SUMMARY

1. ^3H -labelled chylomicra were obtained by feeding [^3H]stearic acid to young goats with cannulated intestinal lymphatic ducts. Most of the radioactivity was in the triglyceride fraction.

2. After the intravenous injection of ^3H -labelled chyle into lactating goats the half-life of radioactivity in the blood was 7.5–11.5 min. The milk fat was maximally labelled at 4 and 7 hr., and 67 and 38 % of the total activity was recovered in the milk fat in 22 hr.

3. In isolated perfused goat mammary glands, ^3H -labelled chyle or washed chylomicra were infused in quantity and 28 % of the radioactivity infused appeared in milk fat in 22 hr. in the presence of heparin.

4. In lactating goats killed 16 and 21 min. after an injection of ^3H -labelled chylomicra, 19 and 50 % of the radioactivity was found in the udder; in one gland perfused *in vitro* 29 % of the radioactivity present was transferred to the milk in 21 hr.

5. It is concluded that the udder of the lactating goat rapidly removes a large proportion of chylomicra entering the circulation.

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Variations in the Concentration of Phytic Acid in the Blood of the Domestic Fowl

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The presence of phytic acid (*myo*-inositol hexaphosphate) in the erythrocytes of chicken blood was first reported by Rapoport (1940), and later Rapoport & Guest (1941) showed that, in the 12 species of birds studied, the concentration of phytate phosphorus varied from 49 to 87 mg./100 ml. of erythrocytes. Phytic acid is not present in the blood of fish, amphibia or mammals,

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and among reptiles Rapoport & Guest (1941) found it only in the erythrocytes of turtles. Nothing definite is known about the function of phytic acid in avian blood cells, and the object of the present study was to investigate the effects of age, sex and reproductive activity on the concentration of phytic acid in the blood of chickens in the hope that the results would indicate the lines along which metabolic studies might most profitably proceed.

EXPERIMENTAL

Determination of phytic acid

Extraction from blood. The method recommended by Rapoport & Guest (1941) for blood containing nucleated erythrocytes was used. Whole blood (2 ml.) containing heparin (Boots Pure Drug Co. Ltd.) as anticoagulant was added to 4 ml. of 20% (w/v) trichloroacetic acid (a grade 'suitable for blood precipitation' obtained from British Drug Houses Ltd.) in a centrifuge tube. After the mixture had been shaken, 4 ml. of water was added with stirring and the contents of the tubes were filtered after 3 hr. through a fluted Whatman no. 1 filter paper (9 cm. diam.).

All subsequent stages of the determination were carried out in thick-walled Pyrex test tubes (16 mm. $\times$ 150 mm.), and it was thus possible to precipitate, wash and digest the ferric phytate precipitate in the same tube.

Precipitation. Phytic acid was precipitated as ferric phytate from 5 ml. samples of the trichloroacetic acid extracts by a procedure based on that of Young (1936). The tubes were heated on a boiling-water bath for 15 min. after the first bubbles appeared to destroy labile phosphate esters and to decrease the concentration of trichloroacetic acid. A few grains of acid-washed sand were added to prevent 'bumping'. After the mixtures had been cooled to room temperature, 1 ml. of ferric chloride (analytical reagent) solution (containing 1 mg. of Fe^{3+} /ml. in *N*-hydrochloric acid) and a few more grains of sand were added, and the tubes were heated on a boiling-water bath for a further 60 min. (With extracts containing amounts of phytic acid greater than 150 μg . precipitation of ferric phytate was complete after boiling for 15 min.) The tubes were cooled in water and centrifuged for 5 min., and the precipitates were washed once with 0.2*N*-hydrochloric acid (5 ml.).

The identity of the ferric phytate was confirmed by hydrolysing samples precipitated in the above manner with 2 ml. of *N*-hydrochloric acid in sealed tubes at 123° for 24 hr. (a period found to be sufficient for complete hydrolysis), and determining *myo*-inositol in neutralized samples of the resulting solution by the microbiological method of Norris & Darbre (1956). The observed phosphorus:inositol ratios ranged from 0.91 to 1.12, the mean being 1.02 ± 0.02 (s.e.m.), showing good agreement with the theoretical ratio of 1.03.

Digestion. The ferric phytate precipitates were digested with a mixture of conc. sulphuric acid (analytical reagent) and conc. nitric acid (analytical reagent) (1 ml. of each). The tubes, still containing the sand, were heated in a flame until brown fumes appeared and the digestion was continued in a preheated drilled aluminium block at 275° for a further 45 min., during which all the nitric acid was volatilized.

