of the hydrochloric acid, the two preparations (about 20 μ mc of each) were run side by side by ionophoresis at pH 3.5. Both preparations gave very similar patterns (Fig. 1), the only difference being the presence of a few extra positively charged bands in the *Serratia* ionogram.

The main bands of both preparations were cut out and subjected to ionophoresis in a second dimension at pH 6.5. This permitted a check of the matching of the individual peptides by ionophoresis at two different pH values. The dipeptides and tripeptides matched very closely in both

proteins. Also, the yields were apparently very similar, except for some components of bands 15 and 16. The tripeptides present in 11, 4 and 7, closely matching in both enzymes, include five amino acids around the reactive serine residue (Milstein, 1964). We conclude that the sequence (Thr or Ser)-Asp-SerP-Ala-neutral residue is probably common to both proteins.

The extra radioactive peptides present in the partial acid hydrolysate of the *Serratia* enzyme as compared with the *E. coli* enzyme (Fig. 1) suggest a difference in the amino acid sequence near the common pentapeptide described above. Close similarity in the amino acid sequences at the active site in the same enzyme of different origins has been found in other cases (Milstein, 1961; Perham & Harris, 1963) and indicates the importance of those amino acids for the normal function of the enzyme.

Fig. 1. Radioautograph obtained after ionophoresis at pH 3.5 (at 35 v/cm. for 2.5 hr. on Whatman no. 52 paper) of partial acid hydrolysates of ³²P-labelled alkaline phosphatases of: A, *S. marcescens*; B, *E. coli*.

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Sequential Changes in Liver and Heart Lipids after Giving Linoleate or Linoleate plus Pyridoxine to Rats Depleted of Fat and Pyridoxine

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Despite considerable investigation, the role of pyridoxine in the metabolism of linoleic acid and arachidonic acid is still not clear (Mueller & Iacono, 1963). In general, studies of the effect of pyridoxine deficiency on the utilization of dietary linoleate have been of two types. The first has been concerned with the effects of pyridoxine deficiency on the utilization of linoleate by fat-deficient animals. Witten & Holman (1952), in a study of this type, observed that total body arachidonate (mg./rat) was greater in rats supplemented with linoleate and pyridoxine for 8 weeks than in rats given only linoleate. Before supplementation, the rats had been depleted of both fat and pyridoxine by giving a fat-free pyridoxine-free diet. From these observations it was suggested that 'pyrid-

oxine is involved in the conversion of linoleate to arachidonate'. Kirschman & Coniglio (1961) also used this type of approach and likewise found more arachidonate in the carcass fat of rats supplemented with linoleate and pyridoxine for 3 weeks than in that of rats given only linoleate. The ratios of arachidonate to fat-free carcass weight, however, were similar in both groups of rats, and it was concluded that the increase in arachidonate in the rats given linoleate plus pyridoxine was the result of tissue growth and not a specific effect of pyridoxine in the conversion of linoleate into arachidonate.

The second type of approach has involved the effects of pyridoxine deficiency on linoleate utilization, uncomplicated by recovery from dietary fat deficiency. Swell, Law, Schools & Treadwell (1961) found that the development of pyridoxine defi-

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ciency in rats given a diet containing 10 % of 'vegetable' oil produced small decreases in both the proportion and the amount of arachidonate in liver phospholipids and triglycerides. They concluded that pyridoxine deficiency impaired the conversion of linoleate into arachidonate to some extent, although they obtained no evidence that the conversion directly involved pyridoxine. In contrast, Kirschman & Coniglio (1961) reported that the development of pyridoxine deficiency in rats given a diet containing 20 % of hydrogenated cottonseed oil (Crisco) did not alter the proportions of linoleate and arachidonate in total liver fatty acids. The fatty acid composition of individual lipid fractions, however, was not determined.

Observations by Williams & Scheier (1961) showed that large increases in liver linoleate and arachidonate occurred after only 1 week of giving a source of these fatty acids to fat-deficient rats. These increases suggested that, if pyridoxine deficiency did decrease the conversion of linoleate into arachidonate, as suggested by Witten & Holman (1952) and by Swell *et al.* (1961), the effect might be more pronounced during the first week of recovery from fat deficiency when there was a greater need for this conversion. The following experiment, therefore, tested the effect of pyridoxine deficiency on the rate of increase of linoleate and arachidonate in liver-lipid and heart-lipid fractions during the first week of giving methyl linoleate to rats previously given a pyridoxine-free fat-free diet.

EXPERIMENTAL

Animals and diets. Male 21-day-old rats (Long-Evans), raised in our Laboratory, were caged separately in galvanized screen-wire cages, and given a fat-free casein-

sucrose diet (Williams & Scheier, 1961) with pyridoxine hydrochloride omitted from the vitamin supplement. The dams of these rats were also given a pyridoxine-deficient diet (Williams, Gunning & Pertel, 1962) 1 week before the young rats were weaned, to prevent their obtaining pyridoxine from the mothers' food.

