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The Serum Ferroxidase System and the Effect of Estrogen on Plasma Iron *

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Estrogen effects on plasma iron and ferroxidase activity in some mammals and domestic fowl are studied, to investigate a possible estrogen mechanism on iron through its action on the ferroxidase system.

Although estrogen generally induces ceruloplasmin, iron mobilization, characterized by a rise in plasma iron, was evident only in rats and chickens. Gonadotrophin treatment confirmed these results. A decreasing affect on plasma iron was noted in rabbits and guinea-pigs, substantiated by some bibliographical data.

Ferroxidase activity increased and a copper-dependent factor was evident in copper injected species. Iron mobilization, however, was produced only in rats and chickens.

D-penicillamine treatment considerably lowered ferroxidase activity in rats and suppressed the estradiol increasing plasma iron effect. This response to the copper-chelating drug did not take place in the other species. This phenomenon could be explained by the presence of two copper-dependent ferroxidases (ferroxidase I or ceruloplasmin and ferroxidase II) in rat plasma, as recently published.

Present and previous results in fowl are confirmed by a newly discovered protein (phosvitin), non copper-dependent but induced by estrogens, in the chicken ferroxidase system.

No plausible explanation has been found for rabbits and guinea-pigs special response to both estrogens and for rats response to diethylstilbestrol.

From the initial work of FRIEDEN and his group (13), ceruloplasmin has been considered as a ferroxidase (E.C.1.12.3.1) and its role in the iron metabolism in pigs (14, 20, 22) and rats (5, 29) has been clearly demostrated. This copper-containing enzyme (ferroxidase I) is copper dependant so that Cu injections stimulate its synthesis (3) and Cu-deficient diets in rats (5), pigs (20) and chickens (19) or diets with copper antagonistic metals such as Ag, Zn, Hg, Cd (19, 28) produce a

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decisive reduction in the ceruloplasmin oxidase activity. Thus this enzyme is considered to be the molecular link between copper and iron metabolism (6).

However, there is some evidence that ceruloplasmin is not the exclusive component in plasma that has a ferroxidase activity (15, 26, 27, 30) and for these reason it must be considered as a complex system.

It is known that estrogens produce a significant increase in ceruloplasmin and copper content in rats (4, 25) and in man (23, 24). In chickens it has been assumed (18, 19) that plasma iron is increased by estrogen as a consequence of previous stimulation of the ferroxidase activity in plasma. However, the estrogen effect on plasma iron is not uniform in mammals (10, 11, 17) where possibly the action point of estrogens is not the ceruloplasmin but another component in the ferroxidase system.

For this reason in this paper the effect of estrogens on plasma iron and on ferroxidase activity is analyzed in order to gain better understanding of its different behaviour among some mammals.

Materials and Methods

Different lots of animals were used, employing a commercial diet and water *ad libitum*. Wistar rats and white Leghorn chickens were used, but rabbits and guinea-pigs were not of such uniform breeds.

Blood samples were obtained before overnight fasting by cardiac puncture in rats and guinea-pigs, on barbiturate anaesthesia, or by direct puncture of the ear veins in rabbits or of the radial veins in chickens.

The ferroxidase activity was determined according to JOHNSON *et al.* (9) using a Hitachi-Perkin Elmer espectrophotometer, Mod. 139, adapted to recorder, Mod. 56. The reaction mixture was 1.475 ml (0.050 ml of chicken serum or 0.025 ml of rat plasma or 0.010 ml of guinea-pig or rabbit serum; 0.600 ml acetate buffer 0.36 M, pH 6; 0.375 ml apo-transferrin 1 %, Boehringer; 0.450 ml Fe (II) 400 μM). All the tests were performed on fresh serum or plasma no more than 24 hours old.

The plasma iron was determined by RAMSAY's method (21) adapted to 250 μl.

Table I. *Plasma iron and ferroxidase activity in different animals.* Mean±S.D. number of speciments in brackets.

Species	Plasma Iron μg Fe/100 ml	Ferroxidase activity μM Fe (II)/min/ml plasma
Domestic fowl *		
♂ (6)	115±5	27±5
♀ (8)	145±31	76±31
Rabbit		
♂ (11)	188±49	573±151
♀ (10)	211±48	431±168
Rat		
♂ (22)	140±10	330±11
♀ (10)	207±23 ($P < 0.01$)	306±14
Guinea pig **		
♂ (21)	184±45	496±107
♀ (29)	256±67 ($P < 0.001$)	891±228 ($P < 0.001$)

* The sex difference has not analyzed because it was clearly established in a previous paper (18,19).
** These lots of animals are of different source.