Colour development. After the mixture had been cooled, water was added and the diluted digest together with washings was transferred to a 50 ml. volumetric flask. The solution was neutralized with 5 ml. of 20% (w/v) sodium hydroxide and the phosphate was determined as the molybdo-vanadate by the method of Hanson (1950). The colours were allowed to develop overnight and read in a Unicam SP. 500 at 420 $\mu\mu$.

Testing the precision and limitations of the method. Since a pure salt of phytic acid was not available, a large volume of blood was precipitated according to the above procedure and samples were analysed for phytate phosphorus.

Other determinations

Packed-cell volume. This was determined by centrifuging heparin-treated blood in Wintrobe tubes at 2100*g* for 30 min. at 5°.

Total *myo*-inositol. Equal volumes (2 ml.) of a trichloroacetic acid extract of whole blood and 1.5*N*-hydrochloric acid were added to a Pyrex tube (as used for the other determinations) to give a solution of total acidity approx. 1*N*. The tube was sealed and heated in an oven at 123° for 24 hr., and inositol was determined in triplicate samples of the neutralized solution by the method of Norris & Darbre (1956).

Free *myo*-inositol. A sample (5 ml.) of a trichloroacetic acid extract of whole blood or plasma (extracted by the procedure used for whole blood) was heated on a boiling-water bath for 3 hr. to destroy most of the trichloroacetic acid, which would otherwise have interfered with the microbiological assay. After adjustment of the pH to 4.8 the extract was made up to 25 ml. and the subsequent assay was carried out as for total *myo*-inositol.

Experimental birds and treatments

All birds used were from the same strain of Rhode Island Red $\times$ Light Sussex. Chicks were housed in tier brooders until 8 weeks of age and during this time they were fed on a chick crumb containing penicillin at a low concentration. They were then transferred to laying batteries and fed on a 'grower's ration'. From 18 weeks of age both pullets and cockerels were fed on 'laying pellets'. All diets were standard commercial products (British Oil and Cake Mills Ltd.). No grit was offered at any time.

Birds of both sexes were bled at 1 day of age and thereafter at 1, 2, 3, 4, 6, 10, 15, 17, 21 and 26 weeks. Up to the age of 4 weeks chicks were bled from the jugular vein after an intravenous injection of heparin (30 units/100 g. body wt.). Older birds were bled from the wing vein by using a heparin-treated syringe.

In a separate experiment total inositol and phytic acid were determined in the blood of chicks 1, 7, 14 and 21 days of age, and on the blood of embryos incubated for 20 days. Embryos were bled from the vitelline artery and it was necessary to combine the blood from 10–15 embryos for each sample. With chicks 1 week old or younger, blood from two chicks was taken for each analysis. In another experiment free inositol was determined in the plasma and whole blood of chicks 1, 7, 14 and 21 days of age.

The effect of reproductive activity on the concentration of phytic acid in the blood was studied in nine pullets that were bled every 3 days from 18 weeks of age until at least 8 days after the laying of the first egg.

RESULTS

The results of the investigation into the precision and limitations of the method employed for the precipitation of ferric phytate are given in Table 1. The method gave highly reproducible results. The minimum amount of phytate phosphorus that could be quantitatively precipitated was about 150 μg . No precipitate could be obtained when less than 30 μg . of phytate phosphorus was present. The

Table 1. *Precipitation of phytic acid as ferric phytate from samples of a trichloroacetic acid extract of chicken blood*

Experimental details are given in the text. Amounts of phytate calculated to be present are based on the amount precipitated from 5 ml. samples. Determined values are means $\pm$ S.E.M., with the numbers of determinations in parentheses.

Vol. of sample (ml.)	Phytate P (μ g.)		Recovery (%)
	Calculated	Determined	
5	270.5	270.5 $\pm$ 1.6 (13)	100
3	162.3	164.1 $\pm$ 1.1 (12)	101.1
2.4	129.8	125.0 $\pm$ 0.7 (12)	96.3
1.8	97.4	91.6 $\pm$ 0.4 (12)	94.0
1.5	81.2	69.2 $\pm$ 0.4 (12)	85.2
1.2	64.9	49.2 $\pm$ 0.4 (12)	75.7
0.9	48.7	28.4 $\pm$ 0.5 (12)	58.3
0.75	40.6	22.9 $\pm$ 0.7 (10)	56.3
0.6	32.5	15.7 $\pm$ 0.2 (10)	48.3

Table 2. *Concentrations of phytic acid in the blood of male and female chickens from 1 day of age to maturity*

Experimental details are given in the text. The concentrations are expressed as mean weights (mg.) of phytate phosphorus/100 ml. of blood cells $\pm$ S.E.M., with the numbers of birds in parentheses.

