Studies on 'Orotic Acid Fatty Liver'

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The development of fatty liver on feeding orotic acid to weanling rats was first reported by Standerfer & Handler (1955). The increase in fat was found to be entirely in the neutral-lipid fraction. An increase in liver cholesterol in addition to the neutral-lipid fraction in the rats fed with orotic acid has been observed in our laboratory in preliminary experiments by D. S. R. Sarma, K. V. Rajagopalan and P. S. Sarma. Choline, methionine and vitamin B₁₂, which are known to have a pronounced effect on neutral lipid (Best, Channon & Ridout, 1934; Best & Ridout, 1936), failed to exert their lipotropic action on 'orotic acid fatty liver' (Standerfer & Handler, 1955). Similarly, inositol, which is known to influence amounts of cholesterol in rats (McHenry & Patterson, 1944; Rajalakshmi, Srinivasan & Sarma, 1960), was also found to be ineffective in our preliminary trials. This inability of choline as well as of inositol to protect the infiltration of fat in the livers of orotic acid-fed rats, together with the observations of Handschumacher, Creasey, Jaffe, Pasternak & Hankin (1960) that only adenine supplementation in the diet restored the concentration of liver lipid to normal, indicated that 'orotic acid fatty liver' is entirely different from other types of fatty liver so far reported in the literature.

Though the accumulation of fat in the livers of rats fed with orotic acid suggested a direct effect on lipid metabolism, yet it also appeared to be due to certain alterations in the metabolism of nucleic acid, protein and carbohydrate. Since orotic acid is a pyrimidine precursor (Arvidson *et al.* 1949) it is reasonable to expect a primary effect on nucleic acid metabolism on feeding orotic acid to animals. In the present investigations, the effect of orotic acid feeding in rats on nucleic acid, protein and carbohydrate contents of the liver was examined. To ascertain whether the accumulation of cholesterol was due to increased synthesis, increased mobilization or decreased oxidation, the incorporation of [1-¹⁴C]acetate into liver cholesterol *in vivo* was studied. Also the cholesterol content of the plasma, carcass and liver, and the pyridine nucleotide content of the liver, were determined.

METHODS

Animals and diets. Albino rats weighing 45–50 g. were divided into different groups of ten, each consisting of five male and five females. Rats in group 1 received the basal

diet of the following composition: sucrose 75%, casein 18%, salt mixture 5% (Hubbell, Mendel & Wakeman, 1937), gingly oil 2%. To each 1 kg. batch of diet were added the following vitamin supplements: thiamine hydrochloride 12.5 mg., inositol 250 mg., pyridoxine hydrochloride 12.5 mg., riboflavin 25 mg., calcium pantothenate 62.5 mg., niacin 250 mg., *p*-aminobenzoic acid 125 mg., vitamin K 12.5 mg., vitamins A and D as Adexolin 1 ml. (Glaxo Laboratories Ltd.). Rats in group 2 received in addition to the basal diet 1% of orotic acid (obtained from Sigma Chemical Co.). Rats in group 3 received 1.5% of orotic acid as a supplement to the basal diet. From the third week onwards half of the animals in group 3 received 0.3% of adenine (Roche) as an additional supplement and these animals constituted group 4. The animals were housed in individual cages and were given various diets *ad libitum* for 4 weeks. At the end of the experimental period, the animals were killed by decapitation. The liver, plasma and carcass were saved for analysis.

Methods of analysis. Liver lipid was extracted by the method of Handler (1948) and that of carcass by the method of McHenry & Gavin (1938). Cholesterol was determined according to the method of Sperry & Webb (1950). The residue which remained after the removal of lipid was treated with cold trichloroacetic acid repeatedly to remove acid-soluble nucleotides. The trichloroacetic acid-treated residue was analysed for adenine by the method of Woodhouse (1950), for pyrimidines by the method of Soodak, Pircio & Cerecedo (1949), for protein nitrogen by direct nesslerization and for glycogen by the anthrone method of Carroll, Longley & Roe (1956). The pyridine nucleotide content was estimated by fluorimetry as described by Carpenter & Kodicek (1950).