As growth failure occurred because of pyridoxine depletion (not fat depletion; Williams & Scheier, 1961), the rats were placed in the treatment groups shown in Table 1. The zero-time controls received no supplements. The rats in the other groups were given either 100 mg. of methyl linoleate daily or 100 mg. of methyl linoleate plus 30 μ g. of pyridoxine daily for 1, 2, 4 or 6 days. (The methyl linoleate was A grade, chromatographically pure, from California Corp. for Biochemical Research, Los Angeles.) Another group was given 100 mg. of methyl linoleate plus 10 μ g. of pyridoxine daily for 6 days. A dose of 10 μ g. of pyridoxine/day will allow maximum growth in rats 3-7 weeks of age (National Academy of Science, 1962). The rats in the pyridoxine-deficient and the pyridoxine-supplemented groups were paired by weight, and each rat given pyridoxine was pair-fed to the food intake of the corresponding rat in the pyridoxine-deficient group (Table 1).

Lipid extraction. The rats were killed by decapitation without anaesthesia, and the livers and hearts were quickly removed and weighed. Portions of fresh liver (2-3 g.) were homogenized in 95 % (v/v) ethanol. Quinol (0.1 ml. of a 0.1 % solution in absolute ethanol) was added to the homogenates as an antioxidant. After 1 hr. at room temperature the supernatants were filtered and the residues were re-extracted with 95 % ethanol and then with absolute ethanol at room temperature for 1 hr. The ethanolic extracts were combined and the ethanol was removed by evaporation under reduced pressure on a rotary evaporator. The lipids were dissolved in light petroleum (b.p. 30-60°) and stored overnight at -20°. The residue was extracted for at least 12 hr. with redistilled diethyl ether in a Soxhlet apparatus. The ether extract was added to the corresponding light petroleum solution and the combined solvents were evaporated immediately under vacuum. The lipid residue was dissolved in light petroleum and stored in the dark at 0° until analysed.

Table 1. *Average weight gain before and after treatment with methyl linoleate or methyl linoleate plus pyridoxine*

Experimental details are given in the text. Values for liver wt. are given as means $\pm$ s.e.m.

Supplement			No. of rats	Weaning wt. (g.)	Depletion		After supplementation		Total food intake (g.)
Time (days)	Methyl linoleate (mg./day)	Pyridoxine (μ g./day)			Time (days)	Wt. (g.)	Final wt. (g.)	Liver wt. (g.)	
0	0	0	10	51	20	64	—	2.84 $\pm$ 0.27	—
1	100	0	7	49	18	61	60	2.87 $\pm$ 0.09	5
1	100	30	6	48	18	60	65	3.54 $\pm$ 0.25	5
2	100	0	6	49	18	61	61	2.94 $\pm$ 0.31	9
2	100	30	7	52	17	62	65	3.02 $\pm$ 0.32	10
4	100	0	6	54	18	70	73	3.62 $\pm$ 0.28	20
4	100	30	6	53	19	70	78	3.40 $\pm$ 0.27	20
6	100	0	5	50	23	66	69	3.41 $\pm$ 0.18	30
6	100	30	6	55	18	69	81	3.02 $\pm$ 0.18	33
6	100	10	5	53	23	72	86	4.10 $\pm$ 0.41	32

The hearts were homogenized with a mortar and pestle. The lipids were extracted and fractionated by the methods used for liver.

Fractionation of lipids on silicic acid. The lipids of the light petroleum extracts were fractionated on silicic acid micro-columns (Lis, Tinoco & Okey, 1961) into four fractions: I, cholesterol esters; II, triglycerides; III, free cholesterol, free fatty acids, monoglycerides and diglycerides; IV, phospholipids. Approx. 15 mg. of lipid was applied to each micro-column. The eluting solvent was evaporated, and the lipid residue was immediately dissolved in the saponification medium (fractions I and III) or the esterification medium (fractions II and IV). Results for fraction III have not been given since it contained less than 5% of the total fatty acids.

Saponification. Fractions I and III were saponified with 0.3 ml. of 33½% (w/w) potassium hydroxide in 3 ml. of absolute ethanol at 37° for 30 min. The flasks were cooled, 3 ml. of distilled water and 10 ml. of light petroleum were added to each, and the mixtures were left overnight at room temperature. The light petroleum phase was decanted off and the aqueous phase was washed several times with light petroleum. The aqueous phase was acidified to pH 3-4 with 0.3 ml. of 9N-sulphuric acid, and 10 ml. of light petroleum was added to extract the fatty acids. The fatty acids were methylated by heating in 1% (v/v) sulphuric acid in absolute methanol for 30 min. on a steam bath.

Fractions II and IV were transesterified in the same way but without previous saponification.

Determinations. Total lipid was determined by a modification of the dichromate oxidation method of Bloor (1928).

Free cholesterol was measured by a modification (Okey & Lyman, 1954) of the method of Sperry & Webb (1950). Total cholesterol was measured by the method of Abell, Levy, Brodie & Kendall (1952).

Phosphorus was determined by a modification of the method of Sumner (1944). Samples containing 0.02-0.06 mg. of phosphorus were digested in 1.4 ml. of 9N-sulphuric acid. Samples were transferred to 15 ml. glass-stoppered centrifuge tubes and diluted to 10 ml. with water. Then 1 ml. of 6.6% (w/v) ammonium molybdate was added, followed by 1 ml. of freshly made ferrous sulphate solution (5 g. of FeSO_4 plus 1 ml. of 9N-sulphuric acid in 50 ml. of solution). After 45 min. the extinction was measured at 800 m μ .

