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LIPID METABOLISM AND THE LAYING HEN

I. PLASMA-FREE FATTY ACIDS AND THE ONSET OF LAYING IN THE DOMESTIC FOWL

P. J. HEALD AND H. G. BADMAN
Twyford Laboratories Ltd., London N.W. 10 (Great Britain)

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SUMMARY

It has been found that the onset of laying in the domestic fowl is preceded by large increases in the plasma- free fatty acids, total lipids and phosphoproteins, the quantities of these components decreasing markedly when laying commences. No such changes were detected in the cockerel on coming to maturity. When intermittent egg laying was induced by decreasing the duration of exposure to light a cyclical rise and fall of the plasma components was found to be associated with oviposition. The clearance rates of [^{14}C]palmitate from the plasma of the laying hen were not markedly different from those of the immature pullet; the flux was related to the concentration of free fatty acids in the plasma. It is suggested that the decrease in the concentration of the plasma fractions results indirectly from a decreased gonadotropic stimulus of the ovary following ovulation and is not simply due to the removal of lipid and phosphoprotein as egg-yolk materials.

INTRODUCTION

Although it is well known that the concentration of lipids in the plasma of the domestic fowl is considerably increased during laying[1] there appears to be no information regarding the free fatty acids of the plasma under similar circumstances. In the mammal it has been firmly established that the free fatty acids of the plasma are a primary source of the plasma lipids and that changes in their concentration occur under a variety of altered physiological conditions[2,3]. Such changes are usually accompanied by changes in the levels of plasma lipids. Part of the work in this laboratory is concerned with a study of lipid metabolism in relation to laying in the domestic fowl and it was therefore of considerable interest to determine whether the marked variations in lipid concentration of the plasma immediately preceding and following the onset of laying were accompanied by similar variations in the concentrations of the circulating free fatty acids.

MATERIALS AND METHODS

Materials

Birds: The birds used were a White Leghorn Cross (CRM × CCA) supplied by Thornber Bros. Ltd., Mytholmroyd, Yorkshire, Great Britain. The strains used in

the cross were derived from breeding lines of known genetic background and performance and these gained weight at closely similar rates. They were fed a commercial baby-chick feed from day old to 6 weeks and from then on fed a growers ration until the first eggs were laid. Both rations were obtained from The British Oil and Cake Mills Ltd. Food and water was available at all times. They were maintained under artificial day-light for 10 h per day from 8.0 *a.m.* to 6.0 *p.m.* at a constant temperature of 55–60° F from 6 weeks of age.

Blood samples: Samples of blood (5 ml) were obtained *via* the wing vein with a heparinised syringe. Care was taken not to inject heparin before the sample was withdrawn. The blood was placed in oxalated tubes[4] and after removal of a sample into Wintrobe tubes the remaining blood was centrifuged to obtain as much plasma as possible.

Analytical methods

Haematocrits: These were determined by centrifuging at 2260 × *g* at 5° for 30 min taking the reading and centrifuging for a further 30 min. The second reading, which usually differed little from the first, was taken as the red-cell volume. The volumes of the red cells and of the white "buffy coat" were recorded separately.

Total lipids: Plasma (0.5 ml) was mixed with 9.5 ml of chloroform–methanol (2:1, v/v) and the mixture filtered. The residue was washed with a small volume of chloroform–methanol and the total filtrates brought to 10.0 ml. Water (2 ml) was added and after shaking, the aqueous phase was removed and discarded. The lower chloroform phase was then evaporated to dryness in a weighed vessel.

Phospholipoprotein: This fraction, the appearance of which is characteristic of the onset of laying, was determined essentially as described by McINDOE[5]. Thus, 0.5 ml of plasma was diluted with 4.5 ml of water and after standing in ice for 1 h the precipitate was centrifuged at 10000 × *g* for 20 min. With extremely lipemic plasmas the precipitate usually rose to the surface as a tightly packed solid on centrifuging but on some occasions no movement could be obtained on centrifuging at 144000 × *g* for 1 h. In these cases the determination was abandoned. The precipitate was taken up in H_2O (3.0 ml) and reprecipitated by the addition of 0.5 ml 0.1 M NaCl. It was finally taken up in water and dried to constant weight at 110°.

Free fatty acids: These were determined by the method of DOLE AND MEINERTZ[6] using 1.0 ml of plasma and reducing the proportions of the other reagents by the appropriate amounts.