Incorporation of [1-¹⁴C]acetate into liver cholesterol *in vivo*

At the end of the experimental period 10 μ Ci of sodium [1-¹⁴C]acetate (obtained from The Radiochemical Centre, Amersham, Bucks.) was injected intraperitoneally into rats of groups 1 and 2. These animals were killed after 4 hr. by decapitation and the livers were chilled in ice immediately. The cholesterol was isolated as its digitonide by the method of Srere, Chaikoff & Dauben (1948). The digitonide was cleaved by anhydrous pyridine after purification (Schönheimer & Dam, 1933). Cholesterol was dissolved in peroxide-free ether. A sample was assayed for cholesterol and the remainder was plated on an aluminium planchet and its radioactivity was determined in a proportional gas-flow counter connected to a Berkeley scaler.

RESULTS

Results presented in Table 1 show that animals in groups 2 and 3 had decreased growth rate. There was also a significant increase in liver weight and

total lipid. The data in Table 2 summarize the effect of orotic acid on cholesterol content of the liver, plasma and carcass. The figures show that rats in groups 2 and 3 had an enhanced liver cholesterol content with a concomitant decrease in both plasma and carcass. The inclusion of adenine in the diet restored concentrations of plasma and carcass cholesterol to normal, though the liver cholesterol was not completely reduced. However, the liver weight and total lipid values were restored to normal. In Table 3 are given the results of incorporation of [^{14}C]acetate into liver

cholesterol *in vivo*. It is obvious that there was no increase in the incorporation of [^{14}C]acetate into liver cholesterol. There appeared to be a decrease in cholesterol synthesis in the livers of rats fed with orotic acid. No significant change could be noticed in the incorporation of [^{14}C]acetate into the liver fatty acids. In Table 4 are presented the data on the effect of orotic acid on pyridine nucleotide and protein-nitrogen contents of the liver as well as the adenine and the pyrimidine (uracil + cytosine) contents of the defatted liver. A marked decrease in adenine, protein nitrogen and pyridine nucleo-

Table 1. *Effect of orotic acid on liver fat of rats*

For details see Methods section. Values are given $\pm$ s.d.

Group no.	Supplement to the basal diet	Average gain in weight during the experimental period (g.)	Liver wet wt. (g.)	Liver total lipid (% wet wt.)
1	Nil	74.0 $\pm$ 6.0	5.1 $\pm$ 0.4	8.4 $\pm$ 0.3
2	1% of orotic acid	64.0 $\pm$ 9.0	7.8 $\pm$ 0.8	21.8 $\pm$ 1.1
3	1.5% of orotic acid	56.0 $\pm$ 7.0	8.2 $\pm$ 1.4	19.5 $\pm$ 1.6
4	1.5% of orotic acid + 0.3% of adenine	66.0 $\pm$ 8.0	5.6 $\pm$ 0.2	8.9 $\pm$ 0.3

Table 2. *Effect of orotic acid on total cholesterol content of plasma, liver and carcass of rats*

For details see Methods section. Values are given $\pm$ s.d.

Group no.	Supplement to the basal diet	Plasma (mg./100 ml.)	Liver (mg./100 g. wet wt.)	Carcass (mg./100 g. wet wt.)
1	Basal diet	82.9 $\pm$ 5.6	314.8 $\pm$ 10.3	333.4 $\pm$ 15.4
2	1% of orotic acid	42.8 $\pm$ 6.5	554.6 $\pm$ 30.6	298.7 $\pm$ 7.4
3	1.5% of orotic acid	42.0 $\pm$ 2.0	602.9 $\pm$ 17.4	255.1 $\pm$ 15.5
4	1.5% of orotic acid + 0.3% adenine	105.8 $\pm$ 3.3	488.6 $\pm$ 4.7	324.2 $\pm$ 7.8

Table 3. *Incorporation of [^{14}C]acetate into liver cholesterol in vivo*

Each rat received 10 μC by intraperitoneal injection and was killed after 4 hr. The activity values were corrected self-absorption. For further details see Methods section.

