The Synthesis of Phosvitin *in vitro* by Slices of Liver from the Laying Hen

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1. Slices of liver from laying hens incorporated $\text{Na}_2^{14}\text{CO}_3$ and $\text{NaH}_2^{32}\text{PO}_4$ into phosvitin. Slices of liver from immature birds did not do so to any appreciable extent. The ^{32}P was incorporated into *O*-phosphorylserine in the phosvitin molecule. 2. Kidney, spleen, muscle, large and small intestine, ovary and oviduct from laying birds did not incorporate $\text{Na}_2^{14}\text{CO}_3$ into phosvitin. 3. Slices of liver from laying hens carried out a net synthesis of phosphoprotein under the standard conditions of incubation. Slices from the livers of immature pullets did not do so. 4. Liver from the laying hen incorporated $[2\text{-}^{14}\text{C}]\text{glycine}$, $[3\text{-}^{14}\text{C}]\text{serine}$ and $[2\text{-}^{14}\text{C}]\text{glutamic acid}$ into phosvitin. Part of the glycine was shown to be present as serine in the final product. 5. Slices of liver from immature birds treated with oestradiol synthesized phosvitin from $[2\text{-}^{14}\text{C}]\text{glycine}$, but the addition of oestrogens *in vitro* to slices from untreated immature birds did not promote synthesis during a 3 hr. incubation period.

Although it has been known for many years that the plasma, or serum, of the laying hen contains phosphoprotein-like material (Sturkie, 1954), until recently there has been no precise knowledge of its identity or of the site of its synthesis.

Roepke & Bushnell (1936) showed that a crude phosphoprotein fraction from the sera of laying hens was immunologically similar to a fraction found in egg yolk. More recently Hosoda, Abe & Kaneko (1961) showed that the 'lipovitellin' (or phosphoprotein) fraction from plasma (Laskowski, 1935; McIndoe, 1959) reacted with antisera prepared in rabbits to microsomal or supernatant material from the livers of laying hens, but not with antisera prepared from similar fractions from other body tissues or from the liver of the cockerel. Further, impairment of liver function by the administration of *p*-nitro-*o*-toluidine (Hosoda, Kaneko, Mogi & Abe, 1955) reduced the quantities of plasma phosphoprotein in laying birds. It was also demonstrated that neither ovariectomy nor splenectomy affected the production of phosphoprotein in immature birds treated with oestrogen. These experiments were considered to show that the plasma phosphoprotein fraction was synthesized by the liver.

However, the plasma fraction is complex (McIndoe, 1959), containing large amounts of lipid and protein other than phosphoprotein. Further, it has been shown that, whereas such a complex produces in the rabbit antisera capable of forming a

precipitate with the antigen (Francis & Wormall, 1948), the purified phosphoprotein, phosvitin, does not do so (Francis, 1952). It is therefore not possible to conclude that the immunological response of the crude phosphoprotein preparations was due to the phosphoprotein present or to decide its site of synthesis.

Flickinger & Rounds (1956) injected radioactive phosphate into two laying hens and determined the specific radioactivity of phosphoprotein phosphorus in the liver, the blood and the yolk from various ova, 6 and 12 hr. after injection. Since the specific radioactivities decreased in the order liver, blood and yolk, it was concluded that the liver synthesized the yolk phosphoprotein. No other organs were examined and the nature of the phosphoproteins studied was not determined. In a different type of experiment Vanstone, Dale, Oliver & Common (1957), using a technique of functional hepatectomy (Ranney, Chaikoff & Dobson, 1951), showed that removing the liver from the circulatory system in the oestrogen-treated cockerel decreased or prevented the incorporation of radioactive phosphorus into plasma phosphoproteins. However, these results, as well as those of Flickinger & Rounds (1956), are not unequivocal since from them it cannot be decided whether the liver forms plasma phosphoproteins unaided or whether it constitutes merely a link in a process involving other tissues. This possibility has again been raised by the demonstration (Lush, 1963) that the plasma of the non-laying

bird contains a protein fraction which disappears when laying commences and at the time that the plasma phosphoprotein increases in quantity.

Heald & McLachlan (1963a,b) isolated and identified phosvitin as the major phosphoprotein in plasma, both from laying birds and from immature birds treated with an oestrogen. Similar techniques have now been applied to an investigation of the site of synthesis of phosvitin in tissues of the laying bird *in vitro*.

