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 considerable 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).

Recently it was shown that the phosphoprotein in the plasma of the laying bird was phosvitin (Heald & McLachlan, 1963), and it was therefore decided to determine whether the phosphoprotein in the plasma of the immature pullet treated with oestrogen was phosvitin also.

MATERIALS AND METHODS

Birds. The birds used were a White Leghorn cross (CRM × CCA) supplied by Thornber Bros. Ltd., Mytholmroyd, Halifax, Yorkshire. They were maintained on a baby chick feed from 1 day to 3 weeks of age and then on a growers' ration. All rations were obtained from British Oil and Cake Mills Ltd., London. The birds were maintained at an average temperature of 55° F from 6 weeks of age and subjected to 10 hr. of light in each 24 hr.

Treatment and isolation of phosvitin. Birds 9–12 weeks of age were injected intramuscularly with 2 mg. of an oestradiol preparation (Dimenforman, Organon Laboratories, London, W.C. 2) on each of five alternate days. They were then bled and the plasma was obtained as described by Heald & McLachlan (1963). The plasma was bright yellow and extremely lipaemic. Crude phosphoprotein fractions were isolated and subjected to further fractionation on columns of DEAE-cellulose as described by Heald & McLachlan (1963). The fractions eluted from such columns were dialysed extensively against water before being freeze-dried.

Analytical methods. Total phosphorus, phosphorus labile in alkali, and total nitrogen were determined as described by Heald (1962).

For the determination of serine alone protein samples were hydrolysed with 6N-HCl in sealed tubes for 24 and 48 hr. The acid hydrolysate was evaporated to dryness over P_2O_5 and KOH, and the residues were dissolved in water. Serine was determined as described by Frisell, Meech & Mackenzie (1954). Since serine comprises half the total amino acids of phosvitin, alanine was added to the total acid digests as recommended by Rees (1946). Serine standards were subjected to the entire procedure to provide a correction for losses during hydrolysis.

For the determination of total amino acids samples were hydrolysed at 100° in 6N-HCl for 24 hr., or, for the fraction eluted with 0·30M-NaCl, for 24, 66 and 90 hr., and analysed by a method identical with that of Moore, Spackman & Stein (1958) by using the EEL–Guiness analyser.

RESULTS

When the crude phosphoprotein fraction, obtained from plasma, was separated on DEAE-cellulose by using the technique of gradient elution as described by Heald & McLachlan (1963), the bulk of the phosphoprotein was eluted in two fractions which overlapped; however, these could be resolved by stepwise elution (Fig. 1). Most of the phosphorus was eluted in a sharp fraction with 0·30M-sodium chloride and a smaller quantity with 0·25M-sodium chloride. No other major peaks were seen, and little additional phosphorus was removed with N-sodium hydroxide. The fractions were dialysed against water and freeze-dried before further analysis.

The total recovery of phosphoprotein phosphorus was similar to that found by Heald & McLachlan (1963) during the isolation of phosvitin from the plasma of laying hens (Table 1). Thus, in

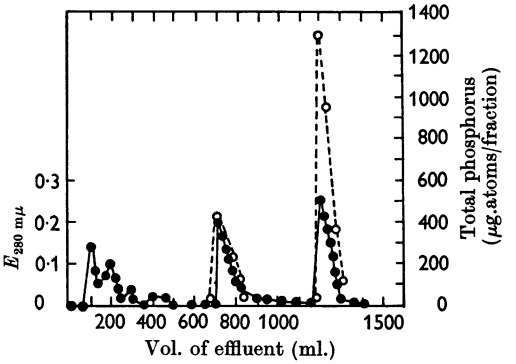

Fig. 1. Elution of plasma phosphoprotein fractions from a DEAE-cellulose column by stepwise elution with NaCl. The concentrations of NaCl in 5 mM-tris buffer, pH 8·0, and the volumes of effluent collected were: 0·15M-NaCl, 0–200 ml.; 0·20M-NaCl, 200–600 ml.; 0·25M-NaCl, 600–1200 ml.; 0·30M-NaCl, 1200–1500 ml. ●, $E_{280\ m\mu}$; ○, total phosphorus. Some experimental points have been omitted for clarity.

Table 1. *Recovery of phosphoprotein phosphorus during isolation from the plasma of the laying hen*

Plasma was collected and processed as described by Heald & McLachlan (1963), and samples were analysed for phosphoprotein as described by Heald (1962).