Phospholipid was calculated as mg. of P $\times$ 25, and fatty acids in phospholipids as phospholipid (mg./g.) $\times$ 0.72.

The recovery of cholesterol and phosphorus after fractionation was 90% or greater.

Differences between means are reported as significant at the 5% level (Student's *t* test; Snedecor, 1957).

Gas-liquid chromatography. An Aerograph A-90-C gas-liquid-chromatography apparatus (Wilkins Instrument Co., Walnut Creek, Calif., U.S.A.) with a thermal-conductivity detector was used. The liquid phase was diethylene glycol succinate polyester supported on Chromosorb W (60-80 mesh) with helium at 50-70 ml./min. at 160-180°. Relative response factors (Horrock, Cornwell & Brown, 1961) were derived from known mixtures of pure methyl esters. The factors were obtained by dividing the amount (mg.) of injected methyl esters by their peak areas (mm.²) and then relating these to the factor for palmitate (1.20). The areas of peaks were measured by planimetry. The amount (mg./g.) of each fatty acid in a fraction was calcu-

lated as a percentage of the total fatty acid (mg./g.) in the fraction. The methyl esters were identified from the retention values (Orr & Callen, 1958; Lipsky, Landowne & Godet, 1959) and by chromatographing before and after hydrogenation of the samples.

Solvents. Ethanol, both 95% (v/v) and absolute, was redistilled in glass; light petroleum (b.p. 30-60°) was redistilled in glass over metallic sodium; ethyl ether was reagent grade, anhydrous, stored at 4°; methanol was reagent grade, anhydrous.

RESULTS

Liver phospholipids. The fatty acid composition of liver phospholipids is given in Table 2. In the pyridoxine-deficient groups, the proportion of arachidonate increased significantly from 23.6% to 31.4% after 1 day and then remained relatively constant at 31-32%. The proportion of linoleate increased significantly from 4.7% to 10.6% after 1 day and then rose gradually to 13% after 6 days. The amounts of arachidonate and linoleate (mg./g. of liver or mg./liver) also increased significantly after 6 days.

With the rats given 30 μ g. of pyridoxine, the proportion of arachidonate increased only 2.6% after 1 day, a significantly smaller rise than that of the rats given linoleate alone, but continued to rise to 39.3% after 6 days, a value significantly higher than that of the pyridoxine-deficient group. In the rats given 10 μ g. of pyridoxine, the proportion of arachidonate was 37.7%. The proportion of linoleate in the pyridoxine-fed groups increased significantly after 1 day and then remained approximately constant at a level significantly lower than in the pyridoxine-deficient group.

In all groups little change occurred in the proportions of other fatty acids with the exception of oleate, which decreased significantly after 1 day of linoleate treatment. Over 95% of the total liver arachidonate and 64-83% of the linoleate were in the phospholipid fraction, with the remainder chiefly in triglycerides (Scheier, 1963). The proportion of the unknown acid, presumed to be the C₂₀ trienoic acid characteristic of fat deficiency (Fulco & Mead, 1959), also declined quite rapidly.

The percentages of laurate and myristate were measured for all fractions, but these values have been omitted from the Tables since the sum of these two fatty acids was always less than 5% of the total fatty acids. In most cases only trace amounts of laurate and myristate were present. Also, fatty acids containing more than 20 carbon atoms appeared only in trace amounts.

Liver cholesterol. After 1 day, the concentration of free cholesterol in the pyridoxine-deficient group was significantly greater than that of the pyrid-

oxine-fed group. After 2 days, however, there were no significant differences (Table 3). The concentration of cholesterol esters after 1 day was also somewhat higher in the pyridoxine-deficient group, but after 2 days was consistently lower; after 6 days, the decrease was statistically significant. The proportion of arachidonate in cholesterol esters increased from 3.6% to 5.9% after 1 day, to 17-18% after 2 days, and then remained at that level. The proportion of linoleate increased from 6.7% to 9.1% after the first day, then rose to 10-11% for the remainder of the treatment period.

In the rats given 30 μ g. of pyridoxine daily, the proportion of arachidonate in cholesterol esters increased from 3.6% to 19% after 4 days. The proportion of linoleate increased from 6.7% to about 8-9%.

Liver triglycerides. In the pyridoxine-deficient groups, the concentration of triglyceride (Table 4) increased significantly after 1 day, then declined after 2 days to 5.2 mg./g., and remained at approximately that level after 4 and 6 days. Very little arachidonate was present in triglyceride fatty acids.

In the rats given 30 μ g. of pyridoxine, triglycerides increased significantly after 1 day, reached a maximum value of 22.3 mg./g. after 2 days, and declined to 5.5 mg./g. after 6 days. The highest triglyceride concentration, 22.5 mg./g., appeared in the rats given 10 μ g. of pyridoxine for 6 days.

Hearts. The phospholipid content of the tissue decreased significantly in the pyridoxine-deficient groups after 6 days and increased slightly after 6 days in the rats given 30 μ g. of pyridoxine, in comparison with the zero control group (Table 5). In the rats given 10 μ g. of pyridoxine the phospholipid content decreased.