MATERIALS AND METHODS

Birds. The birds used were a White Leghorn cross (CRM × CCA) supplied by Thornber Bros. Ltd., Mytholmroyd, Halifax, Yorkshire. They were reared initially on a commercial chick feed and, later, on a growers' ration until the first eggs were laid, when they were transferred to a layers' ration. All rations were obtained from British Oil and Cake Mills Ltd. (Unilever House, Blackfriars, London, E.C.4). From 6 weeks of age to point-of-lay the birds were subjected to 10 hr. of light in each 24 hr. and from point-of-lay onwards to 14 hr. of light in each 24 hr. Food and water were supplied *ad lib*. The average room temperature was about 15°. The effect of an oestrogen on the ability of liver to synthesize phosvitin was studied in 7-week-old pullets given oestradiol benzoate (Dimenformon; Organon Laboratories Ltd.), 2 mg./day for 4 consecutive days. The birds were killed 24 hr. after the last injection.

Incubation of tissues. The birds were killed by severing the spine and neck vessels, and the liver and other tissues were removed as rapidly as possible. Slices of liver, kidney and spleen were cut and weighed as described by Heald (1962). Each slice was trimmed roughly with a scalpel and, after being weighed, was placed in one of six or eight 50 ml. flasks containing 5 ml. of a bicarbonate-buffered medium (Krebs & Henseleit, 1935). Slices were placed in each flask in rotation until approx. 1 g. of tissue had been added. Tissues such as intestine and oviduct were not sliced. The oviduct, principally the magnum, was slit open and small pieces (100–150 mg.) were cut off with a razor blade. The pieces were washed in the medium, drained and weighed before placing in the appropriate flasks. A similar procedure was adopted for intestine, the pieces consisting of large and small intestine mixed at random. Each piece was washed twice before being drained and weighed. Ovarian tissue was obtained from the larger follicles. These were slit open with fine scissors and as much yolk as possible was drained out. The remaining tissue was washed gently in the saline medium to remove adhering yolk, before finally being weighed and transferred to the incubation flasks. The flasks and contents were then flushed with $O_2 + CO_2$ (95:5). Additions of radioactive materials were made at this stage. The flasks were rapidly closed with rubber stoppers and were shaken at 41.3° for 2.5–3.5 hr. At least two, and sometimes four, flasks were used for each radioactive compound studied. In all instances sodium pyruvate was added to a final concentration of 20 mM, since this substrate supports a steady respiration for at least 3 hr. (Heald, 1962).

Isolation of phosvitin. After incubation the contents of similar flasks were pooled and, after a centrifuging to remove slices and debris, the supernatants were dialysed, first against two changes of 0.05 M-EDTA (sodium salt),

pH 7.4 (2 l.), and then against two changes of water. The dialysed solutions were transferred to separating funnels and sufficient M-sodium acetate solution, pH 4.0, was added to produce a final concentration of 0.05 M, and sufficient solid $(NH_4)_2SO_4$ to produce a final concentration of 0.4 M. Phosvitin (50 mg.) was added to each funnel and the solutions were thoroughly shaken with 1 vol. of diethyl ether and allowed to stand overnight. The aqueous phase was removed and kept. The ether layer was discarded and the gelatinous interfacial layer was centrifuged. The aqueous layer was again carefully removed and combined with the previous one. The gelatinous layers were extracted twice by shaking with 10 ml. of 0.4 M- $(NH_4)_2SO_4$ in 0.05 M-sodium acetate, pH 4.0; 5 ml. of diethyl ether was added to the first extraction. The aqueous washings were removed and added to those obtained previously. The combined aqueous layers were shaken twice with diethyl ether (25 ml.) and filtered after each shaking. The filtrates were dialysed against two changes of water (2 l.) and freeze-dried.

The freeze-dried samples were taken up in 5 mM-tris-HCl buffer, pH 8.0, and separated on a column of DEAE-cellulose (Whatman DE50), treated as described by Peterson & Sober (1962), equilibrated with 0.1 M-NaCl in 5 mM-tris-HCl buffer, pH 8.0 (Connelly & Taborsky, 1961), and poured to form columns 0.9 cm.² × 33 cm. After eluting first with 200–250 ml. of 0.25 M-NaCl in 5 mM-tris-HCl buffer, pH 8.0, which removed any traces of albumin, phosvitin was eluted with 0.3 M-NaCl in tris-HCl buffer, pH 8.0. Usually 100 ml. sufficed to remove all the phosvitin, which was precipitated from solution as the manganese salt by addition of 0.6 M-MnCl₂ solution to yield a final concentration of 0.03 M-MnCl₂ (Rabinowitz & Lipmann, 1960). After standing for 1–2 hr. at 2° the precipitate was centrifuged, dissolved in 2–3 ml. of 0.5 M-EDTA, pH 7.4, and dialysed against three or four changes of water. Samples were then taken for the determination of phosphorus and radioactivity.

