(70.5 mg./100 ml.) and then more slowly up to the age of 10–15 weeks when the mature value is reached (76.1 mg./100 ml.).

2. It is suggested that the rapid increase may be due to the replacement of embryonic-type erythrocytes low in phytic acid by adult-type erythrocytes high in phytic acid.

3. There are no differences between the sexes in the concentration of phytic acid in the blood cells, and the concentration is not influenced by reproductive activity.

4. The mean concentration of free inositol in the plasma of chicks 3 weeks of age (5 mg./100 ml.) is at least 5 times as high as in human plasma.

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Metabolic Aspects of 'Orotic Acid Fatty Liver'

NUCLEOTIDE CONTROL MECHANISMS OF LIPID METABOLISM

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Fatty liver in rats given a purified diet containing 1% of orotic acid is not prevented by usual lipotropic factors such as folic acid, vitamin B₁₂, choline, methionine and inositol (Standerfer & Handler, 1955; Rajalakshmi, Sarma & Sarma, 1961). Only supplementation with adenine restored the liver-lipid concentration to normal (Hanschumacher, Creasey, Jaffe, Pasternak & Hankin, 1960a). These findings, together with other observations, show that in this type of fatty infiltration there were some changes in the metabolism of pyrimidines; this led to the hypothesis that 'orotic acid fatty liver' may be due to alterations in nucleotide metabolism (Hanschumacher, Creasey & Hankin, 1960b).

In the present paper, the effect of high doses (1% of the diet) of orotic acid on lipid, phospholipid, acid-soluble nucleotide and nucleic acid contents in the liver of rats given a purified diet is reported. Also, RNA and DNA turnover, liver adenosine-deaminase, xanthine-oxidase and carbamoyl phosphate-aspartate-transcarbamoylase activities and the concentration of nicotinamide nucleotides in the liver were determined. The results of comparative studies on the effect of lower dietary doses (0.001% of diet) of orotic acid and of supplementation with adenine are also given; previous studies have shown that 0.001% of orotic acid is unable to produce an abnormal infiltration

of fat into the liver of rats (Marchetti, Puddu & Caldarera, 1962a) and of vitamin B₁₂-deficient chicks (Marchetti, Puddu & Caldarera, 1962c). However, it can induce certain changes in the concentrations of free nucleotides in the livers of these animals (Marchetti, Caldarera & Moruzzi, 1962b; Marchetti, Caldarera & Puddu, 1962d).

MATERIALS AND METHODS

Male albino rats of the Wistar strain weighing 45–50 g. were divided into four groups of 30 animals each. Rats of the first group received the purified diet (Standerfer & Handler, 1955) of the following composition: sucrose, 75%; vitamin-free casein, 18%; salt mixture, 5%; corn oil, 2%; vitamin supplements, 0.2%; sulphadiazine was also added to the diet. The other three groups were fed on the same diet supplemented with 0.001% of orotic acid, 1% of orotic acid and 1% of orotic acid plus 0.25% of adenine sulphate respectively. The animals were housed in individual cages and were given different diets and water *ad libitum* for 4 weeks.

At the end of the experimental period 24 rats of each group were decapitated, and the livers were saved for chemical analysis and for preparing histological slides. Total lipids were extracted twice by homogenizing livers in 20 vol. of ethanol-diethyl ether (3:1, v/v) in a glass Potter-Elvehjem homogenizer, and the homogenates were kept for 24 hr. at 37°. The residue obtained after centrifugation was re-extracted three times with fresh portions of

solvent. The pooled extract was then evaporated to dryness *in vacuo* and the residue dissolved in light petroleum (b.p. 30–50°). The total lipids were measured gravimetrically and portions were taken for photometric analysis of lipid phosphorus by the method of Fiske & Subbarow (1925). Protein nitrogen was determined in each sample by the usual micro-Kjeldahl technique.

Acid-soluble nucleotides in the liver were determined by chromatography on ion-exchange resin, as described by Hurlbert, Schmitz, Brumm & Potter (1954), and RNA and DNA were determined by the method of Schneider (1946). The purine base:pyrimidine base ratio was determined by the method described by Marchetti, Caldarera & Moruzzi (1960). Liver adenosine-deaminase and xanthine-oxidase activities were estimated by the methods outlined by Kalekar (1947) and by Litwack, Bothwell, Williams & Elvehjem (1953) respectively.

