Retinal in the Blood and Liver of the Fowl in Relation to Sex and Maturity

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1. Concentrations of retinal (vitamin A₁ aldehyde) in the plasma and liver of laying hens, mature cockerels, immature pullets and pullets undergoing sexual maturation have been measured. 2. The plasma of laying hens contained about 8 µg. of retinal/100 ml., about ten times that found in the plasma of mature cockerels and immature pullets. In laying hens that had received large doses of retinyl palmitate 8–4 weeks previously, the mean concentration of retinal was 18.3 µg./100 ml. of plasma. 3. The appearance of significant amounts of retinal in the plasma of maturing pullets coincided with hypertrophy of the oviduct, increase in concentration of plasma lipid and onset of egg-laying. 4. Retinal was present in the livers of all types of fowl examined and the concentrations, which ranged from 0.2 to 5.8 µg./g. wet wt., were highly correlated ($r=0.79$) with the concentrations of liver retinyl esters, which ranged from 92 to 1530 µg./g. wet wt.

The occurrence of retinal (vitamin A₁ aldehyde) in eggs of the hen (Plack, 1960, 1963) led us to look for the origin of this particular form of vitamin A. It is unlikely that it is produced in the egg, e.g. by enzymic oxidation of retinol (see Plack, 1963), and the alternative is that retinal as such is put into the egg by the hen. This view is supported by the finding of retinal in the blood of laying hens, together with retinyl esters and retinol, and also in their livers (Plack, 1963). The blood of mature cockerels and immature pullets contained only traces of retinal. A preliminary report of these results has been published (Plack, Miller & Ward, 1964a).

MATERIALS AND METHODS

Birds. (a) Laying hens and mature cockerels, about 1 year old, were from the Institute's breeding stock of Light Sussex birds. They were given *ad lib.* a breeder's mash containing maize and dried grass and added retinyl ester equivalent to 2.5 mg. of retinol/kg. of diet, so that a bird consuming 100 g. of diet/day would receive 250 µg. of vitamin A/day together with further amounts from the provitamin A carotenoids. (b) Four other Light Sussex hens in lay, receiving the above supplemented breeder's mash, were also given by mouth high doses of retinyl palmitate (equivalent in sum to 420 mg. of retinol; see Plack, 1963) during

the period 8–4 weeks before they were killed. (c) Three Light Sussex pullets (14 weeks old) received a grower's mash (Plack, Miller & Ward, 1964b) with added retinyl ester equivalent to 2.5 mg. of retinol/kg. of diet. (d) Maturing pullets were from the flock described by Plack *et al.* (1964b) and received a diet low in vitamin A and in provitamin A carotenoids. From the age of 7 weeks, samples from one bird were analysed each week.

The birds were bled from the jugular vein and the blood, 30–140 ml. according to the size of the bird and the efficiency of bleeding, was collected in a beaker containing heparin. The birds were killed by pulling the neck; their livers were then removed, washed and blotted dry. Oviducts were taken from the maturing pullets, any partly formed eggs removed and the organs weighed.

Chemicals. A.R. grade chemicals were used as supplied, except that diethyl ether and SbCl₃ were purified as described by Plack & Kon (1961).

Extraction of vitamin A. Blood samples were centrifuged and the plasma (20–75 ml.) was removed. Either 40 ml. or, when the volume was less, the whole of the plasma from each sample was extracted in 20 or 10 ml. lots. Each 20 ml. was poured slowly into a blender containing 300 ml. of chloroform-methanol (2:1, v/v) in an atmosphere of N₂. During the addition the blender was run at half speed until the precipitated protein was finely dispersed. Blending was then continued for 2 min. at full speed. The contents of the blender, with rinsings, were transferred to an amber-coloured flask, the volume was made to about 500 ml. with chloroform-methanol (2:1, v/v) and the mixture left to stand at room temperature for 15 min. (Bragdon, 1960). For each 10 ml., or less, of plasma, half the above volumes of solvents were used. The combined extracts from the

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sample were filtered through a Whatman no. 54 paper, and the paper and precipitate washed with chloroform-methanol (2:1, v/v). The filtrate was washed with water by the procedure of Folch, Lees & Sloane-Stanley (1957). Methanol was added to the residual extract to produce a single phase and the solvents were removed at a temperature of about 50° with a rotary evaporator under reduced pressure. The last 20 ml. were inevitably cloudy owing to the presence of water, but the addition and removal, under reduced pressure, of two or three small quantities of ethanol produced a clear residue, which was weighed under N₂ and dissolved in *n*-hexane.