	Fraction	Plasma volume (ml.)	Phosphoprotein phosphorus (mg./fraction)	Phosphoprotein phosphorus (% of that in original plasma)
Expt. 1	Plasma	330	56·4	100
	Ppt. with $CaCl_2$ after extraction with NaCl and dialysis	—	45·5	80·5
	Ether-extracted $(NH_4)_2SO_4$ soln.	—	30·8	53·0
Expt. 2	Plasma	170	31·7	100
	Ppt. with $CaCl_2$ after extraction with NaCl and dialysis	—	31·6	100
	Ether-extracted $(NH_4)_2SO_4$ soln.	—	18·8	59·0

the two experiments, 53 and 59% respectively of the phosphoprotein phosphorus present in the plasma was recovered in the crude fractions before separation on the DEAE-cellulose columns. Of the phosphorus added to the columns, the total recoveries in fractions eluted with 0·25M- and 0·30M-sodium chloride comprised, in four experiments, 89·9, 83·3, 92·5 and 106·4%. Of these 19–31% was eluted with 0·25M-sodium chloride.

Fraction eluted with 0·30M-sodium chloride. This fraction, in two separate experiments, contained 9·5 and 8·6% of phosphorus, all of which was labile in dilute alkali at 37° for 18 hr. The nitrogen content was 9·75% for both preparations. The nitrogen:phosphorus ratios of 2·22 and 2·51 respectively agreed well with those of 2·4–2·7 found for various specimens of phosvitin (Mecham & Olcott, 1949). The serine content of the preparations was 3·16 and 3·33 moles/10^3 g. of protein, which were within the range 3·1–3·7 moles/10^3 g. of protein found by Mecham & Olcott (1949) and Taborsky & Allende (1962) for egg-yolk phosvitin. The serine:phosphorus molar ratios were 1·0 and 1·19, which are also similar to the ratios found by the above authors for egg-yolk phosvitin and by Heald & McLachlan (1963) for plasma phosvitin (Table 2). An amino acid analysis of the protein of a specimen from one experiment is given in Table 3. These results, together with the fact that the fraction was eluted from the DEAE-cellulose columns at a point identical with that at which phosvitin was eluted from similar columns, left little doubt that this material was phosvitin.

Fraction eluted with 0·25M-sodium chloride. The protein of this fraction was clearly different from that eluted with 0·30M-sodium chloride. The nitrogen content was higher and the phosphorus content lower, yielding nitrogen:phosphorus molar ratios of 3·83 and 4·76 respectively (Table 2). Further, the serine content was markedly lower

Table 2. *Gross analysis of phosphoprotein fractions from the plasma of oestrogen-treated immature pullets*

Fractions were eluted from DEAE-cellulose columns as described in the text by using stepwise elution (Connelly & Taborsky, 1961). The major fractions eluted with 0·25M- and 0·30M-NaCl were recovered as described in the Materials and Methods section. In the 0·25M- and 0·30M-NaCl fractions of Expt. 1 and in the 0·30M-NaCl fraction of Expt. 2 serine was determined directly (see the Materials and Methods section); in the 0·25M-NaCl fraction of Expt. 2 the value for serine was obtained from the total amino acid analysis (see Table 3); both methods gave identical results.

	Fraction	N (%)	P (%)	P (moles/10^3 g. of protein)	Serine (moles/10^3 g. of protein)	N:P molar ratio	Serine:P molar ratio
Expt. 1	0·25M-NaCl	10·9	6·3	2·03	2·03	3·83	1·0
	0·30M-NaCl	9·75	9·5	3·14	3·16	2·22	1·08
Expt. 2	0·25M-NaCl	11·50	5·34	1·72	1·35	4·76	0·79
	0·30M-NaCl	9·74	8·6	2·78	3·32	2·51	1·19

Table 3. *Amino acid composition of phosphoprotein fractions from the plasma of oestrogen-treated pullets*

Samples were hydrolysed at 100° in 6N-HCl for 24 hr., or, for the 0·3M-NaCl fraction, for 24, 66 and 90 hr., and analysed in an EEL amino acid analyser. The values obtained were plotted and extrapolated to zero hydrolysis time. The values for egg-yolk phosvitin are from Mecham & Olcott (1949) and Taborsky & Allende (1962).