Age	Male	Female
1 day	24.2 $\pm$ 1.3 (8*)	25.0 $\pm$ 2.1 (8*)
1 week	52.1 $\pm$ 3.8 (8*)	53.6 $\pm$ 2.5 (8*)
2 weeks	62.1 $\pm$ 1.5 (5)	60.0 $\pm$ 0.7 (4)
3 weeks	70.2 $\pm$ 0.7 (4)	70.7 $\pm$ 0.6 (4)
4 weeks	72.5 $\pm$ 1.4 (4)	72.3 $\pm$ 2.3 (4)
6 weeks	72.3 $\pm$ 0.6 (5)	72.3 $\pm$ 2.0 (5)
10 weeks	76.4 $\pm$ 3.0 (6)	72.9 $\pm$ 1.5 (6)
15 weeks	75.0 $\pm$ 2.3 (6)	69.4 $\pm$ 1.6 (6)
17 weeks	78.7 $\pm$ 4.7 (6)	73.4 $\pm$ 2.2 (6)
21 weeks	77.1 $\pm$ 2.4 (6)	77.9 $\pm$ 2.6 (12)
26 weeks	78.2 $\pm$ 2.7 (5)	74.4 $\pm$ 3.1 (5)

* Blood from two chicks combined for analysis.

results obtained in the present study were used to correct observed values of less than 150 μ g. of phytate phosphorus obtained in subsequent experiments. (This correction was only necessary with blood from embryos and 1-day-old chicks.)

Since phytic acid is present only in the blood cells and since the packed-cell volume of chicken blood varies from 25 to 40%, changes in the concentration of phytic acid are best demonstrated when the results are expressed as mg./100 ml. of cells. No attempt was made to investigate whether or not phytic acid was present in the leucocytes, which account for approx. 1% of the total packed-cell volume in normal birds.

Changes in the concentration of phytic acid in the blood cells from 1 day to 26 weeks of age, when both pullets and cocks were sexually mature, are shown in Table 2. No sex differences were observable at any age. The concentration of phytic acid increased rapidly from hatching up to the age of 3 weeks, when a mean concentration of 70.5 mg. of phytate phosphorus/100 ml. of cells was observed. The mean value (117 determinations) for the concentration of phytate phosphorus in the blood cells of mature birds was 76.1 mg.atoms/100 ml., and the results suggest that there was a gradual increase in phytic acid from the age of 3 weeks until this concentration was reached at 10–15 weeks.

The mean concentration of free and total inositol in whole blood, plasma and cells, and of phytate phosphorus in the whole blood, of embryos and of chicks up to the age of 3 weeks are given in Table 3. The marked increase in the phytic acid content of the blood cells during the first 3 weeks after hatching was confirmed, and this was accompanied by increases in the concentration of free inositol in both plasma and cells. The inositol combined as phytate was calculated by difference

Table 3. *Mean concentrations of free and total myo-inositol and phytate phosphorus in the blood of embryos and chicks*

Experimental details are given in the text. The concentrations are expressed as mg./100 ml. of plasma, cells or whole blood.

Age	No. of samples	Plasma. Free inositol	Blood cells. Free inositol	Whole blood				
				Total inositol	Free inositol	Phytate*	Phytate P	Phytate P:phytate inositol ratio
Embryos 20 days	2	—	—	2.19	—	—	1.78	—
Chicks 1 day	3	1.98	3.53	10.95	2.41	8.54	7.97	0.93
Chicks 1 week	3	4.55	5.70	21.00	4.91	16.09	15.93	0.99
Chicks 2 weeks	3	4.15	6.64	22.60	4.95	17.65	18.47	1.05
Chicks 3 weeks	3	5.00	8.45	31.40	6.02	25.38	22.53	0.89
Mean								0.97
P:inositol ratio for phytic acid								1.03

* Total inositol minus free inositol.

Table 4. *Concentrations of phytic acid in the blood of nine pullets before and after laying*

Experimental details are given in the text. The concentrations are expressed as mean weights (mg.) of phytate phosphorus/100 ml. of blood cells $\pm$ S.E.M., with the numbers of birds in parentheses.