Livers were extracted as described by Plack (1963).

Determination of retinal. Retinal was separated by gradient-elution chromatography as described by Plack, Kon & Thompson (1959), and modified by Plack & Kon (1961). The whole of a plasma extract was distributed between several columns so that the load on one column did not exceed 0.5 g. of lipid. A portion of the liver extract equivalent to 1 g. of lipid was subjected to mild saponification (Plack & Kon, 1961), extracted, and the whole used for the separation of retinal by gradient-elution chromatography. Fractions containing retinal were combined, the solvent was removed and the residue weighed. A suitable volume of chloroform, based on the values of E_{370} for the separate fractions, was added and allowance was made for the volume of the residual lipid, which was calculated from its weight and an approximate density of 0.9 g./ml. Retinal was then determined by the Carr-Price reaction with an Optica CF4 double-beam recording spectrophotometer (Plack & Kon, 1961). Negligible amounts of dehydroretinal were present.

Determination of retinyl esters and retinol. The whole of each plasma extract was subjected to gradient-elution chromatography (see above). The fractions preceding those containing retinal contained retinyl esters, and the succeeding fractions, together with the eluate obtained with 50 ml. of diethyl ether, contained retinol. Retinyl esters and retinol were separated from a portion of the liver extract by chromatography and this method, and subsequent procedures for the determination of the two forms of vitamin A, were as described by Plack (1963).

Statistical method. Student's *t* test was used to determine the significance of differences between groups.

RESULTS

Table 1 gives values for the three forms of vitamin A in the plasma of laying hens, mature cockerels and immature pullets. The cockerels and pullets had less than 1 µg. of retinal/100 ml. of plasma, but laying hens had about ten times as much. There were no significant differences between these three types of bird in the concentrations of retinol or retinyl esters in the plasma, and the high variability of the concentrations of the esters probably reflected the intake of vitamin A in the food. The results for individual birds showed that there was no correlation between the concentrations of any of the three forms of vitamin A in the plasma.

Concentrations of plasma lipid were four or five times as great in the laying hen as in the mature cockerel or immature pullet.

The four laying hens that had received large doses of retinyl palmitate 8–4 weeks previously had a concentration of retinal in the plasma about double that of undosed laying hens (Table 1). The mean retinyl ester concentration was also doubled but this value may have been affected by the food recently consumed. Retinol and lipid were present in the plasma at concentrations similar to those found in undosed hens.

The hens, cockerels and pullets all had measurable amounts of retinal in their livers (Table 1) and differences in the plasma concentrations of retinal were not reflected in the liver concentrations. For these three groups, and for the hens dosed with retinyl palmitate, the ratios of the mean concentrations of retinyl esters and of retinal in the livers were similar. The results for individual birds, plotted in Fig. 1, show the concentrations of these two forms of vitamin A in the liver to be highly correlated ($r=0.79$, $P=0.01$), the relationship being independent of variations in the retinol

Table 1. Mean values with their standard errors for concentrations of lipid, retinal, retinyl esters and retinol in the plasma and liver of hens, cockerels and pullets

Numbers of samples analysed are shown in parentheses.

