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Distribution of Unsaturated Fatty Acid in Pyridoxine-Deficient Hypercholesterolaemia

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The present study was prompted by the suggestion of Sinclair (1956) that the development of atherosclerosis may be related to a deficiency of unsaturated fatty acids. Since pyridoxine is known to be a factor for the conversion of linoleate into arachidonate (Witten & Holman, 1952), the atherosclerosis observed in pyridoxine-deficient monkeys (Rinehart & Greenberg, 1959) may be related to the deficiency of arachidonate. To test the validity of this hypothesis, a detailed study has been made of the unsaturated fatty acid content of the blood and arteries of pyridoxine-deficient rats.

Whether the hypercholesterolaemia observed was due to increased mobilization of lipids from liver or to some intrinsic metabolic aberration was tested by determining the cholesterol content of bile, which is known to reflect faithfully the status of cholesterol synthesis by the liver (Friedman & Byers, 1954).

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EXPERIMENTAL

Induction of pyridoxine deficiency. Rats (60) of 60-85 g. body wt. were fed on a purified diet containing vitamin-free casein (22.0%), sucrose (66.5%), fat (coco-nut oil; 9.0%), salt mixture (2.5%) and sulphaguanidine for depletion of vitamins. After 3 weeks the animals were divided into three groups of 20 each. Animals of group 1 served as control and received the basal diet supplemented with a vitamin mixture containing (mg./kg. of diet) the following: calcium pantothenate (44); vitamin A concentrate (200 units/ml.; 99); α -tocopherol (225.5); inositol (301.4); menadione (4.4); biotin (0.7); niacin (595.5); *p*-aminobenzoic acid (22); riboflavin (22); pyridoxine hydrochloride (46); thiamine hydrochloride (22); choline chloride (601.0); folic acid (0.2 μ g./kg.). Group 2 animals (inanition group) also received the same diet but it was adjusted in amount so that their weight gain corresponded to that of group 3 rats (deficient group), which received the same diet with the exception of pyridoxine hydrochloride. Body-weight gains were recorded daily and after 9 weeks the animals were killed. Blood was drawn directly from the heart after

opening the thorax and serum obtained from two animals of the same group was pooled for subsequent analyses.

Determination of unsaturated fatty acid of serum.

(a) Preparation of lipid extract. A portion (2 ml.) of serum was pipetted dropwise into 10 ml. of ethanol-ether mixture (3:1, v/v) and refluxed under nitrogen for 8 hr. Samples were filtered and the residue was further extracted with 200 ml. of ethyl ether for an additional 8 hr. under nitrogen. The two extracts were combined and dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure and the total lipids were re-extracted with light petroleum (b.p. 60–70°).

(b) Separation of lipids on silicic acid column. A column for operation under slight but constant pressure of nitrogen (2 cm. Hg above atmospheric pressure) according to the design of Herb, Witnauer & Riemenschneider (1951) was used. The glass column (21 mm. $\times$ 300 mm.) was packed by introducing silicic acid (E. Merck, analytical grade, suitable for chromatography), prepared by boiling 22 g. of silicic acid in 100 ml. of light petroleum (b.p. 45–60°) for 5 min., with slow stirring with a stainless-steel rod. The column, when settled, was about 19 cm. high. About 10 ml. of the lipid solution was then placed at the top of the column with a separating funnel. This solution was washed down the column with 10 ml. of the solvent. The first eluting solvent was then added at the top of the column and the flow rate adjusted at a rate of about 150 ml./hr. by increasing or decreasing the nitrogen pressure at the top of the column. The order of elution was (1) hydrocarbons: 50 ml. of light petroleum (+1% of ethyl ether); (2) sterol esters: 300 ml. of light petroleum (+1% of ethyl ether); (3) glycerides: 300 ml. of light petroleum (+4% of ethyl ether); (4) sterol and free acids: 350 ml. of light petroleum (+8% of ethyl ether); (5) phospholipids: 250 ml. of methanol-ethyl ether (25:75, v/v) followed by 250 ml. of methanol-ethyl ether (50:50, v/v) (Luddy, Barford & Riemenschneider, 1958).