As a check on the isolation procedure the following experiment was carried out. Slices of liver (10 g.) from the laying hen were incubated with $Na_2^{14}CO_3$ as described above and after the supernatant solution had been dialysed and filtered it was divided into three equal portions, to which were added 25, 50 and 100 mg. respectively of purified phosvitin. Each solution was separately run through the isolation procedure and the specific radioactivities of the isolated phosvitin fractions were determined.

In such a system the specific activity of the phosvitin is given by $n/(y+x)$, where n = total counts present, y = phosvitin added and x = phosvitin originally present. Thus from the specific activities of two of the samples the third is readily calculated. The results of this experiment yielded products with specific activities 432, 267 and 146 counts/min./mg. of phosvitin for solutions to which 25, 50 and 100 mg. of phosvitin had been added. The calculated values are 430, 268 and 153 counts/min./mg. respectively.

Treatment of tissue residues. The tissue residues were washed twice with saline medium and extracted three times with trichloroacetic acid (10%, w/v). Usually 20 ml. of trichloroacetic acid/g. wet wt. of tissue was employed. The residue was then extracted three times with 20 ml. of ethanol and dried *in vacuo*. A weighed quantity was prepared and counted in a liquid-scintillation counter as described by Brown & Badman (1961). Results were expressed as counts/min./mg. of protein residue.

Analytical methods

Phosphorus. Total phosphorus and inorganic phosphorus were determined as described by Heald (1962).

Phosphoprotein phosphorus. After incubation of the liver slices, the slices and medium were centrifuged and the tissue residues washed once with fresh saline medium. The combined supernatants and washings and the tissue residues were separately treated with trichloroacetic acid to a final concentration of 10% (w/v). After extraction of the residues with approx. 20 vol. of chloroform-methanol (2:1, v/v), the residues were digested in *N*-NaOH at 37° for 18 hr. as described by Heald (1962) to liberate phosphoprotein phosphorus. Phosphoprotein phosphorus was calculated as mg. of phosvitin from the relationship mg. of phosvitin = mg. of P × 10.

Radioactivity. The radioactivity of phosvitin samples was measured by adding 0.2 ml. of an aqueous solution to 4.0 ml. of a scintillator NE. 220 [Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh] and counted in a liquid-scintillation counter. Radioactivity was expressed as counts/min./mg. of phosvitin.

RESULTS

Incubation system. It was originally shown by Peters & Anfinsen (1950) that the liver of the domestic fowl incorporates $^{14}\text{CO}_2$ into glutamic acid and aspartic acid, which are subsequently incorporated into serum albumin. Phosvitin also contains small quantities of glutamic acid and aspartic acid (Mecham & Olcott, 1949), and the incubation medium and conditions of Peters & Anfinsen (1950) were accordingly used without modification.

In a laying bird producing one egg per day the quantity of phosvitin synthesized (assuming it is produced by the liver) can be calculated roughly not to exceed 0.2–0.5 mg./g. of tissue/3 hr. Since this quantity was too small for isolation, and since the isolation procedure itself can lead to substantial losses (Heald & McLachlan, 1963*a*), phosvitin was added as a non-radioactive carrier to the pooled incubation media at the end of the experiment.

Incubation with $\text{Na}_2^{14}\text{CO}_3$. When slices of liver from birds in full lay were incubated with $\text{Na}_2^{14}\text{CO}_3$, the phosvitin subsequently isolated from the incubation medium was radioactive (Table 1). When slices of liver from immature pullets were used very little radioactivity was found in the phosvitin. When the $\text{Na}_2^{14}\text{CO}_3$ was replaced by $\text{NaH}_2^{32}\text{PO}_4$, the phosvitin contained appreciable radioactivity only when isolated from media in which slices from the liver of laying hens had been incubated. The phosphorus was organically bound in the phosvitin molecule. Thus when the phosvitin, from the experiment described in Table 1, was hydrolysed at 100° in 2 *N*-hydrochloric acid for 7 hr. and phosphorylserine isolated from the hydrolysate as described by Heald (1962), the specific radioactivity of the phosphorylseryl phosphorus was 6.25