Group no.	Supplement to the basal diet	Liver cholesterol (mg./g. wet wt. of liver)	Specific activity of cholesterol (counts/min./mg.)	Total counts recovered in cholesterol fraction (counts/min./g. of liver)
1	Nil	2.87	82	236
2	1% of orotic acid	4.38	18	79

Table 4. *Effect of orotic acid on rat-liver pyridine nucleotides, protein, ribonucleic acid, adenine and pyrimidines*

Values are given $\pm$ s.d.

	Basal diet	Basal diet + 1% of orotic acid	Basal diet + 1.5% of orotic acid
Total pyridine nucleotides ($\mu\text{g./g.}$ of wet liver)	578 $\pm$ 31.0	350 $\pm$ 9.3	—
Protein nitrogen (% of defatted liver)	14.8 $\pm$ 0.6	10.4 $\pm$ 1.4	—
RNA (mg./g. of defatted liver)	54.6 $\pm$ 2.8	40.3 $\pm$ 4.4	26.7 $\pm$ 5.2
Adenine (mg./g. of defatted liver)	3.9 $\pm$ 0.2	3.0 $\pm$ 0.3	1.8 $\pm$ 0.4
Uracil + cytosine (mg./g. of defatted liver)	5.8 $\pm$ 1.8	7.0 $\pm$ 2.1	7.0 $\pm$ 1.9

tides was noticed together with an increase in the content of pyrimidines. The glycogen values were highly erratic and so have not been included in the table.

DISCUSSION

Standerfer & Handler (1955) were the first to report the accumulation of fat in the livers of albino rats fed with orotic acid. The increase was mainly in the neutral-lipid fraction. The present experiments revealed that orotic acid exerted a definite and consistent effect on cholesterol metabolism. Orotic acid feeding to rats resulted in the accumulation of cholesterol in the liver, accompanied by a simultaneous decrease in the concentrations of cholesterol of both plasma and carcass. This explains the observation of Fillios & Andrus (1960) that orotic acid lowered cholesterol in animals fed with uracil. Measurement of the incorporation of [1-¹⁴C]acetate *in vivo* showed a decrease in cholesterol synthesis in the liver. This is in agreement with the general conception that liver-cholesterol content determines rate of synthesis. Beher & Baker (1959) observed decreased incorporation of radioactive acetate into liver cholesterol *in vivo* of rats given cholic acids. Schade & Saltman (1959) reported similar observation in the liver slices of rabbits fed with nicotinic acid. Recently Siperstein & Guest (1960) suggested that cholesterol feeding exerted a 'feed-back' effect on the conversion of β -hydroxy- β -methylglutaryl-coenzyme A into mevalonate. The cholesterol accumulation in the liver caused by orotic acid feeding in the present set of experiments appears to have exerted similarly a homeostatic effect by depressing cholesterol synthesis.

The low liver-protein-nitrogen values induced by orotic acid feeding may be due either to a decrease in the synthesis of protein or to an increase in protein catabolism. It is clear from the results in Table 4 that the pyrimidine content exceeds the adenine content in the ribonucleic acid (RNA) of the livers of animals fed with orotic acid. In other words, the results indicate a possible alteration in purine/pyrimidine ratio in RNA of the livers of rats fed with orotic acid. The inclusion of 1% of orotic acid, a pyrimidine precursor, in a synthetic diet will increase the pyrimidine pool and might cause a drain on the available adenine if the organism is to maintain a normal purine and pyrimidine ratio in the RNA of the liver. This might in turn affect the concentration of adenine-containing coenzymes and might account for the observed fall in pyridine nucleotides. However, a fall in the pyridine nucleotides was observed by Dianzani (1955) in choline deficiency. As this author found no change in synthesis *in vitro* of diphosphopyridine nucleotide (DPN) through the

Kornberg reaction, he explained the decrease as due to a displacement of DPN from the cell before the onset of fat accumulation. In rats fed with orotic acid, the RNA per unit weight of defatted liver showed a decrease. The RNA content of the livers of rats fed with orotic acid showed an increase if expressed on the basis of the weight of the whole liver, as was observed by Handschumacher *et al.* (1960).