	Amino acid content (moles/10^3 g. of protein)			Amino acid:aspartic acid molar ratio		
Amino acid	0·25M-NaCl fraction	0·30M-NaCl fraction	Phosvitin	0·25M-NaCl fraction	0·30M-NaCl fraction	Phosvitin
Aspartic acid	0·76	0·32	0·32–0·36	1·0	1·0	1·0
Threonine	0·28	0·03	0·07–0·12	0·37	0·1	0·20–0·34
Serine	1·35	2·78	2·86–3·7	1·77	8·75	8·40–10·90
Glutamic acid	0·66	0·14	0·23–0·34	0·87	0·43	0·65–0·97
Proline	0·28	Trace	0·0–0·09	0·37	—	0·0–0·25
Glycine	0·14	0·18	0·16–0·21	0·18	0·56	0·45–0·60
Alanine	0·57	0·08	0·1–0·20	0·75	0·25	0·28–0·56
Valine	0·12	0·06	0·03–0·06	0·16	0·19	0·08–0·17
Methionine	0·08	0·0	0·0–0·14	0·10	—	0·0–0·48
Isoleucine	0·04	0·04	0·04–0·09	0·05	0·10	0·11–0·25
Leucine	0·05	0·07	0·07–0·15	0·07	0·21	0·20–0·4
Tyrosine	Trace	0·01	0·01–0·07	—	0·03	0·03–0·2
Phenylalanine	0·10	0·03	0·06–0·07	0·13	0·09	0·17–0·20
Lysine	0·38	0·32	0·31–0·45	0·49	1·0	0·91–1·32
Histidine	0·07	0·29	0·28–0·31	0·09	0·90	0·80–0·91
Arginine	0·18	0·23	0·28–0·31	0·24	0·72	0·80–0·88
Cysteine + cystine	0·0	0·0	0·0	—	—	—

than that of phosvitin. The fraction was also soluble in 6N-hydrochloric acid, whereas phosvitin was precipitated. The amino acid analysis is presented in Table 3.

DISCUSSION

The results leave little doubt that the major phosphoprotein isolated from the plasma of the oestrogen-treated pullet is phosvitin. In immature birds the ova are undeveloped and are not increased in size by treatment with oestrogen. In such circumstances it seems reasonable to consider that the plasma phosphoprotein is synthesized at a site other than the ovary, the liver being a probable source (cf. Sturkie, 1954).

In addition to phosvitin a second phosphoprotein, not detected in the plasma of the laying hen (Heald & McLachlan, 1963), was obtained in quantities smaller than that of phosvitin. The amino acid composition of this phosphoprotein, together with its appearance in the plasma at the same time as phosvitin, strongly suggests a close relation between the two. Thus cysteine or cystine, which are absent from phosvitin, was absent from this second phosphoprotein also. Methionine, tyrosine, phenylalanine, leucine and isoleucine, which are present in only small quantities in phosvitin, were also found in only small quantities in the second phosphoprotein fraction. On the other hand alanine, threonine, glutamic acid and aspartic acid were present in this fraction in markedly greater proportions than are found in phosvitin. The differences in amino acid composition of the fraction when compared with phosvitin (either from plasma or egg yolk) are emphasized by calculation of the proportion of each amino acid relative to aspartic acid (Table 3). In both phosphoproteins the major amino acid was serine, comprising 27% of the second fraction, compared with 50–55% in phosvitin. Such a comparison suggests that the second phosphoprotein fraction represents either a material involved in the synthesis of phosvitin or a breakdown product. Of these alternatives the latter is considered to be the more probable. Thus, in the absence of growing ova, which normally remove phosvitin from the plasma, some alternative form of removal must exist, for cessation of oestrogen treatment leads to a rapid decrease in the concentrations of phosphoprotein in the plasma. Further, if such a phosphoprotein is an intermediate in the synthesis of phosvitin it might be expected that a similar material would be present in the plasma of the laying hen. No such fraction has in fact been detected (Heald & McLachlan, 1963), though a phosphopeptide, other than phosvitin, has been found in the plasma of laying birds. Thus Pin, Bai & Thoai (1960*a*) isolated a phosphopeptide of small molecular weight containing the amino acids serine, glycine, alanine and glutamic acid only. The amount present in the plasma was not stated. The phosphorus was shown to be present as phosphorylserine and the peptide itself was identical in composition with a peptide isolated from milk (Pin, Bai & Thoai, 1960*b*). A function for these materials was not suggested.

The quantities of each phosphoprotein isolated from the plasma were calculated; in the two experiments the overall recoveries for phosvitin were 32–45% of the total plasma phosphoprotein phosphorus, and, for the second phosphoprotein fraction, were 10–16%. As in the study by Heald & McLachlan (1963), the major losses of phosphoprotein occurred during the stages involving the precipitation and removal of excess of protein from the initial calcium complex, and it has been assumed that equal proportions of each phosphoprotein were lost at each stage. Though this assumption may not be valid, for the loss could also be attributed to the removal of an unidentified phosphoprotein, the major conclusion of the present work, that phosvitin is synthesized by the bird when treated with an oestrogen and that it forms the bulk of the plasma phosphoprotein, appears to be firmly established.