Table 1. Incorporation of ^{14}C and ^{32}P into phosvitin by slices of liver from the domestic fowl

Slices were incubated in the medium and under the conditions described in the Materials and Methods section. Radioactive substances (40 μC of $\text{Na}_2^{14}\text{CO}_3$ or 5 μC of $\text{NaH}_2^{32}\text{PO}_4$ /vessel) were added immediately before incubation, which was carried out at 41.3° for 3 hr. under $\text{O}_2 + \text{CO}_2$ (95:5). Phosvitin was isolated and the radioactivity determined as described in the Materials and Methods section. Laying birds were 30–45 weeks old; non-laying immature birds were 9–13 weeks old.

Expt. no.	Physiological state of the bird	Labelled compound added	Radioactivity of phosvitin (counts/min./mg.)
1	In lay	$\text{Na}_2^{14}\text{CO}_3$	768.0
	Non-laying	$\text{Na}_2^{14}\text{CO}_3$	34.0
2	In lay	$\text{Na}_2^{14}\text{CO}_3$	679.0
	Non-laying	$\text{Na}_2^{14}\text{CO}_3$	74.0
3	In lay	$\text{Na}_2^{14}\text{CO}_3$	1937.0
	Non-laying	$\text{Na}_2^{14}\text{CO}_3$	41.0
4	In lay	$\text{NaH}_2^{32}\text{PO}_4$	1430.0
	Non-laying	$\text{NaH}_2^{32}\text{PO}_4$	20.0

counts/min./ μg . of phosphorus, which was almost identical with that (6.85 counts/min./ μg . of phosphorus) found for the original phosvitin phosphorus. In one experiment both $\text{Na}_2^{14}\text{CO}_3$ and $\text{NaH}_2^{32}\text{PO}_4$ were incubated with slices from the liver of a laying hen. The phosvitin subsequently isolated contained both ^{14}C and ^{32}P , the ratios of the radioactivities being 1.08 after the first manganese chloride precipitation and 1.04 after a subsequent reprecipitation. These results appear to establish that the liver of only the laying hen incorporated appreciable ^{14}C or ^{32}P into phosvitin.

However, in these experiments the age and physiological state of the laying birds were different from those of the immature pullets. Thus the failure of the liver from the immature birds to incorporate appreciable amounts of $^{14}\text{CO}_2$ into phosvitin could conceivably be attributed to differences in the permeability of the tissue to $^{14}\text{CO}_2$ or to differences in the size of the amino acid pools within the two tissues. Accordingly, comparisons were made between the specific radioactivities of the residual tissue proteins and of the phosvitin synthesized by both tissues after incubation with $\text{Na}_2^{14}\text{CO}_3$. The results are presented in Table 2. Though more ^{14}C was incorporated into the total liver proteins of the laying birds than into those of the immature birds, the relative degree of incorporation was considerably less than that found in phosvitin isolated from the incubation media. It was concluded that the increased incorporation of $^{14}\text{CO}_2$ into phosvitin by the liver of the laying hen was not attributable to differences in tissue permeability or the size of the intracellular amino acid pool. The greater specific

Table 2. *Incorporation of $^{14}\text{CO}_2$ into phosvitin and tissue proteins by slices of liver from laying and immature pullets*

Slices were incubated with $\text{Na}_2^{14}\text{CO}_3$ under the conditions described in Table 1. After incubation phosvitin and the total tissue proteins were treated as described in the Materials and Methods section. Laying birds were 25–30 weeks old; non-laying immature birds were 16–17 weeks old.

Expt. no.	Physiological state of the bird	Specific radioactivity (counts/min./mg.)		Ratio $\frac{\text{sp. activity in laying bird}}{\text{sp. activity in immature bird}}$	
		Tissue protein	Phosvitin	Tissue protein	Phosvitin
1	Immature	1758	312	1.49	4.3
	In lay	2626	1344		
2	Immature	1882	229	1.4	7.0
	In lay	2635	1600		
3	Immature	1558	239	2.42	9.0
	In lay	3770	2160		

Table 3. *Incorporation of $\text{Na}_2^{14}\text{CO}_3$ into phosvitin by tissues of the laying hen*

Tissues were prepared and incubated with $\text{Na}_2^{14}\text{CO}_3$ as described in the Materials and Methods section, at 41.3° under $\text{O}_2 + \text{CO}_2$ (95:5). Phosvitin was isolated and the radioactivity determined as described in the Materials and Methods section. Values listed are the radioactivity of phosvitin in counts/min./mg.