Determination of clearance rates of [1-^{14}C]palmitic acid: [1-^{14}C]Palmitic acid (0.1 mg containing 10 $\mu C^{14}C$) was dissolved by addition of an equivalent of NaOH (0.05 N) and complexed with bovine serum albumin (10 mg crystalline albumin in 1.0 ml of H_2O, adjusted to pH 8.5 with NaOH) by warming at 41° for 15 min. Birds were anaesthetised with sodium pentobarbital, and, after a zero-time sample had been taken for the determination of free fatty acids, the [^{14}C]palmitate complex was injected rapidly *via* the femoral vein. Blood samples were taken from the wing vein and stored in oxalated tubes. The free fatty acids were extracted from samples of plasma as described above, 0.5 ml of the heptane extract was mixed with 4 ml of scintillator, NE. 213 (Nuclear Enterprises (G.B.) Ltd., Sighthill, Edinburgh) and counted in a liquid scintillation counter[7].

RESULTS

The plasma of the laying hen differs markedly in composition from that of the non-laying hen[1] and it was considered desirable to re-establish that the procedure for extraction of free fatty acids was effective with avian plasma under these different conditions. The recoveries of [1-^{14}C]palmitate and [2-^{14}C]stearate when added to plasma from the laying hen, the non-laying pullet and the cockerel are shown in Table I. Since the quantities of [^{14}C]fatty acids added to each ml of plasma were approx. 2–10 μg, it was considered unlikely that they would effect the extraction procedure by virtue of a greatly increased concentration. The results showed that the extraction was virtually complete under these conditions and that the presence of increased quantities of lipids and proteins present in the plasma of the laying hen was without effect.

TABLE I

RECOVERY OF PALMITIC AND STEARIC ACIDS FROM AVIAN PLASMA

Plasma (1.0 ml) was incubated with [1-^{14}C]palmitic acid (20 mμC) or [2-^{14}C]stearic acid (20 mμC) for 10–15 min at 37° and extracted by the method of DOLE AND MEINERTZ[6]. The heptane phase was removed and 0.2 ml counted for radioactivity. The amount originally added was determined by adding to the heptane phase of a blank extraction, 20 mμC of ^{14}C-labeled acid and determining the radioactivity in a 0.2-ml sample.

Expt. No.	*Source of plasma*	*[^{14}C]Fatty acid*	*[^{14}C]Fatty acid*		*Recovery (%)*
			Added (counts/min)	*Found (counts/min)*	
1	Laying hen	Palmitic acid	1739	1624	93.5
			1739	1629	
	Pullet	Palmitic acid	1739	1655	95.2
			1739	1658	
	Cockerel (immature)	Palmitic acid	1739	1672	97.4
			1739	1711	
2	Laying hen	Stearic acid	1503	1482	98.7
			1503	1486	
	Pullet	Stearic acid	1503	1483	98.6
			1503	1482	

Changes in plasma constituents during the onset of laying

The volume of blood in the domestic fowl has been assessed as 9–10% of the body weight[1]. The weight of the birds at the start of the experiment averaged 1100 g. The blood volume was thus approx. 100–110 ml. It has been found that the removal of an average of 20–25 ml blood over a period of 5 days results in a steady decline in the red-cell volume. Accordingly it was decided to take samples of 5 ml blood at intervals of 3–4 days. This procedure did not lead to any change in the haematocrit readings over a period of several weeks.

The pattern of the changes in plasma free fatty acids, total lipids and phospholipoprotein as the birds came into lay is shown in Fig. 1, which is typical of results from groups of four birds studied both in August and November. As the birds approached the point of lay the levels of the free fatty acids, total lipids and phospholipoprotein began to rise, the levels falling sharply when laying commenced. Thereafter,

the plasma components, though present in greater quantities than in the immature pullet, never reached the values found immediately before laying.

The extent and rapidity of the increase varied from bird to bird, the highest values at point of lay for free fatty acids being 3700 μequiv/l plasma and the lowest being 1250 μequiv/l plasma. The levels of total lipid and phospholipoprotein at point of lay also varied similarly the maxima being 10.0 and 13.0 g per 100 ml plasma respectively and the minima being 4.0 and 6.0 g per 100 ml plasma respectively. Such ranges of values in plasma lipids have been noted by other workers[1] and emphasise the variability in birds of apparently identical stock maintained under identical conditions. The red-cell volume remained unchanged throughout the experiment showing that the volume of blood taken was not excessive.