	Plasma				Liver		
	Lipid (g./100 ml.)	Retinal (µg./ 100 ml.)	Retinyl esters (µg./ 100 ml.)	Retinol (µg./ 100 ml.)	Retinal (µg./g. wet wt.)	Retinyl esters (µg./g. wet wt.)	Retinol (µg./g. wet wt.)
Laying hen (5)	2.23 ± 0.38	8.2 ± 1.1	12.9 ± 4.7	56.8 ± 5.1	1.8 ± 0.8	460 ± 92	3.7 ± 0.9
Mature cockerel (4)	0.53 ± 0.09	0.9*	18.1 ± 5.6	59.8 ± 6.9	4.5 ± 0.6	892 ± 244	2.6 ± 0.7
Immature pullet (3)	0.42 ± 0.04	0.7*	3.3 ± 0.2	37.8 ± 8.2	0.5 ± 0.2	128 ± 29	1.2 ± 0.3
Laying hen, after dose of retinyl ester† (4)	2.91 ± 0.57	18.3 ± 1.5	25.0 ± 8.4	62.5 ± 4.8	1.7 ± 0.3	459 ± 71	7.0 ± 0.8

* Trace amount; see the Discussion section.

† See the Materials and Methods section.

concentration. There was no correlation between the concentrations of retinal and retinol in the livers.

The difference in concentration of plasma retinal between immature pullets and laying hens led us to examine pullets during the period of sexual maturation and Table 2 gives the results of this investigation. Since sexual maturation does not occur at a given age, the results have been arranged by weight of oviduct rather than by age of bird. The increase in oviduct weight, from 1 g. to more than 50 g., was associated with a two- to four-fold increase in plasma lipid, the appearance in the plasma of retinal in measurable amounts, when only traces had been present in the plasma of immature pullets, and the onset of egg-laying. Concentrations of retinyl esters and of retinol in the plasma were variable but were not related to the maturity of the birds.

DISCUSSION

The high concentration of plasma lipid in the laying hen, in comparison with the mature male and the immature female, has been reported by other workers (Lorenz, Entenman & Chaikoff, 1937-38) and need not be commented on here. We were concerned primarily with the occurrence of retinal and some remarks on the methods used at present for the determination of this compound are called for. Values for blood retinal of 0.5 µg./100 ml. of plasma correspond to extinctions at 666 mµ of the Carr-Price blue colour of about 0.01. This extinction is just detectable as a slight hump on the absorption curve of the coloured product but is not measurable with accuracy, and concentrations of retinal of this order should perhaps be described as traces. The higher values of about 5 µg./100 ml. of plasma correspond to extinctions of the order of 0.1 and give rise to absorption curves of the Carr-Price coloured product with satisfactory peaks at 666 mµ. This characteristic peak and the chromatographic behaviour of the substance confirm the presence of retinal. Liver contained definite amounts of retinal, since the samples used for its determination were equivalent to about 20 g. of liver, but the method is at present only roughly quantitative (see Plack & Kon, 1961) and the values given here are about 80% of the true values.

In previous work (Plack, 1963; Plack *et al.* 1964*b*) we found that, under conditions of high and low dietary vitamin A, the concentrations of retinal, retinol and retinyl esters in hen's eggs varied above and below the concentrations found in the eggs of birds receiving an adequate amount of vitamin A in the diet. It was also deduced that the quantities of the three forms of vitamin A present in eggs reflected their concentrations in the blood. A comparison of the results for laying hens on a normal diet and on the same diet supplemented

Fig. 1. Relationship between the concentrations of retinal and of retinyl esters in the livers of individual laying hens (●), mature cockerels (▲), immature pullets (△) and laying hens that had previously received a large dose of retinyl esters (○).

Table 2. Age, weight of oviduct, concentrations of lipid, retinal, retinyl esters and retinol in plasma, and liver stores of retinyl esters plus retinol for pullets during sexual maturation

Pullets nos. 15-18 had produced no eggs, pullets nos. 19-23 were in lay.										
Pullet no....	...	17	15	18	16	19	22	20	21	23
Age (days)		158	144	165	151	173	193	179	186	209
Weight of oviduct (g.)		0.27	0.70	1.17	17.2	53.3	56.4	66.5	72.5	99.0
Lipid (g./100 ml. of plasma)		0.71	0.48	0.87	2.03	3.44	1.40	2.36	2.10	2.55
Retinal (μg./100 ml. of plasma)		0.6	0.3	0.5	0	7.7	4.6	5.7	2.7	6.6
Retinyl esters (μg./100 ml. of plasma)		6.7	1.7	2.4	3.3	4.4	0.9	2.2	1.8	2.6
Retinol (μg./100 ml. of plasma)		30.8	29.8	57.6	31.3	33.2	14.3	22.4	25.2	32.7
Retinyl esters + retinol (μg./liver)		668	393	334	287	2653	227	273	183	1460