Liver carbamoyl phosphate-aspartate-transcarbamoylase activity was estimated by the method of Bresnick & Hitchings (1961). Determinations of nicotinamide nucleotides were carried out by the fluorimetric method described by Dianzani (1955).

The remaining animals of each group were injected intraperitoneally with 0.5 mc of [32 P]phosphate (obtained from The Radiochemical Centre, Amersham, Bucks.). The rats were killed after 10 hr. and the livers were used for the measurement of [32 P]phosphate incorporation into the nucleic acids by the method described by Bhargava, Simkin & Work (1958). The specific radioactivity of each sample was corrected for differences in dosage and body weight. As used in the present work, specific radioactivity is defined as $100 \times \text{counts/min.}/\text{mg. of RNA or DNA}$ divided by the counts/min. administered/g. body wt., i.e. biological concentration coefficient (Schulman & Falkenheim, 1950). To relate the fractions more directly to their precursor, the radioactivities of RNA and DNA fractions were reported as relative specific radioactivities. The relative specific radioactivity of a fraction is defined as $100 \times \text{the specific radioactivity of that fraction divided by the specific radioactivity of the acid-soluble phosphorus}$. The total relative radioactivity is defined as the amount of RNA (or DNA)/g. of defatted liver multiplied by its relative specific radioactivity. All the results were analysed for statistical significance by Fisher's *t* test, a difference between two mean values being regarded as significant if *P* was no greater than 0.01.

RESULTS

Histological examination showed that the structure of the liver of rats given 1 % of orotic acid in

the diet was well preserved and the cells were full of fat, whereas no fatty infiltration could be observed in the livers of rats receiving 0.001 % of orotic acid or 1 % of orotic acid plus 0.25 % of adenine sulphate. These histological observations were confirmed by the following analytical results. Table 1 shows that the total lipid content of rats given 1 % of orotic acid was markedly higher than that of animals given the purified diet ($P < 0.001$). No significant variation was noted in animals given 0.001 % of orotic acid ($P < 0.2$) or 1 % of orotic acid plus 0.25 % of adenine sulphate ($P < 0.2$). On the contrary, liver phospholipids were decreased only in rats receiving 1 % of orotic acid ($P < 0.001$).

In rats given 1 % of orotic acid the content of protein nitrogen in the liver was decreased by 15 %; no significant difference was noticed in animals given 0.001 % of orotic acid or 1 % of orotic acid plus 0.25 % of adenine sulphate.

It appears from Tables 2 and 3 that the presence of 1 % of orotic acid in the diet resulted in significant changes in the content of acid-soluble nucleotides in the liver. Purine nucleotides, and in particular adenine nucleotides, showed a remarkable decrease ($P < 0.001$). However, an increase in pyrimidine nucleotides was noted. This increase was due almost entirely to the uridine nucleotides ($P < 0.01$); the cytidine nucleotides showed a decrease ($P < 0.01$). In those rats given 0.001 % of orotic acid cytidine nucleotides increased ($P < 0.01$). Supplementation with adenine restored in part the concentrations of free nucleotides in the liver to normal. In fact no significant differences in the concentrations of cytidine nucleotides ($P < 0.2$) and adenine nucleotides ($P < 0.1$) in the liver were noted between the control rats and the animals given 1 % of orotic acid plus 0.25 % of adenine sulphate; a scarcely significant difference in the concentration of uridine nucleotides ($P 0.05$) was observed.

The RNA content (Table 4) of the livers of rats given 1 % of orotic acid was higher ($P < 0.01$) than that in the liver of rats that had received the purified diet alone or the same diet plus 0.001 % of orotic acid, when expressed on the basis of the weight of the whole liver. No significant variation

Table 1. *Effect of orotic acid and adenine on the total lipids and phospholipids of rat liver*

Each value is given as the mean $\pm$ S.E.M. of ten determinations on different animals. Experimental details are given in the text.