Tissue	Expt. no....1	2	3	4	5
Liver	207.0	935.0	627.0	1196.0	1091.0
Spleen	8.6	0.0	12.5	—	—
Ovary	19.3	0.0	31.3	—	—
Kidney	5.7	21.6	30.0	—	—
Intestine	0.0	0.0	22.0	—	—
Oviduct	—	—	—	6.2	5.8
Muscle	—	—	—	4.2	9.0

activity of phosvitin produced by tissue from immature birds in these experiments as compared with Table 1 is considered to be due to their closer approach to maturity. Two birds of this group came into lay at 19 weeks of age.

Tissues of the laying hen, other than the liver, did not incorporate $^{14}\text{CO}_2$ into phosvitin (Table 3). Thus kidney, spleen, muscle, large and small intestine, ovary and oviduct were all largely inactive in this respect when incubated under conditions similar to those of the liver slices.

Attempts to demonstrate a total synthesis of phosvitin. The experiments above did not provide unequivocal evidence that a synthesis of phosvitin had taken place. Thus it could be argued that any radioactive glutamic acid or aspartic acid formed from $\text{Na}_2^{14}\text{CO}_3$ was simply exchanging with similar residues in phosvitin already present in the system, either in the liver cells or in blood remaining in the tissue. This latter was considered improbable since tissues such as kidney or spleen, which contained

Table 4. *Effect of increasing concentrations of phosvitin on the incorporation of $^{14}\text{CO}_2$ into phosvitin by liver slices from the laying hen*

Slices of liver were incubated with the concentrations of phosvitin given below under the conditions described in the Materials and Methods section. $\text{Na}_2^{14}\text{CO}_3$ was added immediately before incubation. After incubation the concentrations of phosvitin in the various media were made equal and the phosvitin was isolated and counted as described in the Materials and Methods section.

Expt. no.	Phosvitin added to the incubation medium (mg.)	Radioactivity of phosvitin isolated (counts/min./mg.)
1	0.0	109.3
	50.0	59.0
	200.0	45.3
2	0.0	400.0
	195.0	211.0

visibly more blood than the liver, did not incorporate $^{14}\text{CO}_2$ into phosvitin to any appreciable extent. Since the direct measurement of the quantity of phosvitin produced by the system was not possible, for reasons given above, two types of experiment were performed in attempts to resolve this point. Slices of liver from laying hens were incubated with $\text{Na}_2^{14}\text{CO}_3$ in the absence and in the presence of different quantities of phosvitin and, after incubation, the phosvitin contents of the incubation media were adjusted to the same amounts before finally isolating the phosphoprotein and determining the radioactivity. It was found (Table 4) that increasing the quantity of phosvitin in the medium reduced, rather than increased, the amount of ^{14}C incorporated whereas the reverse might reasonably have been expected had incorporation been largely a process of exchange with phosvitin occluded by the tissue slice.

Table 5. *Synthesis of phosphoprotein by the liver of the domestic fowl in different physiological states*

Slices of liver were incubated in a bicarbonate-buffered medium as described in the Materials and Methods section. The substrate was sodium pyruvate (20 mM). At various times flasks were removed from the shaking bath and both the supernatant solutions and the tissue residue were taken for the determination of phosphoprotein phosphorus. *R*, Phosphoprotein ($\mu\text{g./g.}$ wet wt. of tissue) in the tissue residue; *S*, phosphoprotein ($\mu\text{g./g.}$ wet wt. of tissue) in the supernatant solution; *T*, total of supernatant and residue phosphoprotein ($\mu\text{g./g.}$ wet wt. of tissue).