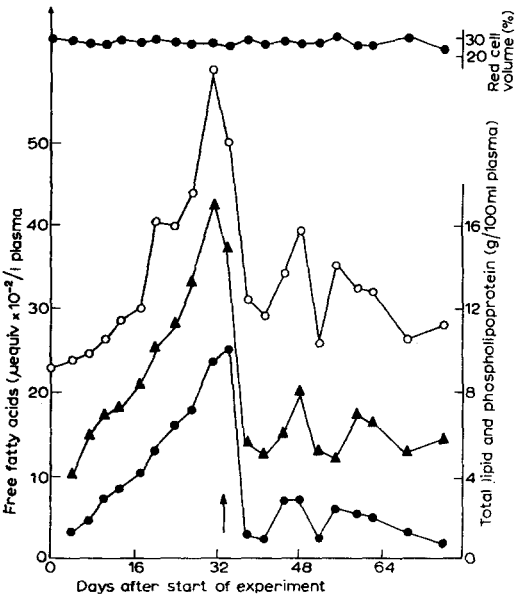

Fig. 1. Plasma levels of free fatty acids, total lipids and phospholipoprotein during the onset of laying in the domestic fowl. O—O, plasma free fatty acids; ●—●, total lipids; ▲—▲, phospholipoprotein. The values for free fatty acids have been increased by 2000 μequiv per litre of plasma and those for phospholipoprotein by 9.0 g per 100 ml of plasma to avoid marked overlapping of the individual values. Egg laying commenced where indicated by the arrow.

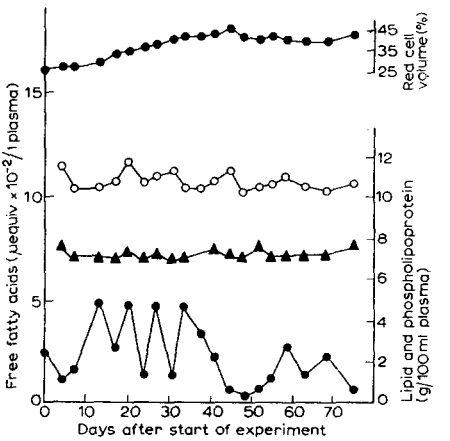

Fig. 2. Plasma levels of free fatty acids, total lipids and phospholipoprotein in the plasma the cockerel during the onset of sexual maturity. ●—●, plasma free fatty acids; O—O, total lipids; ▲—▲, phospholipoprotein. The values for total lipids have been increased by 10 g/100 ml and those of phospholipoprotein by 7 g/100 ml to avoid a confusion of experimental points.

In contrast to the pullets, cockerels of the same age and group, maturing at the same time showed no such changes in the plasma constituents (Fig. 2). Thus the free fatty acids and total lipids remained low, the values being similar to those found in immature pullets. Phospholipoprotein was absent, a finding similar to that of McINDOE[5]. The cockerels became mature during the experiment as was seen both by the physical appearance and marked comb growth and by the increase in the red-cell volume. It is well established that the red-cell volume of the mature cockerel is

considerably greater than that of the immature cockerel[8,9] and that this change is associated with the increasing production of androgens by the maturing testis.

Effect of frequency of laying on the plasma components

The marked decrease in levels of the plasma constituents accompanying the onset of laying (Fig. 1) is usually observable once only in birds coming into lay and it was considered desirable to see whether this phenomenon occurred as a regular cyclical feature preceding the laying of an egg by an individual bird. The frequency of laying was therefore reduced in a group of birds which were laying regularly by decreasing the hours of exposure to light to six only in each twenty-four. Under these conditions egg laying became intermittent. Changes in the plasma component of one of the group, which showed the most intermittent egg production, are presented in Fig. 3.

It will be seen that after an egg had been laid the plasma component rose to a higher level and dropped again shortly before a second egg was laid. The levels rose once again and decreased preceding the onset of a more regular laying period. Thereafter the total plasma lipids remained at a low level though the free fatty acids increased before finally dropping again as egg laying became more regular.