The solvent was removed from the eluted fractions under reduced pressure and transferred to round-bottomed flasks with light petroleum. This solvent was then removed *in vacuo* and the residue saponified with ethanolic KOH. The fatty acids were then extracted quantitatively with light petroleum after acidification and made to standard volume with light petroleum.

(c) Isomerization. A portion (15 ml.) of the solution was then transferred to reaction tubes designed according to Pikaar & Nijhof (1958). The solvent was evaporated in a water bath under a flush of nitrogen. A portion (0.5 ml.) of the KOH-ethylene glycol reagent was added and the tube heated to 180° for exactly 16 min. The tubes were then cooled and the volume was made to 10 ml. with methanol. Extinction values were measured in a Unicam SP. 500 spectrophotometer at 233, 268, 315, 346 and 374 $m\mu$, against a blank similarly treated. The concentrations of polyethenoid fatty acids were calculated by using the equations of Herb & Riemenschneider (1952).

In the determinations of unsaturated fatty acids, it has been found desirable not to warm the reagent before addition of the sample. Nevertheless, the application of the formulae of Herb & Riemenschneider (1952) remains justifiable, as pointed out by several observers (Pikaar & Nijhof, 1958).

Fatty acids of arterial tissue. These were determined by the method of Dam & Engel (1958). Samples (10 ml.) of the light petroleum extracts were evaporated in reaction tubes and isomerized with KOH-ethylene glycol followed by

determinations of extraction in the u.v. region. The total fatty acid content was estimated by weighing the residue after evaporation and also by titrating a sample dissolved in benzene with 0.005N-sodium ethoxide with phenolphthalein as indicator.

Iodine value. Samples of a solution of weighed fatty acids were transferred to glass-stoppered 50 ml. flasks; the solvent was evaporated under a stream of nitrogen and the contents were redissolved in chloroform. Exactly 1.0 ml. of 0.2N-Wijs solution was added, and the flasks were stoppered and kept in the dark for 1 hr. After this reaction time 0.5 ml. of 15% KI was added, followed by 5 ml. of water. The iodine was then titrated with 0.01N-sodium thiosulphate with 4–8 drops of 1% starch as indicator.

Bile. This was collected from fistulae made on rats under urethane anaesthesia, and bile and serum cholesterol were determined by the method of Mookerjee & Sadhu (1955).

RESULTS

The fatty acid deposition of the animals in general appear to be a little low when compared with results obtained by others, which is probably due to the inclusion of coco-nut oil as the dietary source of fat. There was, however, no appreciable deficiency in respect of essential fatty acids of the animals, as judged morphologically, and the differences between the control and the experimental groups in respect of the polyenoic acid concentration of the sterol-ester fractions of blood serum are significant (Tables 1 and 2). That coco-nut oil itself has an insignificant effect on the cholesterol of the rat is borne out by the observations of Nath, Weiner, Harper & Elvehjem (1959). Blood cholesterol (free) was 43.0 ± 4.8 mg./100 ml. in controls and 80.0 ± 3.1 mg./100 ml. in the experimental animals, the corresponding values for the ester fraction being 62.0 ± 2.7 and 118.0 ± 5.3 mg./100 ml. The aortic arch and the thoracic aorta have cholesterol values of 210.0 ± 8.5 mg./100 g. in the controls and 325.0 ± 12.5 mg./100 g. in the deficient animals.

DISCUSSION

There is increased cholesterol deposition in the arteries of pyridoxine-deficient rats, accompanied by esterification of sterol esters of blood with more saturated fatty acids, supporting the thesis of Sinclair (1956). Whether the arterial-sterol deposition is due to esterification with more saturated fatty acids cannot be said definitely as yet. Pyridoxine deficiency affects arachidonic acid formation and if cholesterol deposition in aortae of pyridoxine-deficient rats is due to more saturated sterol esters, there may be less cholesterol arachidonate in the aorta wall. This, combined with the increased amount of saturated fatty acids deposited with cholesterol in the arterial wall, will be reflected in the total fatty acid spectrum of the aortae in such a way as to lower its iodine value and arachidonate

Table 1. *Polyenoic fatty acid composition of arterial tissue of control rats and rats made deficient in pyridoxine*

Analysis on fatty acids recovered after saponification and removal of unsaponifiable material. Total fatty acid is expressed as g. and cholesterol as mg./100 g. wet wt. of tissue. Results are means $\pm$ S.D. for 20 rats in each group.