Group	Physiological state	Period of incubation (hr.)	Expt. 1			Expt. 2			Expt. 3			Expt. 4		
			<i>R</i>	<i>S</i>	<i>T</i>	<i>R</i>	<i>S</i>	<i>T</i>	<i>R</i>	<i>S</i>	<i>T</i>	<i>R</i>	<i>S</i>	<i>T</i>
A	In lay	0	121	30	151	59	21	80	64	59	123	52	24	76
	Age 35 weeks	2	101	97	198	92	64	156	75	58	133	38	41	79
		4	85	86	171	64	39	103	112	67	179	47	42	89
	Total change		-36	+56	+20	+5	+18	+23	+48	+8	+56	-5	+18	+13
B	In lay	0	103	27	130	51	29	80	118	24	142	65	16	82
	Age 35 weeks	1	101	37	138	55	55	110	111	41	152	65	26	91
		3	78	54	132	74	49	123	109	43	152	63	53	116
	Total change		-25	+27	+2	+23	+20	+43	-9	+19	+10	-2	+37	+35
C	Non-laying	0	117	0	117	116	0	116	67	8	75	—	—	—
	Age 9-12 weeks	1	101	0	101	118	0	118	36	7	44	—	—	—
		3	83	0	83	77	0	77	58	6	64	—	—	—
	Total change		-34	0	-34	-39	0	-39	-9	-2	-11	—	—	—

Table 6. *Incorporation of different amino acids into phosvitin by the liver of the laying hen*

Slices of liver from laying hens were incubated in the medium and under the conditions described in the Materials and Methods section. Radioactive amino acids were added to yield 5 $\mu\text{C/g.}$ wet wt. of tissue. $\text{Na}_2^{14}\text{CO}_3$ was added to yield 40 $\mu\text{C/g.}$ wet wt. of tissue. After incubation for 3 hr. phosvitin was isolated and counted as described in the Materials and Methods section.

Expt. no.	Addition	Radioactivity of phosvitin isolated (counts/min./mg.)
1	[2- ^{14}C]Glycine (10 $\mu\text{g./ml.}$)	2200
	[3- ^{14}C]Serine (20 $\mu\text{g./ml.}$)	918
	DL-[2- ^{14}C]Glutamic acid (14 $\mu\text{g./ml.}$)	596
	$\text{Na}_2^{14}\text{CO}_3$	1200
2	[2- ^{14}C]Glycine (10 $\mu\text{g./ml.}$)	3900
	[3- ^{14}C]Serine (20 $\mu\text{g./ml.}$)	1160
	DL-[2- ^{14}C]Glutamic acid (14 $\mu\text{g./ml.}$)	266
	$\text{Na}_2^{14}\text{CO}_3$	2980

In the second type of experiment the total phosphoprotein content of the tissue and of the media were measured at the beginning and after different periods of incubation. The results are presented in Table 5. In all except one of eight experiments involving slices of liver from laying birds there was a total gain in the amount of phosphoprotein in the system, which was sometimes, but not always, accompanied by a loss of phosphoprotein from the residue. In the three experiments involving slices from immature non-laying birds

there was a net loss of phosphoprotein, the loss occurring almost entirely in the residue and little, if any, phosphoprotein appearing in the supernatant. It was concluded that slices of liver from laying birds under the conditions of incubation employed carried out a net synthesis of phosphoprotein.

Incorporation of amino acids. Slices of liver from the laying hen incorporated various amino acids into phosvitin (Table 6). Of those examined incorporation was greatest with [2- ^{14}C]glycine. The quantities of glycine and serine added to the medium were 10 and 20 $\mu\text{g./ml.}$ respectively. It seems probable that the greater incorporation achieved with glycine reflects a greater ease of penetration into the tissue. It has been shown (Richert, Amberg & Wilson, 1962) that the liver of the domestic fowl rapidly converts glycine into serine, and it was found that part at least of the glycine carbon atoms appearing in phosvitin had been metabolized to serine before incorporation. Thus phosvitin was isolated from an experiment in which slices of liver were incubated with [2- ^{14}C]glycine. It was partially hydrolysed in conc. hydrochloric acid at 30° for 18 hr. and the phosphorylseryl peptides containing one or more phosphoseryl residues were separated from the hydrolysate by the technique of Williams & Sanger (1959). Each peptide was found to be radioactive, the specific radioactivities varying from 30 to 50% of that of the original phosvitin. It seems clear from this experiment that part at least of the glycine added to the medium was metabolized to serine before incorporation into the phosvitin molecule.

Effect of oestrogens on phosvitin synthesis. It has been shown (Heald & McLachlan, 1963b, 1964) that phosvitin constitutes the major phosphoprotein present in the plasma of immature birds treated with oestradiol, and it was accordingly decided to test whether slices of liver from such treated birds synthesized phosvitin *in vitro*. The results of experiments with three birds are presented in Table 7.

In contrast with these results oestradiol, when added *in vitro* to slices of liver from immature pullets, did not induce phosvitin synthesis (Table 8). Oestrone, which has been detected in the ovaries of laying hens and tentatively identified as a conjugate

in the plasma (Layne, Common, Maw & Fraps, 1958), also had no major effect on phosvitin synthesis.