with high doses of vitamin A (Table 1) shows that the high doses of vitamin A have produced an increase in the concentration of retinal in the plasma that was still apparent 4 weeks after dosing had ceased, when the liver concentrations of vitamin A were no greater than normal. A similar persistence of retinal in plasma has been found with cod after spawning (P. A. Plack & P. M. J. Woodhead, unpublished work).

In addition to this dietary effect on the concentration of retinal in plasma, the sex and maturity of the bird also influenced the concentration. The measurable amounts of retinal in the plasma of laying hens, compared with only traces in the plasma of mature cockerels and immature pullets, suggested that the synthesis, or mobilization, of this substance is related to egg-laying. The results in Table 2 of experiments with maturing pullets support this suggestion, since the appearance of significant amounts of retinal in the plasma coincided with the increase in weight of the oviduct and the onset of egg-laying.

Ideally, it would have been better to follow the sexual maturation of individual birds by taking a series of blood samples, but the large blood sample necessary for the determination of retinal by the methods now available made it necessary to use a flock of birds and kill one for each sample. Since the birds matured at different ages, the time-scale of the experiment was lost, but the hypertrophy of the oviduct has been shown by Common, Rutledge & Bolton (1946-48) to occur over the period of 10-14 days before egg-laying. The use of birds on a diet low in vitamin A may have decreased the concentration of plasma retinal below the normal level, but immature pullets receiving normal amounts of vitamin A (Table 1) still had only traces of retinal in their plasma.

Injection of female sex hormones into immature pullets is known to increase the concentrations of plasma lipid (Lorenz, Chaikoff & Entenman, 1938) and of retinyl esters (Chapman, Gluck, Common & Maw, 1949; Gardiner, Phillips, Maw & Common, 1952). We have attempted to measure the change in concentration of plasma retinal in immature pullets after intramuscular injections of oestradiol monobenzoate but found that the increases in concentrations of plasma lipid and of plasma retinyl esters interfered with the determination of retinal. During sexual maturation the immature pullets (Table 2) showed an increase in the concentration of plasma lipid but no increase in the concentration of plasma retinyl esters, and the latter is possibly produced only by the large, unphysiological, amounts of hormone used in the injection experiments.

All three types of bird had small but definite amounts of retinal in their livers. The correlation

between the concentrations of retinyl esters and retinal in livers was unexpected. The biochemical pathway from the ester to the aldehyde form would have the alcohol as an intermediate, but there was no correlation between the concentrations of retinal and retinol in the livers, and with the mature cockerels the concentration of retinal in the liver exceeded that of retinol. The equilibrium between retinal and retinol *in vitro* in the presence of alcohol-NAD oxidoreductase (EC 1.1.1.1) from horse liver at physiological pH favours retinol (Bliss, 1951), but the combination of retinal or retinol, or both, with protein *in vivo* might be expected to interfere with the equilibrium.

It is unlikely that the retinal in plasma is derived from the food, e.g. from β -carotene by the mechanism proposed by Glover & Redfearn (1954), since orally administered retinal is converted into retinol in the gut (Glover, Goodwin & Morton, 1948), and such a mechanism would not account for the differences observed by us between the laying hen and the mature male and immature female birds. The absence of a correlation between concentrations of retinal in plasma and in liver indicates that the liver may not be the source of the plasma retinal and we can only say, at the moment, that the mobilization or the synthesis of retinal is related to egg-laying. Nevertheless, we have come to the conclusion that the retinal in eggs is of no apparent significance for the development of the chick embryo (Plack *et al.* 1964b), although it may be so for fish embryos since, in the eggs of several marine fish, retinal is the predominant form of vitamin A. Its presence in the hen and hen's eggs may be an example of evolutionary recapitulation.

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