Acid	Pyridoxine-deficient group	
	Control group	
Iodine value	63.5	68.7
Total fatty acids	4.3 $\pm$ 0.38	4.2 $\pm$ 0.73
Oleic acid (%)	40.01 $\pm$ 3.86	44.67 $\pm$ 2.31
Dienoic acid (%)	20.09 $\pm$ 1.31	18.38 $\pm$ 0.97
Trienoic acid (%)	2.38 $\pm$ 0.38	3.10 $\pm$ 0.56
Tetraenoic acid (%)	9.87 $\pm$ 0.96	10.13 $\pm$ 0.68
Pentaenoic acid (%)	1.26 $\pm$ 0.93	0.99 $\pm$ 0.39
Hexaenoic acid (%)	Traces	Traces
Cholesterol, free	210.0 $\pm$ 8.5	325.0 $\pm$ 12.5

Table 2. *Polyenoic fatty acid composition of blood-serum sterol esters of control rats, inanition-group rats and rats made deficient in pyridoxine*

Analysis on fatty acids of sterol esters separated from a silicic acid column of Bloor extract. Cholesterol concentration is expressed as mg./100 ml. of blood serum and liver bile. Results are means $\pm$ S.D. for 20 rats in each group.

Acid	Pyridoxine-deficient group		
	Control group	Inanition group	
Iodine value of Bloor extract	42.0 $\pm$ 1.7	39.5 $\pm$ 2.1	21.0 $\pm$ 6.6
Oleic acid (%)	31.65 $\pm$ 1.3	27.10 $\pm$ 1.8	36.1 $\pm$ 3.6
Dienoic acid (%)	41.33 $\pm$ 3.5	38.10 $\pm$ 3.2	48.33 $\pm$ 2.7
Trienoic acid (%)	1.38 $\pm$ 0.4	0.98 $\pm$ 0.2	1.16 $\pm$ 0.4
Tetraenoic acid (%)	5.71 $\pm$ 0.8	4.42 $\pm$ 0.5	2.26 $\pm$ 0.9
Pentaenoic acid (%)	0.97 $\pm$ 0.3	0.78 $\pm$ 0.2	0.69 $\pm$ 0.3
Hexaenoic acid (%)	0.53 $\pm$ 0.2	Traces	Traces
Serum cholesterol, free	43.0 $\pm$ 4.8	38.2 $\pm$ 2.1	80.0 $\pm$ 3.1
Serum cholesterol, ester	62.0 $\pm$ 2.7	55.0 $\pm$ 4.1	118.0 $\pm$ 5.3
Bile cholesterol	12.4 $\pm$ 0.2	9.1 $\pm$ 0.3	10.6 $\pm$ 1.4

concentration. Our data, however, do not show any significant difference of the unsaturated fatty acid spectrum of aorta between pyridoxine-deficient rats and the controls.

The hypercholesterolaemia of pyridoxine deficiency is not a reflexion of any aberration in the synthetic pattern of cholesterol by liver, as evidenced by the constancy of bile cholesterol. If the enzymic pattern for cholesterol synthesis is not affected by pyridoxine deficiency, it may tentatively be assumed that cholesterol synthesis by arteries is also normal and the cholesterol that is deposited in the intima has reached there from the blood stream.

Although the polyethenoid fatty acid, particularly arachidonic acid content, of the serum was lowered in pyridoxine deficiency, the rats did not show symptoms of deficiency of essential fatty acids to any significant extent, as judged by their general appearance. The same order of tetraenoic fatty acid concentration in the aortae of the control and of the deficient rats, despite the significant difference in the blood concentrations of the two groups of

animals, points either to a relatively unimportant role of arachidonic acid in the aortae or to a greater effort of the deficient animals to maintain the tetraenoic acid content of the aortae constant. In either case it is difficult to reconcile it with the hypothesis of Sinclair (1956).

