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Iron Mobilization and Plasma Ferroxidase Factors in Chickens

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Hematological values, metal content (Fe, Cu, Zn, Cd) in plasma and liver, ceruloplasmin (p-phenylendiamine oxidase) and ferroxidase activity in plasma, were analyzed in chicks, fed during 10 weeks on a commercial diet, supplemented by either 5,000 ppm Zn or 100 ppm Cd.

A microcytic and hypochromic anemia was evident in both groups but in the Cd-fed chicks, plasma iron and ceruloplasmin values were normal.

Estrogen administration mobilized iron in the Cd-group but not in the Zn-group. The precipitation of plasma phosvitin reduced (90 %) the ferroxidase activity that had been previously induced by the estrogens.

Correcting copper levels in the Zn-group, by copper injection, restored the ceruloplasmin level. However, the estrogens, in such birds, neither mobilized the plasma iron nor increased the ferroxidase activity.

Plasma citrate was determined in laying, non-laying hens and in estrogenized or normal males. The contribution of citrate to the ferroxidase activity of plasma during the laying period, was negligible.

It was concluded that plasma phosvitin during laying was the main factor responsible for the ferroxidase activity. However, the ceruloplasmin in chickens, could play a secondary role in iron mobilization.

Ceruloplasmin (Cp), a copper metalloenzyme of plasma, presents a ferroxidase activity (12) and its physiological role in iron mobilization has been demonstrated *in vivo* in pigs (16, 19) and rats (4, 27). This protein has been considered to be the molecular link between iron and copper metabolism (6). Deficient copper diets in rats (4) or the interference of Cu absorption by Zn-rich foods (11) have caused ceruloplasmin levels in plasma to drop and have provoked anaemic states.

The presence of ceruloplasmin in bird plasma, detected as p-phenylendiamine (PPD)-oxidase, has been established by SEAL (20). The normally low levels in chickens were increased by cortical steroids (5, 21). STARCHER and HILL (22) isolated and characterized chicken Cp.

The ferroxidase activity (FOX) of chick-

en plasma and the increasing effect of estrogens was first established by PLANAS and FRIEDEN (14). Estrogen induced phosvitin has been considered to be responsible for the majority of the plasma ferroxidase activity in chickens (12). Other factors in plasma, such as, citrate (27), transferrin (1), or ferritin (3), could also present ferroxidase activity.

Thê participation of ceruloplasmin, phosvitin and citrate in the ferroxidase activity of chicken plasma, as well as some aspects of iron metabolism in laying hens and estrogenized males, has been analyzed. The birds were fed either on a normal diet or with metal (Zn or Cd) supplement, which interfered with the copper and iron.

Materials and Methods

White Leghorns, Shaver strain, fed on a commercial diet (Piensos Flotats R.), were used. In two lots of animals, the diet was supplemented with 5,000 ppm Zn or with 100 ppm Cd, for 10 to 15 weeks. The animals had free access to food and water at all times.

Blood samples were obtained, either by wing vein punture with heparinized syringes when the bird was kept for further experiments, or by total exsanguination. The hematocrit was determined in a microhematocrit centrifuge (Gri-Cell) and the hemoglobin concentration with Drabkin's reagent.

The Fe, Cu, Zn and Cd determinations, in plasma and liver, were carried out by atomic absorption. A 2 ml sample was used for plasma but the liver was withdrawn after total exsanguination, washed in destilled water and kept frozen (—20° C) until digestion. A 0.5 g dry liver was placed in an oven at 80° C and processed with perchloride and sulfuric acid. Samples were read in a Perkin-Elmer.

Ceruloplasmin concentration in plasma, according to its p-phenyl-endiamina oxidase activity, was determined by the KUZNETSOV method (10).

Ferroxidase activity of plasma, was measured by the JOHNSON *et al.* (9) method. The non-phosvitin ferroxidase activity was evident by the precipitation of this phosphoprotein by $CaCl_2$, according to HEAL and MCLACHLAND (8). The estrogenized roosters were prepared with 3 intramuscular injections of dietylstilbestrol (DES, Merck) dissolved in 2,4-propilen, 2-4 mg DES/kg body weight, every other day (15).

A comparative analysis of the variation of plasma iron, phosvitin, citrate, ceruloplasmin and ferroxidase activity, was carried out on Shaver hens and estrogenized roosters. The plasma iron and the total iron binding capacity were determined according to RAMSAY (17, 18). The plasma phosvitin was separated and the phosphorus evaluated, according to BEUVING and GRUBER (2). The plasma citrate was determined by the STERN method (23).