Days before (-) or after (+) laying	Phytate P
-23 to -20	75.5 $\pm$ 1.1 (6)
-19 to -17	76.7 $\pm$ 0.8 (8)
-16 to -14	75.8 $\pm$ 1.0 (5)
-13 to -11	77.7 $\pm$ 1.1 (9)
-10 to -8	76.7 $\pm$ 1.3 (8)
-7 to -5	77.6 $\pm$ 0.8 (8)
-4 to -2	75.6 $\pm$ 1.7 (6)
-1 to +1	74.8 $\pm$ 1.6 (5)
+2 to +4	73.8 $\pm$ 1.5 (5)
+5 to +7	75.5 $\pm$ 0.8 (5)
+8 to +10	73.8 $\pm$ 0.8 (3)
+11 to +20	74.0 $\pm$ 1.1 (9)

from the mean values for total and free inositol for each age of chick, and the phytate phosphorus: phytate inositol ratios were also calculated. The mean value for these ratios was 0.97, slightly less than the ratio for phytic acid itself (1.03).

The results from the experiment in which pullets were bled every 3 days before and after laying are presented in Table 4. Mean values were derived for 3-day intervals in relation to the laying of the first egg, and it is clear that the concentration of phytic acid was not influenced by reproductive activity.

DISCUSSION

The mean concentration of phytate phosphorus observed in the blood cells of three chickens (age unspecified) by Rapoport & Guest (1941) was 65.4 mg./100 ml., and, in view of the different analytical techniques used, this value is in reasonably good agreement with the one of 76.1 mg./100 ml. obtained for mature birds in the present investigation.

The most interesting observation made in this study was the remarkable increase in the phytic acid concentration of the blood cells which took place during the first 3 weeks after hatching (Table 2), and it must be assumed that this increase occurred largely if not entirely in the erythrocytes. During the greater part of the embryonic period the yolk sac is the main site of erythropoiesis. The formation of erythrocytes by the bone marrow begins after about 14 days' incubation, and from the time of hatching the marrow is the only source of erythrocytes in the chicken. Mature erythrocytes are larger in the embryo than in the adult, and there is a pronounced decrease in the mean size of the erythrocytes 2 days after hatching, when

erythrocytes of the adult type enter the circulation in large numbers. However, embryonic-type erythrocytes persist for 6-10 weeks (Sandreuter, 1951). It seems reasonable to suggest that the adult-type erythrocyte produced in the bone marrow is rich in phytic acid, whereas the embryonic-type produced mainly in the yolk sac is poor in phytic acid. The mean life-span of the erythrocytes of chickens is 28 days (Hevesey & Ottensen, 1945), and the rapid rise in the concentration of phytic acid observed during the first 3 weeks after hatching, followed by a slow rise, though probably due in the main to the rapid entry into the blood of newly formed erythrocytes rich in phytic acid, was no doubt exaggerated by the destruction of embryonic erythrocytes low in phytic acid.

The mean phytate phosphorus:phytate inositol ratio for the blood of chicks from hatching to the age of 3 weeks was slightly less than the phosphorus:inositol ratio for phytic acid (Table 3), but it is doubtful if this represents a genuine difference in view of the error to which these ratios (derived from three separate determinations) are subject. A ratio substantially less than that for phytic acid would suggest the presence of phosphate esters of inositol lower than the hexaphosphate, but we have been unable to detect any such esters by the paper-chromatographic method of Desjobert & Petek (1956).

The amount of free inositol in the plasma of chick blood increased from 2.0 to 5.0 mg./100 ml. from hatching to 3 weeks of age (Table 3). The latter concentration is five- to ten-fold greater than the values reported for human blood (Malangeau, 1956; Perles & Colas, 1959).

The results from the age-survey (Table 2) show that the concentration of phytic acid in the erythrocytes is not influenced by reproductive activity, and this was confirmed for pullets in the experiment (Table 4) in which birds were bled at regular intervals before and after laying.

In avian erythrocytes phytate appears to replace the diphosphoglycerate found in mammalian erythrocytes (Rapoport & Guest, 1941), and its main role may be associated with the maintenance of ionic balance. If all the phytate present in the blood cells of mature chickens were to be fully ionized it would contribute approx. 50 m-equiv./l. to the total anions of the cells.

SUMMARY

1. The concentration of phytic acid (*myo*-inositol hexaphosphate) in the blood cells of chickens has been studied from hatching to maturity. The concentration is low at hatching (24.6 mg. of phytate phosphorus/100 ml. of cells) and rises rapidly up to the age of 3 weeks

(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