Group	Supplement to basal diet	Total lipids (mg./g. wet wt. of liver)	Phospholipids (mg./g. wet wt. of liver)
1	None	54.6 $\pm$ 2.27	26.24 $\pm$ 0.57
2	Orotic acid (0.001%)	51.3 $\pm$ 1.37	23.93 $\pm$ 0.50
3	Orotic acid (1%)	153.5 $\pm$ 11.39	16.76 $\pm$ 0.60
4	Orotic acid (1%) + adenine sulphate (0.25%)	56.3 $\pm$ 2.39	22.11 $\pm$ 0.45

Table 2. *Effect of orotic acid and adenine on the free nucleotides of rat liver*

Each value is given as the mean $\pm$ s.e.m. of eight determinations on different animals. Experimental details are given in the text.

Group	Supplement to basal diet	Free nucleotides (μ moles/100 g. of defatted liver)							UDP-acetyl-glucosamine
		CMP	AMP	GMP	IMP	UMP	ADP		
1	None	59 $\pm$ 4.5	153 $\pm$ 11.3	21 $\pm$ 2.8	66 $\pm$ 5.9	36 $\pm$ 4.2	105 $\pm$ 8.8	62 $\pm$ 4.8	
2	Orotic acid (0.001%)	91 $\pm$ 8.7	183 $\pm$ 13.9	40 $\pm$ 4.5	69 $\pm$ 7.1	49 $\pm$ 6.1	107 $\pm$ 6.1	52 $\pm$ 5.1	
3	Orotic acid (1%)	47 $\pm$ 2.4	124 $\pm$ 6.5	33 $\pm$ 4.2	41 $\pm$ 7.8	115 $\pm$ 9.9	38 $\pm$ 2.1	119 $\pm$ 7.4	
4	Orotic acid (1%) + adenine sulphate (0.25%)	81 $\pm$ 9.3	152 $\pm$ 17.2	23 $\pm$ 3.1	38 $\pm$ 8.6	83 $\pm$ 9.2	54 $\pm$ 4.3	104 $\pm$ 13.2	
Free nucleotides (μ moles/100 g. of defatted liver)									
GDP + mixture of unidentified adenosine polyphosphates*									
UDP-glucose									
1	None	73 $\pm$ 3.6	55 $\pm$ 8.2	5 $\pm$ 0.2	49 $\pm$ 6.7	59 $\pm$ 7.1	GTP	UTP	
2	Orotic acid (0.001%)	54 $\pm$ 4.3	59 $\pm$ 7.6	20 $\pm$ 3.1	33 $\pm$ 5.2	50 $\pm$ 7.9	9 $\pm$ 1.1	19 $\pm$ 1.5	
3	Orotic acid (1%)	124 $\pm$ 11.5	48 $\pm$ 3.9	Trace	59 $\pm$ 7.3	24 $\pm$ 1.7	11 $\pm$ 1.4	8 $\pm$ 1.1	
4	Orotic acid (1%) + adenine sulphate (0.25%)	92 $\pm$ 8.7	47 $\pm$ 6.2	Trace	54 $\pm$ 8.9	30 $\pm$ 4.9	Trace	9 $\pm$ 1.6	
							3 $\pm$ 0.5	4 $\pm$ 0.9	
* Cf. Hurlbert <i>et al.</i> (1954).									

Table 3. *Effect of orotic acid and adenine on the free nucleotides of rat liver*

Each value is given as the mean $\pm$ s.e.m. of eight determinations on different animals. The results summarize the values shown in Table 2.

Group	Supplement to basal diet	Free nucleotides (μ moles/100 g. of defatted liver)						Total purine nucleotides
		Uridine nucleotides	Cytidine nucleotides	Total pyrimidine nucleotides	Adenine nucleotides	Guanine nucleotides	Inosine nucleotides	
1	None	239 $\pm$ 21.8	64 $\pm$ 4.6	303	317 $\pm$ 27.4	85 $\pm$ 12.6	66 $\pm$ 5.9	468
2	Orotic acid (0.001%)	196 $\pm$ 22.1	111 $\pm$ 11.9	307	340 $\pm$ 27.9	110 $\pm$ 13.7	69 $\pm$ 7.1	519
3	Orotic acid (1%)	423 $\pm$ 37.7	48 $\pm$ 2.3	471	186 $\pm$ 10.3	82 $\pm$ 8.7	41 $\pm$ 7.9	309
4	Orotic acid (1%) + adenine sulphate (0.25%)	338 $\pm$ 39.3	82 $\pm$ 9.3	418	236 $\pm$ 27.2	73 $\pm$ 9.8	38 $\pm$ 8.6	346

Table 4. *Effect of orotic acid and adenine on the content of nucleic acid in rat liver*

Each value is given as the mean $\pm$ S.E.M. of six determinations on different animals. Experimental details are given in the text.