SUMMARY

1. Pyridoxine deficiency induces a significant degree of hypercholesterolaemia in rats and there is deposition of cholesterol in aortae (thoracic and abdominal).

2. The tetraenoic fatty acid level of serum is lowered in pyridoxine-deficient hypercholesterolaemia in rats, but the unsaturated fatty acid spectrum of aortae is not significantly altered in pyridoxine deficiency.

3. The hypercholesterolaemia is probably due to increased mobilization of cholesterol from the liver.

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The Inhibition of Photoreactions of Chloroplasts by 2-Alkyl-4-hydroxyquinoline N-Oxides

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An antagonist of streptomycin action in several bacteria was shown to consist of a mixture of 2-alkyl-4-hydroxyquinoline N-oxides (Cornforth & James, 1956). The same compounds were demonstrated to be effective inhibitors of electron transport in cell-free preparations of heart muscle and some bacterial preparations (Lightbown & Jackson, 1956; Jackson & Lightbown, 1958). The site of the inhibitory action of 2-heptyl-4-hydroxyquinoline N-oxide in heart-muscle preparations was concluded to be between diaphorase and cytochrome *c*, and most probably between cytochrome *b* and cytochrome *c* (Jackson & Lightbown, 1958).

More recently, the same compound was observed (Smith & Baltscheffsky, 1959) to inhibit completely at $1\ \mu\text{M}$ concentration light-induced phosphorylation in extracts of the photosynthetic bacterium *Rhodospirillum rubrum*. At the same concentration it had no effect on the respiratory activity of the extract. In addition, it produced changes in the light-dependent difference spectrum which were interpreted as an increased oxidation of cytochrome *c*₂ and a decreased oxidation of the *b*-type cytochromes. 2-Heptyl-4-hydroxyquinoline N-oxide has also been used as an accelerator of photo-oxidation (Vernon, 1959) and inhibitor of photo-reduction (Vernon & Ash, 1959) catalysed by bacterial chromatophores. A communication describing its inhibition of photophosphorylation catalysed by spinach chloroplasts has recently appeared (Baltscheffsky, 1959).

In view of the potency of these compounds in specifically inhibiting the bacterial photophosphorylative system, it was thought of importance to

determine their effect on the different photoreactions catalysed by higher-plant chloroplasts. Two of the most potent of this group of inhibitors were selected (Lightbown & Jackson, 1956), and a quantitative study of their inhibitory action was undertaken. Experiments designed to test their specificity, site of action, manner of attachment and relation to other inhibitors of photoreactions in chloroplasts are described below.

METHODS

Preparation of chloroplasts. Chloroplasts and chloroplast fragments were prepared from greenhouse-grown Swiss-chard leaves as described by Avron (1960).

Measurement of reactions. The procedure followed and the measurement of the production of adenosine [³²P]triphosphate (AT³²P) were as described by Avron (1960). The reduction of ferricyanide was determined from the decrease of *E* at 420 m μ of a sample of the trichloroacetic acid-denatured supernatant, a molar absorption coefficient of 1.06×10^3 being used. The reduction of triphosphopyridine nucleotide (TPN) and trichloroindophenol was determined from the change of *E* at 340 and 620 m μ respectively of the complete reaction mixture. All changes in *E* were measured against a blank which contained the same reaction mixture, but to which no dye had been added.

All reactions were run at 17°, with air as the gas phase. The reaction cuvettes were placed within the aquarium described by Avron (1960), with an illumination of about 150,000 lux of white light.

Solutions of inhibitor. The inhibitor was dissolved in mm-NaOH solution and added as such. Control samples contained an equivalent amount of mm-NaOH. The concentration of the inhibitor in the stock solution was determined before each experiment by its absorption at 346 m μ (Corn-