Results

The effects, of a ten-week supplemented diet with either Zn 5,000 ppm or Cd 100 ppm, on blood parameters and on metal contents in plasma and in liver, are shown (table I). The Zn and Cd significantly decreased the hematocrit, the hemoglobin concentration and the iron concentration in liver. However, the effect of these metals on plasma iron and copper, on the content of copper in liver and on the ceruloplasmin levels was different, as the cadmium did not alter these parameters. The ferroxidase activity was similar in both groups and comparable to the control animals.

The values, before and after the administration of strogen which induced iron mobilization in chickens fed on commercial food (basal group) or supplemented with Zn or Cd, are shown in table II. The estrogens neither caused iron mobilization nor increased the ceruloplasmin or ferroxidase levels in Zn-treated chickens. In the cadmium group, however, the plasma iron

Table I. *Effects of supplemented diets with Zn or Cd during 10 weeks on hematocrit (Hc), hemoglobin (Hb), metal contents in plasma and liver, and on the ceruloplasmin (Cp) as PPD-oxidase and the ferroxidase activity (FOX) in plasma.*
Mean ± s.d. Number of animals in parenthesis. Significantly different from basal group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Diet	Blood		Plasma						Liver			
			Fe	Cu	Zn	Cd	Cp	Fox	Fe	Cu	Zn	Cd
	Hc %	Hb g %	µg/100 ml plasma				µg PDD/ min/ml	µM Fe (II)/ min/ml	µg/g dry liver			
Basal	30.7±0.7 (12)	10.5±0.3 (12)	119±10 (16)	22.2±2 (16)	184± 8 (16)	0 (16)	1.36±0.07 (20)	104±6 (14)	354±30 (20)	16 ±0.4 (20)	103± 5 (20)	2± 0.2 (20)
Zn 5,000 ppm	22 ±1 (12) ***	7 ±0.6 (12) ***	57± 6 (16) ***	7.7±0.3 (16) ***	486±30 (16) ***	0 (16)	0.67±0.03 (20) ***	100±7 (16)	169±12 (16) ***	9.3±0.5 (16) ***	685±73 (16) ***	2± 0.3 (16)
Cd 100 ppm	25 ±0.6 (12) ***	7.8±0.3 (12) ***	112±10 (16)	20.7±0.9 (16)	125±10 (16) **	5.7±0.6 (16) ***	1.30±0.09 (20)	116±5 (14)	185±20 (20) ***	17.8±1 (20)	158±10 (20) *	253±31 (20) ***

Table II. *Response to estrogen administration (DES, 2 mg/kg b.w.; 3 injection) in plasma iron (Fe) and Copper (Cu), and on the ceruloplasmin (Cp) as PPD-oxidase and the ferroxidase activity (FOX) in chickens fed with a commercial diet or supplemented with Zn 5,000 ppm or Cd 100 ppm, during 10 weeks.*

Mean ± s.d. Number of animals in parenthesis. Significantly different from basal group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Significantly different from non-precipitated phosvitin (Phv): ●●● $P < 0.001$.

Diet	Before estrogen administration				After estrogen administration				
	Fe	Cu	Cp	Fox	Fe	Cu	Cp	Fox	
	μg/100 ml plasma		μg PDD/ min/ml	μM Fe (II)/ min/ml	μg/100 ml plasma		μg PDD/ min/ml	Whole plasma	Without Phv
Basal (10)	113±3 (11)	18 ±1 (11)	1.29±0.12 (11)	179±24 (11)	661±67 (10) ***	25 ± 2 (10) **	1.86±0.14 (10) **	8,417±1,120 (10) ***	866±230 (10) ●●●
Zn 5,000 ppm (8)	62±7 (10)	9.3±0.3 (10)	0.67±0.06 (10)	122±10 (10)	58± 7 (8)	8.4±0.6 (8)	0.80±0.08 (8)	58± 6 (8) ***	60± 5 (8)
Cd 100 ppm (6)	116±8 (6)	22.6±2 (6)	1.17±0.08 (6)	91± 8 (6)	569±26 (6) ***	18.5±2 (6)	1.10±0.10 (6)	2,421± 500 (6) ***	—

Table III. *Effects of copper injections (1 mg Cu/kg b.w.) on chickens, injected every other day during te final 2 weeks of a 15-week Zn (5,000 ppm) supplemented diet with a simultaneous 3-dose estrogen (2 mg DES/kg b.w.) treatment.*
Mean ± s.d. Number of animals per group, 15. Significantly different from the copper injected group: *** $P < 0.001$; ** $P < 0.01$.