Group	Supplement to basal diet	RNA		DNA		Nucleic acid. Purine base: pyrimidine base ratio
		(mg./g. of defatted liver)	(mg./liver)	(mg./g. of defatted liver)	(mg./liver)	
1	None	7.97 $\pm$ 0.14	55.04 $\pm$ 1.02	2.08 $\pm$ 0.063	14.38 $\pm$ 0.46	1.35
2	Orotic acid (0.001%)	7.83 $\pm$ 0.28	57.16 $\pm$ 2.58	1.86 $\pm$ 0.021	12.92 $\pm$ 0.38	1.24
3	Orotic acid (1%)	7.95 $\pm$ 0.21	63.17 $\pm$ 1.97	1.57 $\pm$ 0.021	12.50 $\pm$ 0.80	1.07
4	Orotic acid (1%) + adenine sulphate (0.25%)	9.76 $\pm$ 0.11	69.67 $\pm$ 0.84	1.84 $\pm$ 0.031	13.15 $\pm$ 0.24	1.48

Table 5. *Effect of orotic acid and adenine on the incorporation of [32 P]phosphate into the nucleic acids of rat liver*

Each value is given as the mean $\pm$ S.E.M. of six determinations with different animals. Experimental details are given in the text.

Group	Supplement to basal diet	RNA		DNA	
		Relative sp. radioactivity	Total relative radioactivity	Relative sp. radioactivity	Total relative radioactivity
1	None	3.59 $\pm$ 0.27	28.63 $\pm$ 1.83	0.378 $\pm$ 0.053	0.787 $\pm$ 0.12
2	Orotic acid (0.001%)	3.45 $\pm$ 0.41	26.98 $\pm$ 2.95	0.310 $\pm$ 0.047	0.577 $\pm$ 0.09
3	Orotic acid (1%)	4.44 $\pm$ 0.22	35.38 $\pm$ 1.15	0.343 $\pm$ 0.028	0.539 $\pm$ 0.11
4	Orotic acid (1%) + adenine sulphate (0.25%)	5.24 $\pm$ 0.43	51.14 $\pm$ 4.22	0.517 $\pm$ 0.085	0.952 $\pm$ 0.17

Table 6. *Effect of orotic acid and adenine on the adenosine-deaminase, xanthine-oxidase and carbamoyl phosphate-aspartate-transcarbamoylase activities of rat liver*

Each value is given as the mean $\pm$ S.E.M. of nine determinations on different animals. Experimental details are given in the text.

Group	Supplement to basal diet	Adenosine-deaminase activity (μ mole of adenosine deaminated/hr./mg. of N)	Xanthine-oxidase activity (μ mole of xanthine oxidized/hr./mg. of N)	Carbamoyl phosphate-aspartate-transcarbamoylase activity (μ moles of carbamoyl aspartate formed/hr./g. fresh wt. of tissue)
1	None	0.264 $\pm$ 0.023	0.39 $\pm$ 0.031	33.9 $\pm$ 1.23
2	Orotic acid (0.001%)	0.366 $\pm$ 0.040	0.29 $\pm$ 0.039	34.4 $\pm$ 2.84
3	Orotic acid (1%)	0.486 $\pm$ 0.031	0.51 $\pm$ 0.022	33.2 $\pm$ 1.98
4	Orotic acid (1%) + adenine sulphate (0.25%)	0.306 $\pm$ 0.020	0.53 $\pm$ 0.043	15.0 $\pm$ 2.26

was present if the results were expressed per unit weight of defatted liver ($P < 0.9$).

In the rats given 1% of orotic acid plus 0.25% of adenine sulphate the RNA content of the liver showed an increase whether expressed per unit weight of defatted liver ($P < 0.001$) or on the basis of the weight of the whole liver ($P < 0.01$).