In both the pyridoxine-deficient and the pyridoxine-fed groups, phospholipid linoleate showed the largest change in response to feeding with linoleate. In the pyridoxine-deficient groups the proportion of linoleate rose from 13.0% to 25% after 4 and 6 days. As the proportion of linoleate rose, the proportions of arachidonate, oleate and palmitoleate decreased. In the groups given pyridoxine, the proportion of linoleate remained at a level significantly lower than that of the pyridoxine-deficient rats. The proportion of arachidonate, after a slight decline at 4 days, rose to a level significantly higher than that of the pyridoxine-deficient group. The proportions of oleate and palmitoleate decreased. In the rats given 10 μ g. of pyridoxine, arachidonate did not increase although linoleate increased to a value slightly higher than that of the rats given 30 μ g. of pyridoxine.

In all groups heart cholesterol values (Table 6) were very similar and were lower than the liver

Table 2. *Proportions of fatty acids in liver phospholipids of rats given methyl linoleate or methyl linoleate plus pyridoxine after previous fat and pyridoxine depletion*

Experimental details are given in the text. The results for total phospholipids are given as means $\pm$ s.e.m. For the fatty acid composition, trace quantities of other acids were ignored, and the results are given as means $\pm$ s.e.m. The unknown acid, after hydrogenation, gave a peak identical with that of arachidic acid.															
Supplement			No. of rats	Phospho-lipids (mg./g. of liver)	Fatty acid composition of liver phospholipids (% of total)							$C_{18:2}$ acid		$C_{20:4}$ acid	
Methyl linoleate (mg./day)	Pyridoxine (μ g./day)	$C_{16:0}$ acid			$C_{18:0}$ acid	$C_{16:1}$ acid	$C_{18:1}$ acid	$C_{18:2}$ acid	Unknown acid	$C_{20:4}$ acid	(mg./g. of liver)	(mg./g. of liver)			
0	0	0	10	27.4 $\pm$ 1.4	17.6 $\pm$ 0.2	18.0 $\pm$ 0.5	5.6 $\pm$ 0.2	20.0 $\pm$ 0.7	4.7 $\pm$ 0.5	9.6 $\pm$ 1.1	23.6 $\pm$ 1.3	0.93	2.64	4.65	13.2
1	100	0	7	23.1 $\pm$ 2.0	15.9 $\pm$ 0.9	17.8 $\pm$ 0.5	4.5 $\pm$ 0.2	12.7 $\pm$ 0.4	10.6 $\pm$ 0.5	6.8 $\pm$ 0.7	31.4 $\pm$ 0.9	1.76	5.05	5.21	15.0
1	100	30	6	20.6 $\pm$ 0.8	19.0 $\pm$ 1.1	17.0 $\pm$ 0.7	5.6 $\pm$ 0.7	14.1 $\pm$ 0.8	9.7 $\pm$ 0.4	8.1 $\pm$ 1.0	26.2 $\pm$ 1.1	1.44	5.10	3.88	13.7
2	100	0	6	21.1 $\pm$ 1.9	16.0 $\pm$ 0.8	19.2 $\pm$ 0.7	3.7 $\pm$ 0.2	10.6 $\pm$ 0.6	12.5 $\pm$ 0.4	5.9 $\pm$ 0.6	31.9 $\pm$ 0.9	1.90	5.59	4.85	14.2
2	100	30	7	25.1 $\pm$ 2.0	15.7 $\pm$ 0.9	19.1 $\pm$ 0.7	3.8 $\pm$ 0.2	12.1 $\pm$ 0.7	9.9 $\pm$ 0.3	5.4 $\pm$ 0.5	34.0 $\pm$ 1.4	1.79	5.40	6.15	18.6
4	100	0	6	22.1 $\pm$ 2.4	17.3 $\pm$ 0.5	18.0 $\pm$ 0.3	3.0 $\pm$ 0.3	9.4 $\pm$ 0.2	13.1 $\pm$ 0.6	5.3 $\pm$ 1.0	32.3 $\pm$ 0.6	2.08	7.55	5.14	18.7
4	100	30	6	28.5 $\pm$ 3.1	16.7 $\pm$ 0.6	18.9 $\pm$ 0.4	2.4 $\pm$ 0.1	11.6 $\pm$ 0.5	10.3 $\pm$ 0.4	4.6 $\pm$ 0.8	35.0 $\pm$ 0.9	2.11	7.17	7.18	24.4
6	100	0	5	29.2 $\pm$ 0.1	18.4 $\pm$ 0.8	19.0 $\pm$ 0.8	2.8 $\pm$ 0.4	11.9 $\pm$ 0.5	12.7 $\pm$ 0.2	4.1 $\pm$ 0.8	30.9 $\pm$ 0.5	2.67	9.10	6.49	22.1
6	100	30	6	34.3 $\pm$ 1.1	15.7 $\pm$ 0.4	17.9 $\pm$ 0.5	2.1 $\pm$ 0.2	11.2 $\pm$ 0.5	10.5 $\pm$ 0.4	3.0 $\pm$ 0.5	39.3 $\pm$ 0.6	2.59	7.82	9.71	29.3
6	100	10	5	25.1 $\pm$ 1.9	16.8 $\pm$ 0.5	16.2 $\pm$ 0.4	3.0 $\pm$ 0.2	12.7 $\pm$ 0.5	8.7 $\pm$ 0.7	4.7 $\pm$ 0.7	37.7 $\pm$ 0.5	1.57	6.44	6.82	28.0

Experimental details are given in the text. The results for total phospholipids are given as means $\pm$ s.e.m. For the fatty acid composition, trace quantities of other acids were ignored, and the results are given as means $\pm$ s.e.m. The unknown acid, after hydrogenation, gave a peak identical with that of arachidic acid.