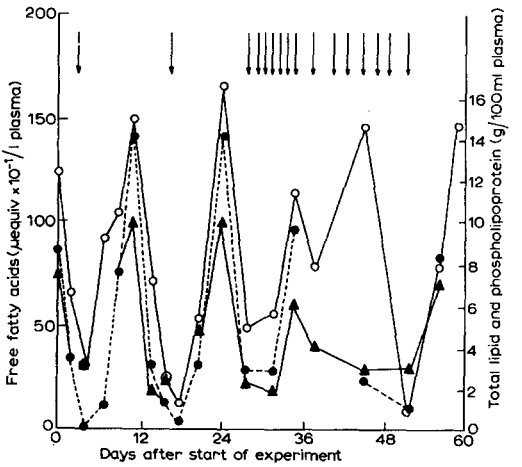

Fig. 3. Plasma levels of free fatty acids, total lipids and phospholipoprotein in the plasma of a laying bird under conditions of restricted lighting. O—O, plasma free fatty acids; ●—●, phospholipoprotein; ▲—▲, total lipids. Arrows indicate the points at which eggs were laid.

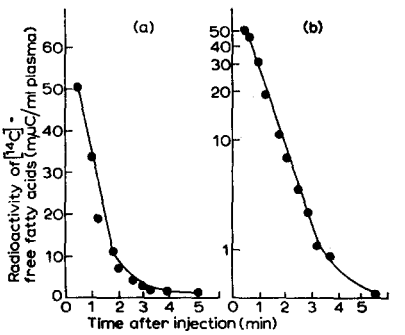

Fig. 4. Rate of removal of [1-^{14}C]palmitate from the plasma of a laying hen. (a), Plasma radioactivity at intervals after injection; (b), the same data plotted on a semi-logarithmic basis.

Clearance rates of plasma free fatty acids

The decreased levels of the plasma constituents following laying could be due to their removal as yolk material and it was thought that measurement of the rates of removal of free fatty acids from the plasma in the pullet when immature, at point of lay and when in lay might reveal significant differences. Clearance rates of [1-^{14}C]-palmitic acid injected into the plasma of birds at these three stages were studied. A typical curve is shown in Fig. 4(a). When plotted on a semilogarithmic scale a curve of the type shown in Fig. 4(b) was obtained. The rate of decrease in radioactivity began

to deviate from linearity between the 3rd and 4th minute presumably owing partly to the conversion of fatty acid to lipid and partly to the return of fatty acid from adipose tissue to the plasma[10]. Accordingly the straight line drawn between the points from the 1st to the 3rd minute was used to calculate the half life. The quantity of free fatty acids leaving the plasma in unit time was calculated from the equations[11]:

$$\log M^* - \log M_0^* = \frac{-Kt}{2.3\,M}$$

and

$$t_{\frac{1}{2}} = \frac{0.623\,M}{K}$$

where M^* and M_0^* represent radioactivities at a time t and when $t = 0$. M, plasma concentration of free fatty acids and K, flux in mequiv free fatty acids/l plasma/min.

The data from several determinations are presented in Table II. It will be seen that though the half life of the injected palmitate was greater in the birds at point of lay than in immature or laying birds, the total flux was also considerably greater being related to the level of the plasma free fatty acid.

TABLE II

THE CLEARANCE RATE OF [1-^{14}C]PALMITATE FROM THE PLASMA OF THE DOMESTIC FOWL

For experimental details see MATERIALS AND METHODS section.

Expt. No.	*Physiological state of bird*	$t_{\frac{1}{2}}$	*Free fatty acids (mequiv/l)*	*K (mequiv/l/min)*
1	Immature pullet	0.60	0.398	0.460
2	Immature pullet	0.445	0.643	1.00
3	Immature pullet	0.8	0.706	0.612
4	Point of lay	1.0	2.030	1.408
5	Point of lay	0.75	2.150	1.984
6	Point of lay	1.25	2.24	1.24
7	In lay	0.60	—	—
8	In lay	0.55	0.911	1.15
9	In lay	0.55	1.390	1.752
10	In lay	0.60	0.745	0.860

DISCUSSION

A major feature of the present work has been the finding of the rise in plasma free fatty acids before the bird comes into lay followed by a sharp drop when laying commences. The accompanying changes in the quantities of total lipid and of phospholipoprotein have been the subject of other studies[5,12] and were included in the present work to establish the relationship of such change to those in the plasma free fatty acids.