Since the total content of DNA was essentially constant in all experimental groups ($P < 0.1$) the higher content of RNA per liver of the rats given 1% of orotic acid or 1% of orotic acid plus 0.25% of adenine sulphate seems to indicate a true increase of RNA synthesis.

The purine base:pyrimidine base ratio showed a considerable alteration only in animals given 1% of orotic acid. The increased synthesis of RNA was also suggested by the higher incorporation of

[32 P]phosphate into liver RNA in rats given 1% of orotic acid ($P < 0.01$) and to a greater extent in those given 1% of orotic acid plus 0.25% of adenine sulphate ($P < 0.01$). No significant difference was found in the incorporation of [32 P]phosphate into DNA among the controls and the animals given 0.001% of orotic acid ($P < 0.2$), 1% of orotic acid ($P < 0.2$) or 1% of orotic acid plus 0.25% of adenine sulphate ($P < 0.5$) (Table 5).

Table 6 shows a significant increase of adenosine-deaminase activity in animals given 1% of orotic acid ($P < 0.001$); smaller and insignificant differences were found in those rats receiving 0.001% of orotic acid ($P < 0.05$) or 1% of orotic acid plus 0.25% of adenine sulphate ($P < 0.2$). A marked increase of xanthine-oxidase activity in rats given 1% of orotic acid ($P < 0.001$) was noted.

Table 7. *Effect of orotic acid and adenine on the concentration of nicotinamide nucleotides in rat liver*

Each value is given as the mean $\pm$ S.E.M. of ten determinations on different animals. Experimental details are given in the text.

Group	Supplement to basal diet	Concn. of nicotinamide nucleotides (μ g./g. of defatted liver)
1	None	1734 $\pm$ 72.9
2	Orotic acid (0.001%)	1634 $\pm$ 174.4
3	Orotic acid (1%)	1344 $\pm$ 157.6
4	Orotic acid (1%) + adenine sulphate (0.25%)	1267 $\pm$ 48.1

Table 6 shows no variation of carbamoyl phosphate-aspartate-transcarbamoylase activity in rats given 0.001% ($P < 0.9$) or 1% of orotic acid ($P < 0.8$); only supplementation of the diet with adenine caused a decrease of enzyme activity ($P < 0.001$).

The concentration of nicotinamide nucleotides in the liver was decreased in rats given 1% of orotic acid ($P < 0.001$) or the same diet plus 0.25% of adenine sulphate ($P < 0.001$) (Table 7).

DISCUSSION

The administration of 1% of orotic acid results in a significant increase in the content of total lipids in the liver and a decrease in that of phospholipids; significant changes in nucleotide metabolism are also found. These results could be interpreted as demonstrating interrelationships between lipid and nucleotide metabolism. Orotic acid, a pyrimidine base precursor, may increase the synthesis of RNA, as suggested by Handschumacher *et al.* (1960*a*). This increased synthesis of liver RNA might cause a drain on any available purine nucleotide, being an attempt by the organism to maintain a normal purine base:pyrimidine base ratio in the nucleic acids.

The increase of liver adenosine-deaminase and xanthine-oxidase activities, which are related to purine metabolism, is in agreement with an increased turnover of these compounds. The fact that supplementation of the diet with adenine causes a significant decrease in liver carbamoyl phosphate-aspartate-transcarbamoylase activity cannot be explained. However, the observed decrease in the enzyme activity may reflect a decreased rate of pyrimidine base synthesis. Contrary to the interpretation involving a decrease in the catabolism of orotic acid, rat-liver orotic acid has been shown to be catabolized primarily to carbon dioxide and β -alanine, after anabolism to UMP, through reactions similar to those for the degradation of uracil (Euler, Rubin & Handschumacher, 1963).

As already suggested, the lower content of adenine nucleotides might be secondary to the drain on purine nucleotides into RNA, and it could be one of the fundamental metabolic damages in determining this type of fatty liver. In agreement with this, low concentrations of adenine in the diet prevent fatty infiltration and the decrease in adenine nucleotides while liver RNA is being synthesized very rapidly. Induction of fatty liver by the adenine analogue, 4-aminopyrazolo[3,4-*d*]-pyrimidine (Henderson, 1963), supports this hypothesis; the possibility therefore exists that purine nucleotide cofactors are required for metabolic processes involving lipids, and that this nucleotide analogue may interfere with their function.