DISCUSSION

The results described above appear to provide good evidence that the synthesis of the egg-yolk phosphoprotein, phosvitin, takes place in the liver of the laying bird. Thus liver slices of birds in full lay incorporated ^{14}C from $\text{Na}_2^{14}\text{CO}_3$, or from ^{14}C -labelled amino acids, into phosvitin to a markedly greater degree than was found with liver slices from non-laying immature birds. Other tissues, such as oviduct, ovary, spleen and kidney, were inactive in this respect. The increased incorporation of $^{14}\text{CO}_2$ into phosvitin was not accompanied by an equal increase in the incorporation of $^{14}\text{CO}_2$ into the total proteins of the liver slices. These results suggest that the increased synthesis of phosvitin by the liver of the laying hen is the result of an increase in a specific synthetic process and not simply due to a variation either in the permeability of the tissue or to the size of the intracellular amino acid pools.

The incorporation is not considered to arise from a simple exchange of radioactive amino acids with similar amino acids of a preformed phosvitin molecule, for the addition of phosvitin to the incubation mixture did not increase, but decreased, the amount of radioactivity incorporated into the final product. The explanation of these results is not clear. A possible reason could be that the increased concentration of the phosvitin in the external medium prevented the extrusion of

Table 7. *Incorporation in vitro of [2- ^{14}C]glycine into phosvitin by the liver of immature pullets treated with an oestrogen*

Immature pullets, 7 weeks of age, were injected with oestradiol benzoate (2 mg.) daily, as described in the Materials and Methods section. Uninjected pullets, of the same group and age, served as the control birds. Slices of liver were cut and incubated with [2- ^{14}C]glycine (1 $\mu\text{C}/\text{ml.}$) and the phosvitin was isolated as described in the Materials and Methods section.

Bird no.	Treatment	Radioactivity in phosvitin (counts/min./mg.)
1	Oestradiol	811
2	Oestradiol	891
3	Oestradiol	493
4	None (control)	36
5	None (control)	34

Table 8. *Effects of oestradiol-17 β and oestrone on the synthesis of phosvitin by slices of liver from immature pullets*

Slices of liver from 8-week-old pullets were incubated under the conditions described in the Materials and Methods section with radioactive amino acids (1 $\mu\text{C}/\text{ml.}$) or $\text{Na}_2^{14}\text{CO}_3$ (8 $\mu\text{C}/\text{ml.}$). Oestrogens were added to the medium at the start of the incubation. After 3 hr. incubation, phosvitin was isolated from the medium as described in the Materials and Methods section.

Expt. no.	Addition	Oestrogen	Quantity added ($\mu\text{g.}/\text{ml.}$)	Radioactivity of phosvitin (counts/min./mg.)
1	[2- ^{14}C]Serine	None	—	50.6
		Oestradiol	2.0	52.2
		Oestradiol	20.0	65.0
2	$\text{Na}_2^{14}\text{CO}_3$	None	—	9.3
		Oestradiol	2.0	76.0
		Oestradiol	20.0	82.5
3	$\text{Na}_2^{14}\text{CO}_3$	None	—	23.6
		Oestrone	4.1	11.5
		Oestrone	20.5	22.4
4	[2- ^{14}C]Glycine	None	—	57.7
		Oestrone	20.0	72.5
5	[2- ^{14}C]Glycine	None	—	87.5
		Oestrone	20.0	112.8

phosvitin synthesized by the tissues. Further, with slices of liver from laying hens there was a consistent increase in the total quantity of phosphoprotein during incubation, whereas a consistent decrease was observed with slices from the livers of immature non-laying hens (Table 5).

The data in Table 5 refer only to total phosphoprotein phosphorus expressed as phosvitin. Thus the decrease in the total phosphoprotein phosphorus found in slices of liver from the immature pullets could represent a metabolic loss of phosphoprotein other than phosvitin, though a partial loss of phosvitin, if present, cannot be ruled out. With other tissues, e.g. cerebral cortex, a total loss of phosphoprotein phosphorus was found when slices were incubated in saline media (Heald, 1959). Since the total quantities of phosphoprotein produced by avian liver were extremely small, the direct isolation and identification of the phosphoprotein released to the medium by the liver of the laying hen was not possible. However, the fact that slices of this tissue synthesized phosphoprotein and incorporated radioactive amino acids or $\text{Na}_2^{14}\text{CO}_3$ into phosvitin points strongly to a total synthesis of phosvitin taking place.