The concentrations of the free fatty acids immediately before laying commenced are considerably in excess of those found normally in the fed mammal, which range from 0.15 to 0.5 mequiv/l in the plasma of man, dog, rabbit and the rat[13–16], and occurred under conditions in which the levels of plasma glucose remain essentially constant[17,18]. In the rat, dog and man, increased plasma concentrations of free fatty acids are frequently accompanied by decreases in blood glucose and it has been well established that the availability of blood glucose is the most probable factor controlling

the release of free fatty acids from adipose tissue[2]. Under conditions in which the free fatty acids level is raised by injection of anterior pituitary preparations, however, the concentration of plasma glucose is unchanged[19]. The extent to which the increased concentrations of free fatty acids in the plasma of the bird may be accompanied by a decreased consumption of glucose cannot be decided directly from experiments of this type, but it seems probable that the increases result from a stimulus leading to the production of materials for yolk synthesis rather than solely to a change in a caloric requirement.

In the laying bird it is well established that the growth of ovarian follicles begins some 8–10 days preceding the onset of laying[20]. Examination of Fig. 1 shows that this period corresponds closely with that in which the greatest rise occurs and that the decrease coincides with oviposition. It has been further shown[21,22] that the phosphoprotein present in the plasma of the laying hen, part of which is represented in the phospholipoprotein fraction, is phosvitin, which is ultimately deposited as the principal phosphoprotein of the yolk. It might therefore seem reasonable to suggest that the decrease in quantities of the plasma constituents is a result of their removal as egg-yolk components.

However, this is almost certainly an over-simplification as regards the free fatty acids. Thus from Table II it can be calculated that the total flux of free fatty acids in a laying bird weighing 1.5 kg and with an average plasma free fatty acids concentration of 1.0 mequiv/l is equivalent to 30–40 g of free fatty acids as palmitate per 24 h. In the bird at point of lay with a plasma free fatty acids content of 3.0 equiv/l the flux becomes 90–120 g/24 h. Since a bird lays only one egg containing 6–7 g lipid each 24 h at the best, it seems clear that the decrease in levels of plasma free fatty acids cannot be ascribed solely to their removal as egg-yolk lipid.

An alternative explanation is a reduction in the intensity of the stimulus causing the initial increase. In the hen as in the mammal the growth and ripening of the ovarian follicles is a result, at least in part, of stimulation by pituitary gonadotrophins[23]. This growth is accompanied by an increased secretion of oestrogenic substances[1] administration of which has been shown to induce marked increase in levels of plasma lipids and phosphoprotein. Thus the increase in levels before the onset of laying could be attributed to the oestrogenic secretion by the growing follicles under the influence of pituitary gonadotrophins. Following ovulation, the presence of an egg in the oviduct has also been shown to inhibit pituitary secretion though not to an extent which completely abolishes the maintenance of the follicle[24]. Under these conditions the quantity of oestrogens secreted is likely to be diminished. This view is partly supported by observations made on a single hen over a period of three months during which period no eggs were laid. Nevertheless the plasma levels of free fatty acids were 1000–2300 μequiv/l and those of phospholipoprotein 3.0–3.5 g per 100 ml, values similar to those of the laying bird. At autopsy a large partly calcified egg was found in the oviduct while the ovary itself presented the appearance of that of a normally laying bird.

Such an explanation requires that the levels of plasma free fatty acids, lipid and phosphoprotein undergo cyclic variation in relation to the frequency of ovulation and in the bird laying at the normal rate of one egg per day with an interval of one day between clutches this is not easy to detect. It seems therefore of considerable significance that such cyclic changes were indeed observed under conditions in which the frequency of oviposition was reduced to intervals of several days by reducing the

intensity of the stimulus (light) normally leading to increased gonadotrophin production (Fig. 3).

The finding of an increased flux of plasma free fatty acids under conditions in which the levels of plasma free fatty acids increase is similar to that found in man[3] and in the dog[25], though the quantities exchanged per kg body weight are considerably greater. Thus assuming a blood volume of 10% of the body weight, the flux values for the bird ranged from 91 to 215 μequiv free fatty acids/kg/min, which may be compared with 11–20 μequiv free fatty acids/kg/min in the dog[25] and 2–21 μequiv free fatty acids/kg/min in man[3].

In the mammal it is well established that the plasma free fatty acids are derived from the adipose tissue in response to a variety of stimuli. Such descriptions do not yet appear to have been made in the bird and the effect of similar factors as they relate to the origin and fate of plasma materials during the process of egg production is under investigation.

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