Finally, in the liver of animals receiving lower doses of orotic acid, when the concentration of adenine nucleotides is unchanged, no change in the content of total lipids and phospholipids was observed.

There are other possible relationships between adenine-containing coenzymes and induction of fatty liver. Other studies on liver acid-soluble nucleotides of rats given 1% of orotic acid or 1% of orotic acid plus 0.25% of adenine sulphate for 3 days (Euler *et al.* 1963) agree with the studies reported above and in preliminary reports of this work (Caldarera, Puddu & Marchetti, 1962; Marchetti *et al.* 1962*c*). In addition to the decrease in the concentration of adenine nucleotides the decrease in the concentration of cytidine nucleotides with the same experimental conditions must also be considered. This phenomenon seems to be connected with a diminished capacity to convert UMP into cytosine derivatives, as suggested by Handschumacher *et al.* (1960*b*). The role of cytidine nucleotides as coenzymes in phospholipid synthesis and the decreasing availability of these compounds in the cell may be relevant in the induction of fatty liver; for instance, in groups given 0.001% of orotic acid or 1% of orotic acid plus 0.25% of adenine sulphate, when the cytidine nucleotide content is normal, changes in the amounts of lipid and phospholipid in the liver were not observed.

Rajalakshmi *et al.* (1961) have shown that in rats given 1% of orotic acid the concentrations of nicotinamide nucleotides, particularly NADP⁺ and NADPH (Euler *et al.* 1963), were decreased. The studies reported above show that the decrease in the concentration of total nicotinamide nucleotides is not prevented if the diet containing 1% of orotic acid is also supplemented with 0.25% of adenine sulphate. The decrease in the concentration of nicotinamide nucleotides is therefore not a fundamental metabolic lesion in the occurrence of fatty liver, even though similar changes have been observed in other conditions of fatty infiltration of

liver, e.g. choline deficiency, carbon tetrachloride poisoning and phosphorus poisoning (Dianzani, 1955).

SUMMARY

1. The effects of 0.001 % of orotic acid, 1 % of orotic acid, and 1 % of orotic acid plus 0.25 % of adenine sulphate in the diet on the lipid and phospholipid contents and on some aspects of nucleotide metabolism in the liver of weanling albino rats have been studied.

2. In animals given 1 % of orotic acid the total lipid content and RNA synthesis in the liver were increased, the phospholipid content was decreased and the nucleic acid purine base:pyrimidine base ratio was altered. DNA was unchanged. The concentration of free uridine nucleotides was increased, whereas those of adenine nucleotides and cytidine nucleotides were decreased. Also, liver adenosine-deaminase and xanthine-oxidase activities were higher than those in control rats, and the concentrations of nicotinamide nucleotides were decreased. In rats given 0.001 % of orotic acid no significant changes were observed.

3. Supplementation of the diet with adenine reversed almost entirely the effects of orotic acid. Only RNA synthesis and xanthine-oxidase activity were higher than in the controls. Liver carbamoyl phosphate-aspartate-transcarbamoylase activity was lower than in rats given only orotic acid.

4. These results are discussed in relation to the nucleotide control mechanisms of lipid metabolism.

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The Isolation of Phosvitin from the Plasma of the Oestrogen-Treated Immature Pullet

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The onset of laying in the domestic fowl is accompanied by changes in the majority of the plasma components, which include increases in the concentrations of the total lipids and of total calcium and the appearance of a phosphoprotein (Sturkie, 1954). Many of these changes can be induced by treatment of the immature pullet, or cockerel, with oestrogens (Sturkie, 1954; Schiede & Urist, 1960), and such work has provided consider-

able support for the view that the changes found in the plasma of the normal laying hen result from the action of oestrogens secreted by the ovary. The precise nature of the circulating oestrogens remains to be established, but secretion of oestrogens by the ovary was demonstrated by Marlow & Richert (1940), and oestradiol, oestrone and oestriol have been detected in the ovary of the laying hen (Layne, Common, Maw & Fraps, 1958).