Table 3. *Proportions of fatty acids in liver cholesterol esters of rats given methyl linoleate or methyl linoleate plus pyridoxine after previous fat and pyridoxine depletion*

Experimental details are given in the text. The results for cholesterol are given as means $\pm$ S.E.M. The values for total fatty acids were obtained by multiplying the values for cholesterol esters by 0.71. For the fatty acid composition, trace quantities of other acids were ignored, and the results are given as means $\pm$ S.E.M.

Supplement			Liver wt. (g.)	Free cholesterol (mg./g. of liver)	Cholesterol esters (mg./g. of liver)	Total fatty acids (mg./g. of liver)	Fatty acid composition of liver cholesterol esters (% of total)					
Time (days)	Methyl linoleate (mg./day)	Pyrid- oxine (μ g./day)					C _{17:0} acid	C _{18:0} acid	C _{18:1} acid	C _{18:2} acid	C _{20:4} acid	
0	0	0	8	2.84	2.34 $\pm$ 0.17	0.24	27.9 $\pm$ 1.0	12.4 $\pm$ 1.8	11.4 $\pm$ 0.9	34.3 $\pm$ 3.0	6.7 $\pm$ 1.5	3.6 $\pm$ 1.8
1	100	0	7	2.87	2.12 $\pm$ 0.08	0.47	24.2 $\pm$ 1.5	12.5 $\pm$ 1.2	14.5 $\pm$ 2.0	32.0 $\pm$ 3.1	9.1 $\pm$ 1.7	5.9 $\pm$ 3.0
1	100	30	6	3.54	1.72 $\pm$ 0.11	0.28	26.9 $\pm$ 1.1	9.6 $\pm$ 0.9	14.0 $\pm$ 1.5	30.4 $\pm$ 2.4	10.4 $\pm$ 1.6	7.1 $\pm$ 1.8
2	100	0	5	2.94	1.88 $\pm$ 0.16	0.19	24.4 $\pm$ 2.7	18.4 $\pm$ 0.2	7.8 $\pm$ 0.3	19.3 $\pm$ 1.6	10.3 $\pm$ 1.8	17.9 $\pm$ 3.1
2	100	30	7	3.02	2.22 $\pm$ 0.15	0.53	25.6 $\pm$ 1.6	10.0 $\pm$ 1.2	12.8 $\pm$ 1.3	32.7 $\pm$ 3.1	7.7 $\pm$ 1.4	9.8 $\pm$ 2.6
4	100	0	6	3.62	1.97 $\pm$ 0.13	0.26	26.2 $\pm$ 3.2	13.2 $\pm$ 1.1	10.5 $\pm$ 5.5	19.2 $\pm$ 3.3	11.2 $\pm$ 1.6	17.4 $\pm$ 5.6
4	100	30	6	3.40	2.13 $\pm$ 0.16	0.36	20.2 $\pm$ 2.4	13.7 $\pm$ 1.1	7.8 $\pm$ 1.7	28.4 $\pm$ 4.2	9.4 $\pm$ 0.5	19.1 $\pm$ 5.1
6	100	0	3	3.41	2.05 $\pm$ 0.06	0.19	21.5 $\pm$ 2.1	16.1 $\pm$ 5.0	7.5 $\pm$ 1.8	20.9 $\pm$ 0.4	11.3 $\pm$ 2.4	16.6 $\pm$ 6.4
6	100	30	6	3.02	2.39 $\pm$ 0.28	0.65	17.8 $\pm$ 0.8	10.8 $\pm$ 2.2	7.1 $\pm$ 1.6	34.3 $\pm$ 7.4	9.4 $\pm$ 0.8	19.7 $\pm$ 6.0
6	100	10	5	4.10	1.97 $\pm$ 0.14	0.56	22.3 $\pm$ 0.3	12.5 $\pm$ 1.4	10.8 $\pm$ 1.0	38.3 $\pm$ 1.7	8.0 $\pm$ 0.4	6.9 $\pm$ 1.4

Table 4. *Proportions of fatty acids in liver triglycerides of rats given methyl linoleate or methyl linoleate plus pyridoxine after previous fat and pyridoxine depletion*

Experimental details are given in the text. The values for total triglycerides were calculated by difference and are given as means $\pm$ S.E.M. For the fatty acid composition, trace quantities of other acids were ignored, and the results are given as means $\pm$ S.E.M. N.D., Not detected.