Treatment	Hc %	Hb g %	Plasma				
			Fe µg/100 ml	Cu µg/100 ml	Cp µg PPD/min/ml	Fox µM Fe (II)/min/ml	
						Whole plasma	After $CaCl_2$ precipitation
Without	22.4±0.6	7.5±0.4	52± 6	7.8±0.5***	0.69±0.04***	100± 7***	—
Cu	23.6±0.8	6.6±0.4	65± 7	18.6±0.6	1.62±0.10	164±16	—
Cu + DES	22.2±1	6.8±0.5	82±10	21.7±1	1.58±0.06	108± 7**	109±5**

Table IV. *Variations of the plasma iron and some ferroxidase factors in laying hens and in estrogenized roosters.*
Mean ± s.d. Number of animals in parenthesis. Significantly different from no-laying hens or normals males:
* $P < 0.05$; ** $P < 0.01$; $P < 0.001$.

Group	Fe µg Fe %	TIBC µg Fe %	Phosvitin mg P %	Citrate mg/100 ml	Ceruloplasmin µg PPD/min/ml	Ferroxidase activity µM Fe (II)/min/ml	
						Whole plasma	Without Phv
Hens							
Non Laying (3)	166± 21	238± 67	0.27±0.10	5.55±1.02	1.46±0.31	186± 38	—
Laying 10-30 % (3)	340±140*	316± 37	2.50±0.44***	2.65±0.78**	2.47±0.23*	825± 340*	97±15
» 30-60 % (5)	679±161***	510±149*	4.04±0.71***	1.69±0.52***	2.43±0.43*	3,055±1,050**	154±46
» 60-90 % (4)	731±167***	532±142**	5.79±1.33***	1.48±0.49***	2.33±0.71*	7,070±1,784***	179±56
Roosters							
Normals (9)	137± 35	187± 24	0.25±0.07	4.68±1.06	1.64±0.5	179± 24	—
Entrogenized[a] (6)	858± 80***	673± 58***	7.67±0.82***	4.03±1.77	2.04±0.6	8,223±2,487***	75±21

[a] Daily injection of 4 mg DES/kg b.w., for 3 days.

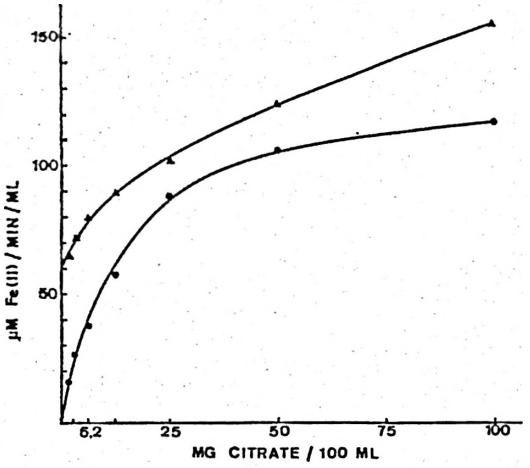

Fig. 1. *Ferroxidase activity of different citrate concentrations in water (●) or added to a chicken plasma (▲).*

presented a 5-fold increase and a 3-fold increase in FOX activity, while the ceruloplasmin was not modified. The precipitation of the phosvitin, from the estrogenised plasma with $CaCl_2$, reduced the ferroxidase values 10 times, but caused no ferroxidase changes in the Zn-group. The low values of copper (in plasma and in liver) and ceruloplasmin, obtained from the Zn-treated chickens, suggested a depletion or a blockage of copper as a possible indirect cause of these results on iron metabolism. Therefore, the administration of estrogen to Zn-fed animals, previously loaded with copper was repeated (table III). The plasma copper and the ceruloplasmin levels recuperated, but the estrogen treatment neither mobilized iron nor increased ferroxidase activity in plasma. Phosvitin precipitation in this plasma did not modify the values of the ferroxidase activity.

The variations of the plasma iron, the total iron binding capacity, plasma citrate, phosvitin, ceruloplasmin (as p-phenolendiamine oxidase) and the ferroxidase activity, in laying and non-laying hens and in estrogenized roosters, is shown in table IV. The increase in plasma iron and in the total iron binding capacity was parallel to the phosvitin concentration and ferroxidase activity, all of which seemed to be related to the intensity of egg production. Ceruloplasmin values rose with laying but presented no proportional variation to the laying intensity. The ferroxidase activity of plasma demonstrated this relationship to laying intensity. However, after phosvitin precipitation, the ferroxidase activity in plasma maintained the same level as the values of non-laying hens.

Citrate in plasma significantly decreased during the laying period. Adult male chickens presented higher citrate values than non-laying hens. The estrogen decreased those values but the differences were not significant. Otherwise, the estrogenized roosters showed similar patterns of variation to laying hens.