The rate of synthesis of phosvitin in the system used was considerably below that likely to occur *in vivo*. Thus, if it is assumed that no degradation of phosvitin occurs during incubation, calculation of the rate of synthesis from the data in Table 4 shows that, in these experiments, the liver was producing 10–20 μg . of phosvitin/g. wet wt./hr. The liver of the adult laying bird weighs 40–45 g., which, from the above-mentioned rate of synthesis *in vitro*, would yield 10–20 mg. of phosvitin/liver/day. The birds used in these experiments were laying at a rate of not less than 60 eggs/100 days. The average phosvitin content of an egg is 200–250 mg., which, at the above-mentioned rate of laying, requires a rate of synthesis of 120–150 mg. of phosvitin/day. The value calculated from the measurements *in vitro* is clearly much lower than this.

The finding that the major synthesis of phosvitin occurs in slices of liver from laying birds is in agreement with previous findings showing that phosphoproteins appear in the plasma in measurable quantities only when the bird comes into lay (Laskowski, 1938; Sturkie, 1954; McIndoe, 1959). Nevertheless, the liver from immature non-laying pullets incorporated small amounts of radioactive amino acids or $\text{Na}_2^{14}\text{CO}_3$ into the phosvitin subsequently isolated. Though this might be attributable to a failure to separate contaminating radioactive material from phosvitin by the purification procedures adopted, it has been shown by histological methods that a slow synthesis of yolk material occurs throughout the life of the bird up to the 2 weeks immediately preceding laying, at which

point synthesis is very greatly increased (Marza & Marza, 1935; Sturkie, 1954).

The methods used for the purification of phosvitin have been shown unequivocally to yield only phosvitin when applied to plasma from the laying hen (Heald & McLachlan, 1963a) and to plasma from the immature pullet treated with oestrogen (Heald & McLachlan, 1963b, 1964), and it was considered unlikely that proteins markedly different from those found in the plasma would be synthesized by liver slices. Further experiments described above showed, first, that when the product isolated was doubly labelled with ^{14}C and ^{32}P the ratio of the specific radioactivities was not altered by reprecipitation of the manganous salt, and, secondly, that the product isolated after incubation of slices with $[2\text{-}^{14}\text{C}]\text{glycine}$ contained sequences of radioactive phosphorylserine indicating a close structural similarity of the synthesized product with phosvitin (Williams & Sanger, 1959). Also, addition of known amounts of unlabelled phosvitin before isolation produced the expected dilution of radioactivity (see the Materials and Methods section), thus providing further support for the assumption that the material isolated was phosvitin.

The synthesis of yolk materials in the bird is considered to be stimulated by the secretion of oestrogens from the developing ovary (Sturkie, 1954; Van Tienhoven, 1961). In the immature bird oestrogen treatment leads to the appearance of phosvitin as the major plasma phosphoprotein (Heald & McLachlan, 1963b, 1964), and it has now been shown that slices of liver from oestrogen-treated immature birds synthesize phosvitin (Table 7). This process could not be stimulated by the addition of oestradiol or oestrone to liver slices incubated *in vitro* for 3 hr. The stimulation of protein synthesis in a variety of tissues by oestrogens or androgens administered *in vivo* has been well demonstrated (for review see Tomkins & Maxwell, 1963), though not by hormones added directly to incubation systems *in vitro*. The results found above for the synthesis of phosvitin in the liver provide another example of this effect. The inability of oestrogens when added *in vitro* to promote phosvitin synthesis suggests either that the action of the oestrogen *in vivo* is an indirect one or that the periods of incubation used *in vitro* were too short for any such action to become apparent.

A final point concerns the possible usefulness of the system described here. Phosvitin itself is a unique molecule containing about 100 phosphorylserine residues, many of which, if not all, are linked in adjacent sequences (Williams & Sanger, 1959). If, as is currently held (see Tomkins & Maxwell, 1963), the synthesis of proteins is dependent on the production of specific messenger RNA, the synthesis of phosvitin by avian liver appears to offer a

natural system in which the structural relations between messenger RNA and specific protein synthesis may be studied. In addition the finding that phosvitin synthesis occurs after treatment of the immature pullet with oestrogen may also afford an opportunity for examining the mechanism of oestrogen action in this relationship.

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