Supplement			Liver wt. (g.)	Triglycerides		Fatty acid composition of liver triglycerides (% of total)					
Time (days)	Methyl linoleate (mg./day)	Pyrid- oxine (μ g./day)		(mg./g. of liver)	(mg./ liver)	C _{16:0} acid	C _{18:0} acid	C _{18:1} acid	C _{18:2} acid	C _{20:4} acid	
0	0	0	10	3.6 $\pm$ 1.6	10.2	28.3 $\pm$ 1.2	2.7 $\pm$ 0.8	9.2 $\pm$ 1.2	53.0 $\pm$ 2.5	4.6 $\pm$ 1.3	0.4
1	100	0	7	10.1 $\pm$ 2.1	29.0	28.7 $\pm$ 0.8	1.6 $\pm$ 0.4	10.6 $\pm$ 1.0	47.6 $\pm$ 1.8	9.1 $\pm$ 1.3	0.5
1	100	30	5	12.6 $\pm$ 2.0	44.6	36.7 $\pm$ 1.4	1.8 $\pm$ 0.2	12.2 $\pm$ 1.0	42.5 $\pm$ 2.2	4.6 $\pm$ 1.0	Trace
2	100	0	6	5.2 $\pm$ 1.3	15.3	31.7 $\pm$ 1.9	2.1 $\pm$ 0.4	8.6 $\pm$ 0.8	43.2 $\pm$ 2.3	12.5 $\pm$ 2.5	N.D.
2	100	30	7	22.3 $\pm$ 8.6	67.3	33.8 $\pm$ 2.5	2.3 $\pm$ 0.9	10.1 $\pm$ 1.6	43.9 $\pm$ 2.3	8.0 $\pm$ 2.4	N.D.
4	100	0	6	4.6 $\pm$ 1.5	16.7	36.1 $\pm$ 3.0	2.6 $\pm$ 0.4	9.5 $\pm$ 1.2	38.0 $\pm$ 1.9	11.2 $\pm$ 4.6	0.3
4	100	30	6	11.8 $\pm$ 4.6	40.1	30.1 $\pm$ 1.1	3.3 $\pm$ 1.5	5.8 $\pm$ 1.8	50.9 $\pm$ 3.5	8.3 $\pm$ 1.8	N.D.
6	100	0	5	5.7 $\pm$ 0.8	19.4	32.1 $\pm$ 1.7	2.5 $\pm$ 0.2	9.0 $\pm$ 1.6	44.8 $\pm$ 2.2	9.4 $\pm$ 1.8	N.D.
6	100	30	5	5.5 $\pm$ 2.0	16.6	30.2 $\pm$ 1.5	2.5 $\pm$ 0.4	5.7 $\pm$ 0.8	53.2 $\pm$ 2.2	7.2 $\pm$ 1.8	0.3
6	100	10	5	22.5 $\pm$ 11.5	92.2	36.0 $\pm$ 0.5	2.4 $\pm$ 0.2	9.5 $\pm$ 0.5	47.5 $\pm$ 1.4	2.6 $\pm$ 0.5	N.D.

Table 5. Proportions of fatty acids in heart phospholipids of rats given methyl linoleate or methyl linoleate plus pyridoxine after previous fat and pyridoxine depletion

Experimental details are given in the text. The results for heart wt. and total phospholipids are given as means $\pm$ s.e.m. For the fatty acid composition, trace quantities of other acids were ignored, and the results are given as means $\pm$ s.e.m. The unknown acid, after hydrogenation, gave a peak identical with that of arachidic acid.													
Supplement				Heart wt. (g.)	Phospholipids		Fatty acid composition of heart phospholipids (% of total)						
Time (days)	Methyl linoleate (mg./day)	Pyrid- oxine (μ g./day)	No. of rats		(mg./g. of heart)	(mg./ heart)	C _{18:0} acid	C _{18:1} acid	C _{18:2} acid	C _{18:3} acid	Unknown acid	C _{20:4} acid	
0	0	0	4	0.310 $\pm$ 0.028	29.7 $\pm$ 1.3	9.2	11.9 $\pm$ 0.9	20.6 $\pm$ 0.7	2.4 $\pm$ 0.4	16.8 $\pm$ 1.4	13.0 $\pm$ 0.8	2.6 $\pm$ 0.8	31.8 $\pm$ 1.4
1	100	0	6	0.283 $\pm$ 0.012	29.4 $\pm$ 0.5	8.3	12.2 $\pm$ 0.4	20.3 $\pm$ 0.5	1.5 $\pm$ 0.2	12.6 $\pm$ 0.6	17.7 $\pm$ 0.2	1.9 $\pm$ 0.4	30.8 $\pm$ 0.9
1	100	30	6	0.327 $\pm$ 0.027	28.8 $\pm$ 1.5	9.4	11.4 $\pm$ 0.4	19.0 $\pm$ 1.0	1.4 $\pm$ 0.1	13.2 $\pm$ 0.4	20.0 $\pm$ 1.0	1.5 $\pm$ 0.2	30.8 $\pm$ 1.1
4	100	0	4	0.356 $\pm$ 0.017	26.5 $\pm$ 1.8	9.4	11.9 $\pm$ 0.6	17.3 $\pm$ 3.7	1.3 $\pm$ 0.1	11.4 $\pm$ 0.6	24.7 $\pm$ 1.0	2.0 $\pm$ 0.2	28.1 $\pm$ 2.6
4	100	30	5	0.315 $\pm$ 0.018	28.0 $\pm$ 1.9	8.8	11.3 $\pm$ 0.5	22.5 $\pm$ 0.8	1.2 $\pm$ 0.1	11.2 $\pm$ 0.6	21.7 $\pm$ 0.9	2.1 $\pm$ 0.4	26.8 $\pm$ 2.3
6	100	0	5	0.384 $\pm$ 0.025	24.4 $\pm$ 1.5	9.4	10.7 $\pm$ 0.4	21.5 $\pm$ 0.8	0.5 $\pm$ 0.7	9.7 $\pm$ 0.3	25.3 $\pm$ 0.8	3.2 $\pm$ 0.6	27.2 $\pm$ 0.7
6	100	30	6	0.298 $\pm$ 0.007	32.5 $\pm$ 3.0	9.7	10.4 $\pm$ 0.6	19.8 $\pm$ 1.1	0.8 $\pm$ 0.2	8.8 $\pm$ 0.4	21.2 $\pm$ 1.0	1.7 $\pm$ 0.5	35.2 $\pm$ 2.6
6	100	10	5	0.328 $\pm$ 0.013	22.0 $\pm$ 1.0	7.2	10.8 $\pm$ 0.4	20.0 $\pm$ 1.4	0.9 $\pm$ 0.3	11.4 $\pm$ 0.7	24.1 $\pm$ 0.9	2.9 $\pm$ 0.8	28.6 $\pm$ 0.9