The ferroxidase activity of the citrate in a wide range of concentrations, acting alone or added to chicken plasma, is shown in figure 1.

Discussion

The role of ceruloplasmin, in iron mobilization and in the Cu-Cp relationship, has been clearly demonstrated in rats (4, 11, 26). This dietary anemic effect of Zn, has suggested an antagonism to copper (11). A reduction of the ferroxidase activity of the serum and a blockage of the iron mobilization by estrogens after penicillamine treatment has been observed (14). It is well known, that an administration of estrogen produces an increase of the ceruloplasmin content and plasma copper (24) in rats. Also, in chickens and turkeys, the injection of estrogens increases the copper and iron and the ferroxidase activity in plasma (13). However, in copper and iron depleted chickens the administration of estrogen did not produce iron mobilization but an increase in the ferroxidase activity. A copper supplement in

these birds raised the plasma iron and the FOX activity (15). A 6 day-treatment of penicillamine (100 mg/kg b.w.) did not block the mobilizing iron response to estrogens (15). However, it was completely blocked in the Zn-fed group (table II). Thus, it seems, that these doses of Zn could produce inhibitory effects on protein biosynthesis.

The addition of either Zn or Cd to commercial food for chickens, produced a microcytic and hypochromic anaemia. However, Cd did not modify the ceruloplasmin levels or the copper content in liver as did the Zn plasma (table I). This difference was confirmed by the administration of estrogens to these experimental groups of birds. Iron mobilization produced by the estrogens in the Cd-group was similar to that of the control group, without any response in the Cp levels.

Phosvitin has presented a ferroxidase activity (25). It has also been demonstrated (12) that concentrations as low as 1 μM-phosvitin were easily detected as ferroxidase activity. Therefore, the increase of the FOX activity after an injection of diethylstilbestrol, as reported by PLANAS and FRIEDEN (15), could also be due to this protein.

A dependence between Cp and copper seemed to be evident, especially in the experiments for refilling copper deposits (table II). However, in chickens it was insufficient to induce iron mobilization by estrogens. In other species, the normal levels of copper in plasma basically maintain the physiological concentration of ceruloplasmin, which for pigs (16, 19), or rats (4, 15, 26) is the crucial factor in the mobilization of iron.

82 % of ferroxidase activity in human plasma has been attributed to the plasma citrate (27), where the mean concentration was 2.2 mg %. In chickens, the plasma citrate was double (table IV) and decreased with laying. The increase of the FOX activity in laying hen plasma, due to the citrate, was negligible as it represented only about 1 %. In non-laying hens or in roosters this percentage was clearly higher (4-20 %) (fig. 1, table IV).

The ferroxidase activity of plasma in chickens, dependend mainly on the phosvitin, which increased greatly during laying periods. The increase of ceruloplasmin, during this period, expressed as PPD-oxidase, was not analyzed, but the plasma copper content was concomitant to iron (13). Arguments which would place the ceruloplasmin in a secondary position, in the mechanism of iron metabolism in birds, could be presented. This conclusion agrees with the present tendency to consider this protein to be multifunctional, with the primary function of copper transport, and with a secondary role in iron mobilization (7).

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Resumen

Se analizan los valores de ceruloplasmina, como p-fenilendiamina-oxidasa, así como la actividad ferroxidasa del plasma en pollitos alimentados durante 10 semanas con dieta comercial o suplementada con 5.000 ppm Zn o 100 ppm Cd. Es evidente una anemia microcítica e hipocrómica en ambos grupos, pero él hierro plasmático sigue normal, así como el nivel de ceruloplasmina, en el grupo del cadmio.

La administración de estrógenos provoca la movilización del hierro en dicho grupo, frente a su bloqueo en los tratados con Zn. La actividad ferroxidásica inducida por el estrógeno se reduce en un 90 % después de la precipitación de la fosvitina plasmática. La inyección de Cu para corregir la depleción de este metal por la alimentación con Zn normaliza los niveles de ceruloplasmina. Sin embargo, los estrógenos tampoco movilizan el hierro ni aumentan la actividad ferroxidasa.

Se analiza el contenido del citrato plasmático en gallinas en puesta y no en puesta, y en machos estrogenizados. Se considera que la contribución de esta sustancia en la actividad fe-

rroxidasa del plasma en las gallinas en puesta es despreciable. La fosvitina es la principal responsable de la actividad ferroxidasa en el plasma durante la puesta. Por otra parte, la ceruloplasmina en la gallina juega un papel secundario en la movilización del hierro.

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