The accumulation of cholesterol in the livers of rats fed with orotic acid occurs despite its decreased synthesis as revealed by tracer studies. The concomitant decrease in carcass and plasma suggest that the accumulation of lipid is due to increased mobilization from extra-hepatic tissues to the liver. Adenine feeding reverses this condition.

SUMMARY

1. Feeding orotic acid to weanling albino rats resulted in the accumulation of cholesterol and total lipid in the liver.
2. The accumulation of cholesterol was not due to increased biosynthesis as revealed by the incorporation of [¹⁴C]acetate into liver cholesterol *in vivo*.
3. The concomitant decrease of cholesterol concentration in plasma as well as in carcass and the low pyridine nucleotide content of the liver suggested that the cholesterol accumulation in liver was due to increased mobilization from extra-hepatic tissues, with further impairment in its metabolism in the liver.
4. Low concentrations of protein nitrogen and ribonucleic acid of the liver further indicated that protein and nucleic acid metabolism also could be affected by orotic acid.
5. Supplementation of the diet with adenine completely reversed the effects of orotic acid on the cholesterol content of plasma, carcass and liver.

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Distribution and Seasonal Variation of *allo*Hydroxy-L-proline in *Santalum album* L.

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In the course of extensive investigations on the occurrence of free amino acids in plants (Radhakrishnan, Vaidyanathan & Giri, 1955), the presence of high amounts of free hydroxyproline was noticed on paper chromatograms of extracts of the leaves of sandal, *Santalum album* L., by Giri, Gopalkrishnan, Radhakrishnan & Vaidyanathan (1952). This observation led to the isolation of the imino acid from leaves of sandal tree by Radhakrishnan & Giri (1954), who identified it as *allo*-hydroxy-L-proline.

The distribution of *allo*hydroxy-L-proline in the sandal tree under a variety of conditions and preliminary results concerning its formation *in vivo* are now given.

EXPERIMENTAL

Sampling. For studying the variation of *allo*hydroxy-L-proline content in leaves from different trees, branches were collected in the morning and the eight pairs of youngest leaves were detached from each branch, shredded, dried in an oven at 50° for 48 hr. and powdered (100 mesh/in.).

For distribution studies the branches were obtained as before and the pair of leaves at each node was detached and mixed with the corresponding leaves from other branches. Thus the leaves from the apical nodes of all branches were pooled and the sample was designated '1st node'. Increasing sample number indicates pro-

gressively older leaves. The flowers and buds were dried and powdered similarly. The fruits were dried and powdered after splitting off the pericarp from the seed. The seed was defatted with light petroleum (b.p. 40–60°) before analysis.

The influence of storage upon the *allo*hydroxy-L-proline content of leaves was determined by collecting a large number of leaves from a healthy sandal tree. Separate portions were then kept in a moist chamber under normal laboratory conditions of temperature (24–26°) and illumination. After 1, 3, 4 and 6 days, the *allo*hydroxy-L-proline content of the leaves was determined, before and after drying.

Effect of amino acids on formation of *allo*hydroxy-L-proline by leaves of excised branches of sandal. Branches containing an equal number of nodes were cut from healthy sandal trees, and the excised ends were placed immediately in distilled water. Each experimental sample contained five branches selected from the main stock. The basal ends of the branches were cut to expose fresh surfaces. The branches were placed in a sterile solution of the appropriate amino acid (200 p.p.m.). A control sample was placed in water. After 54 hr. the leaves were detached and their *allo*hydroxy-L-proline contents were determined after drying. All experiments were conducted in triplicate.

Determination of *allo*hydroxy-L-proline. The method followed was essentially that of Neuman & Logan (1950) as modified by Martin & Axelrod (1953). Each powdered sample (50 mg.) was extracted with aqueous 75% (v/v) ethanol (5 ml.) for 15 min. with occasional stirring. After centrifuging, the residue was again extracted with 75% (v/v) ethanol (5 ml.) and finally washed with 75% (v/v) ethanol (1 ml.). The combined ethanol extracts were evaporated to dryness over CaCl₂ *in vacuo*. The residue was extracted with water and after filtration the volume was

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