SUMMARY

1. Phosvitin has been isolated from the plasma of immature pullets treated with oestradiol. The yields in two major experiments were 32–45% of the total phosphoprotein phosphorus present in the plasma.

2. A second phosphoprotein, of smaller molecular weight, was also isolated. The yields of this were 10–16% of the total plasma phosphoproteins.

3. The second fraction contained less serine per unit weight, and more glutamic acid, aspartic acid, alanine and threonine per unit weight, than did phosvitin. It is considered that this material is a breakdown product of phosvitin which, in the oestrogen-treated immature pullet, cannot be removed by the normal process of yolk secretion by the ovary.

It is a pleasure to thank Thornber Bros. Ltd., Mytholmroyd, Halifax, Yorkshire, for the supply of birds, and Dr J. L. Bailey and Mr L. Lester for the amino acid analyses.

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Studies on Histones

7. PREPARATIVE METHODS FOR HISTONE FRACTIONS FROM CALF THYMUS*

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Previous work from this Laboratory (Johns, Phillips, Simson & Butler, 1960) has shown that calf-thymus histones can be separated into three main groups of basic proteins by column chromatography on CM-cellulose. These three groups were characterized by their total and *N*-terminal amino acid analyses and were designated: lysine-rich histones, f1; slightly lysine-rich histones, f2; and arginine-rich histones, f3. A method was also given for the extraction of fraction f3 directly from the tissue by using a mixture of ethanol and hydrochloric acid.

Somewhat similar fractionations were achieved by starch electrophoresis by Cruft, Hindley, Mauritzen & Stedman (1957), and by chromatography on Amberlite IRC-50 by Rasmussen, Murray & Luck (1962). Many other authors have described lysine-rich and arginine-rich histones (for review see Phillips, 1962).

In a further paper (Johns & Butler, 1962) the preparation of two subgroups of the slightly lysine-rich histones, f2 (a) and f2 (b), was described, making use of a combination of ethanol–hydrochloric acid extraction methods and column chromatography on CM-cellulose. A method for the extraction of the lysine-rich histones, f1, with 5% perchloric acid was also given.

These extraction methods have now been studied further and two preparative methods have been developed each of which enables the four groups of histones, f1, f2 (a), f2 (b) and f3, to be isolated from calf thymus, in large quantities, and does not involve the preparation and subsequent fractionation of whole histone.

A method for the further fractionation of f1 is also described, a preliminary account of which has been given (Johns, 1963).

EXPERIMENTAL AND RESULTS

All operations were carried out at 4° except for the washing and drying of all precipitates. Summaries of the extraction methods employed are given in Schemes 1 and 2.

Method 1. Minced calf thymus (100 g.) was homogenized with 700 ml. of 0·9% sodium chloride at top speed for 2 min. in the MSE Ato-Mix. The homogenate was centrifuged at 1100*g* for 30 min. The supernatant with some fatty debris on the surface was discarded, and the sediment was washed five times more in a similar manner but with homogenizing for 30 sec. and centrifuging for 15 min. The sediment obtained from the last washing was homogenized with 400 ml. of 5% (v/v) perchloric acid (0·74N) in the blender for 2 min. at full speed and the suspension was centrifuged for 30 min. at 1100*g*. The sediment was extracted twice more in the same way with 200 ml. of 5% perchloric acid. The combined supernatants from these extractions were clarified by filtering through a grade-4 sintered-glass funnel. Trichloroacetic acid was then added to give a final concentration of 18% (w/v) (1·1M). The lysine-rich histones, f1, that were precipitated were recovered by centrifugation, washed once in acidified acetone (200 ml. of acetone plus 0·1 ml. of conc. hydrochloric acid) and then three times in acetone, and dried under vacuum. The vacuum was released occasionally during the drying and any lumps were broken up with a glass rod. By drying in this way a fine white powder was obtained which dissolved easily in water to give a clear solution. The yield of lysine-rich histones was 440 mg., which is approx. 20% of all the histones that can be extracted from calf thymus.

The sediment remaining after the extraction of fraction f1 with 5% perchloric acid was then mixed with 200 ml. of ethanol, stirred for 2 min. to break up all lumps, and

* Part 6: Butler & Cohn (1963).