Experimental details are given in the text. The results for heart wt. and total phospholipids are given as means $\pm$ S.E.M. For the fatty acid composition, trace quantities of other acids were ignored, and the results are given as means $\pm$ S.E.M. The unknown acid, after hydrogenation, gave a peak identical with that of arachidic acid.

values. Very little cholesterol ester was present even after 6 days of linoleate treatment. The amounts of triglyceride were too small to measure accurately. The hearts of the pyridoxine-deficient groups were significantly larger after 6 days than those of groups given 30 μ g. of pyridoxine.

DISCUSSION

The formation of arachidonate in rats at least mildly deficient in pyridoxine is clearly shown by the increases in liver-phospholipid arachidonate after feeding with linoleate (Table 2). After only 1 day of linoleate treatment, the rise in the proportion of arachidonate was even significantly greater in the pyridoxine-deficient group than in the pyridoxine-fed group. Further, the amount of arachidonate in the livers of pyridoxine-deficient groups increased after 4 and 6 more days of pyridoxine depletion although the proportion of arachidonate did not increase significantly after the first day. There is no apparent explanation for the smaller increase in arachidonate after 1 day in the group given pyridoxine.

Despite the increase in phospholipid arachidonate and phospholipids in the pyridoxine-deficient groups after feeding with linoleate, both the proportion and the amount (mg./g. of liver and mg./liver) of phospholipid arachidonate were still significantly lower in the pyridoxine-deficient group after 6 days than in the rats given 30 μ g. of pyridoxine. The proportion and the amount (mg./liver) in the pyridoxine-deficient group were also significantly lower than in the rats given 10 μ g. of pyridoxine, although the amounts expressed as mg./g. of liver were similar in both groups. The amounts of phospholipid arachidonate, expressed as mg./g. of liver, in these two groups were lower than in the rats given 30 μ g. of pyridoxine. These lower values reflect the relative hypertrophy of the livers of the pyridoxine-deficient group and the rats given 10 μ g. of pyridoxine after 6 days, in comparison with the rats given 30 μ g. of pyridoxine. The hypertrophy in the rats given 10 μ g. of pyridoxine is not the result of fat accumulation since calculation of arachidonate as mg./g. of fat-free tissue gives similar results. Hypertrophy of the liver has previously been found in pyridoxine-deficient rats (Agnew, 1955).

The similar weight gains for the rats given 10 μ g. of pyridoxine and those given 30 μ g. of pyridoxine for 6 days show that 10 μ g. of pyridoxine/day was sufficient for maximum growth under these conditions. The differences in liver triglycerides and in liver and heart phospholipids, however, show the metabolic differences produced by the higher pyridoxine intake. The rats given 10 μ g. of pyridoxine also had significantly heavier livers.

Table 6. *Heart cholesterol of rats given methyl linoleate or methyl linoleate plus pyridoxine after previous fat and pyridoxine depletion*Experimental details are given in the text. The results are given as means $\pm$ S.E.M.

