima, Y., Metabolism of arachidonic acid in phospholipid, *Keio J. Med.*, **15**, 213, 1966.
39) Stoffel, W. and Schiefer, H. G., Der Stoffwechsel der ungesättigen Fettsäuren VI. Zur β-cxydation der Mono- und Polyenfettsäuren Untersuhungen *in vivo* und *in vitro* mit [^{3}H, ^{14}C]- und [1-^{14}C]- markierten Mono- und Polyenfettäuren, *H. S. Z. f. Physiol. Chem.*, **341**, 84, 1965.
40) Mohrhauer, H., Christiansen, K., Gan, M. V., Deubig, M. and Holman, R. T., Chain elongation of linoleic acid and its inhibition by other fatty acids *in vitro*, *J. Biol. Chem.*, **242**, 4507, 1967.
41) Hubbard, D. D., Allmann, D. W., Mclain, G. S. and Gibson, D. M., Fatty acid synthesis from malonyl CoA in liver from starved rats refed a fat free diet, *Fed. Proc.*, **20**, 274, 1961.
42) Allman, D. W. and Gibson, D. M., Fatty acid synthesis during early linoleic acid deficiency in the mouse, *J. Lipid Res.*, **6**, 51, 1965.
43) Walker, B. L., Recovery of rat tissue lipids from essential fatty acid deficiency: Plasma, erythrocytes and liver, *J. Nutr.*, **92**, 23, 1967.
44) Rahm, J. J. and Holman, R. T., The relationship of single dietary polyunsaturated fatty acids to fatty acid composition of lipids from subcellular particles of liver, *J. Lipid Res.*, **5**, 169, 1964.
45) Stancliff, R. C., Williams, M. A., Utsumi, K. and Packer, L., Essential fatty acid deficiency and mitochondrial function, *Acta. Biochim. Biophys.*, **131**, 629, 1969.
46) Ostwald, R., Bouchard, P., Miljanich, P. and Lyman, R. L., Influence of sex and gonadal hormones on rat liver and carcass lipids during the development of an essential fatty acid deficiency, *Biochem. J.*, **97**, 485, 1965.
47) Wise Jr., E. M. and Elwyn, D., Rates of reactions involved in phosphatide synthesis in liver and small intestine of intact rats, *J. Biol. Chem.*, **240**, 1537, 1965.
48) Kennedy, E. P. and Weiss, S. B., The function of cytidine coenzymes in the bio-

synthesis of phospholipids, *J. Biol. Chem.*, **222**, 193, 1965.
49) Haskell, B. E. and Snell, E. E., Effect of vitamin B_6 deficiency on the composition of yeast lipids, *Acta. Biochim. Biophys.*, **112**, 494, 1965.
50) Fukazawa, T. and Takahashi, Y., Fatty acid metabolism in liver injury II. Arachidonic acid synthesis in liver injury, *Acta. Hepatol. Jap.*, **9**, 479, 1968 (in Japanese).