Time (days)	Supplement		No. of rats	Total cholesterol (mg./g. of heart)	Free cholesterol (mg./g. of heart)
	Methyl linoleate (mg./day)	Pyridoxine (μ g./day)			
0	0	0	4	1.54 $\pm$ 0.07	1.45 $\pm$ 0.05
1	100	0	6	1.49 $\pm$ 0.06	1.38 $\pm$ 0.11
1	100	30	6	1.48 $\pm$ 0.04	1.42 $\pm$ 0.05
4	100	0	4	1.36 $\pm$ 0.09	1.32 $\pm$ 0.05
4	100	30	5	1.42 $\pm$ 0.01	1.37 $\pm$ 0.04
6	100	0	5	1.36 $\pm$ 0.04	1.27 $\pm$ 0.04
6	100	30	6	1.55 $\pm$ 0.04	1.47 $\pm$ 0.02
6	100	10	5	1.32 $\pm$ 0.08	1.27 $\pm$ 0.09

Comparison of liver and heart phospholipids shows that fat depletion affected liver fatty acids more rapidly, as indicated by the higher percentages of the C₂₀ unknown acid and palmitoleate in liver phospholipids at the start of linoleate treatment. Machlin (1961) reported that the proportions of trienoic acids increased more slowly in the heart than in the liver in the initial stages of fat depletion in young chicks. In contrast, in severe fatty acid deficiency in the rat, the proportion of trienoic acids is much greater in the heart than in the liver (Rieckehoff, Holman & Burr, 1949). The heart hypertrophy in the present experiment was the result of pyridoxine deficiency (Agnew, 1955) and not fat deficiency (Portman, Andrus, Pollard & Bruno, 1961).

The decreased percentage of arachidonate in the organ phospholipids after 6 days of pyridoxine deficiency might have resulted from a decreased rate of biosynthesis or an accelerated catabolism, or from a decreased availability of other compounds required for phospholipid formation, e.g. the phospholipid bases. The latter suggestion is plausible since it has been either demonstrated or suggested that pyridoxal phosphate functions in the reactions leading to the formation of serine, ethanolamine, choline and sphingosine (Braunstein, 1960; Greenberg, 1960; Dekker & Maitra, 1962; Crawhall & Watts, 1962).

SUMMARY

1. Analyses were made of liver and heart lipids of male weanling rats, depleted of pyridoxine and fat and given supplements of methyl linoleate with or without 30 μ g. of pyridoxine daily for 1, 2, 4 and 6 days.

2. In the liver, linoleate plus pyridoxine produced a higher concentration of liver cholesterol esters, phospholipids and phospholipid arachi-

donate, and a lower concentration of phospholipid linoleate, than treatment with linoleate alone. Treatment with linoleate significantly increased phospholipids and phospholipid arachidonate in comparison with the initial values.

3. In the heart, treatment with linoleate plus pyridoxine produced higher concentrations of phospholipids, phospholipid arachidonate and cholesterol. Linoleate alone caused a significant decrease in phospholipid arachidonate and increased phospholipid linoleate.

4. Rats given linoleate plus 10 μ g. of pyridoxine daily gained as much weight as those given linoleate plus 30 μ g. of pyridoxine, but differed considerably in the composition of their liver and heart lipids.

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The Preparation of Phosphoarginine: a Comparative Study

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Phosphoarginine was first isolated from fresh-water crab muscle as an amorphous barium salt $[\text{Ba}(\text{C}_6\text{H}_{14}\text{N}_4\text{O}_5\text{P})_2 \cdot 2\text{H}_2\text{O}]$ by Meyerhof & Lohmann (1928). No further work on this compound was reported until the publication by Ennor, Morrison & Rosenberg (1956) of an improved method for its isolation. This method took advantage of the fact that phosphoarginine forms a copper salt of limited solubility in water and a hydrochloride derivative that is insoluble in ethanol. The product differed in composition from that described by Meyerhof & Lohmann (1928) in that it corresponded to the formula $\text{C}_6\text{H}_{13}\text{BaN}_4\text{O}_5\text{P} \cdot \text{H}_2\text{CO}_3 \cdot \text{H}_2\text{O}$.

More recently, synthetic methods have been used to prepare phosphoarginine. Thus Thiem, Thoai & Roche (1962) developed a procedure that involved phosphorylation of arginine with phosphoryl chloride and separation by column chromatography of the monophosphorylated derivative. The latter was obtained as a crystalline lithium salt, $\text{C}_6\text{H}_{13}\text{LiN}_4\text{O}_5\text{P}$. Cramer, Schieffele & Vollmar (1962) have used more specific reactions to obtain the amorphous barium salt of phosphoarginine, $\text{Ba}(\text{C}_6\text{H}_{14}\text{N}_4\text{O}_5\text{P})_2 \cdot 3\text{H}_2\text{O}$. One involved the phosphorylation of N^α -benzyloxycarbonylarginine with bis-(*p*-nitrobenzyl) phosphorochloridate, and the other involved the transfer of the phosphorylated

amidino group of *O*-methyl-*N*-phosphorylisourea to ornithine.

In connexion with plans to carry out detailed kinetic studies of the reaction catalysed by ATP-L-arginine phosphotransferase (arginine kinase, EC 2.7.3.3):

it was essential to have relatively large amounts of pure phosphoarginine. Thus an investigation has been made of those methods which appeared capable of giving good and reproducible yields of pure product. The present paper reports the results of this investigation together with details of an improved method for the isolation of phosphoarginine from crayfish muscle. It also describes a procedure for the enzymic synthesis of the compound.

EXPERIMENTAL

Materials

Chemicals. Unless otherwise stated, AnalaR-grade chemicals obtained from British Drug Houses Ltd. (Poole, Dorset) were used. L-Arginine hydrochloride and ATP (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used without further purification.

Charcoal was Nuchar C granular (West Virginia Pulp and Paper Co., New York, U.S.A.) which was treated by boiling