In some of the samples, the plasma copper was determined by a spectroscopic technique with a Boehringer kit (Mannheim).

Diethylstilbestrol (Merck) dissolved in 1,2-propanediol and estradiol benzoate in the doses reported in table I were injected intramusculary. Equine serum gonadotrophin (Antex Leo), copper sulfate solutions and D-penicillamine (Merck) were administered subcutaneously in doses and sequences as reported in tables.

Results

The ferroxidase activity and the plasma iron in different species was determined (table I). It was observed that the level of plasma iron was higher in females than in males but these differences were only significant in rats and in guinea-pigs. A comparision among the domestic fowl was not made in the present work because it has been clearly demostrated in previous publications (18, 19). A large difference in the ferroxidase activity was observed

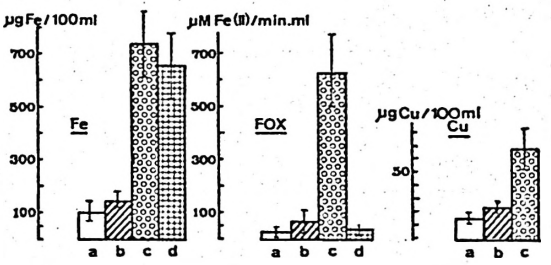

Fig. 1. *Plasma iron (Fe), ferroxidase activity (Fox) and plasma copper (Cu) in immature pullets (a; N = 10), in prelaying hens (b; N = 9), in laying hens (c; N = 12) and in postlaying hens (d; N = 6).* Mean ± S.D.

Table II. *Effect of diethylstilbestrol (DES) or estradiol benzoate (EB) on the plasma iron and the ferroxidase activity (FOX) in several species.*
The intramuscular estrogen administration was made in the last day (immediately after the blood sample for the baseline values) and on the 2nd day; on the 3rd day, the final blood sample. All injections and blood samples have been made between 10 to 12 a.m. Number of specimen in brackets. Mean ± S.D.

Species	Baseline values		Estrogen Doses day/mg/kg	After estrogen injection	
	Plasma Iron µg Fe/100 ml	Fox µM Fe (II)/min/ml		Plasma Iron µg Fe/100 ml	Fox µM Fe (II)/min/ml
Domestic fowl					
♀ (6)	117±40	25± 5	DES 1×2 mg	227± 80 [b]	47± 8 [c]
♂ (12)	115±22	28± 6	DES 2×2 mg	445±100 [d]	60± 28 [d]
♂ (9)	139±25	73± 22	EB 2×1 mg	598±180 [d]	99± 7 [a]
Rabbit					
♂ (4)	242±48	668±113	DES 2×4 mg	166± 15 [a]	624±100
♀ (4)	196±21	515± 82	DES 2×4 mg	138± 28 [a]	708± 76
♂ (4)	185±29	426± 25	EB 2×2 mg	125± 25 [a]	525± 80
♀ (4)	231±48	455±126	EB 2×2 mg	160± 38 [a]	605±107
Rat					
♂ (4)	177±14	347± 14	DES 2×4 mg	130± 26	224± 11 [d]
♀ (4)	223±43	327± 15	DES 2×4 mg	127± 13 [a]	206± 11 [d]
♂ (5)	194±26	360± 22	EB 2×2 mg	250± 19 [a]	371± 27
♀ (7)	233±18	300± 27	EB 2×2 mg	334± 28 [d]	330± 18
Guinea pig					
♀ (4)	321±20	862±144	DES 2×4 mg	191± 44 [d]	1093±238
♀ (6)	—	—	DES 2×4 mg	211± 30	1424±242
♀ (4)	285±20	1037±122	EB 2×2 mg	160± 18 [d]	1502±100 [b]

Probability: a) $P<0.05$; b) $P<0.02$; c) $P<0.01$; d) $P<0.001$.

Table III. *Effect of the serum equine gonadotrophin injection on the plasma iron (Fe) and plasma ferroxidase activity (Fox) in female specimens of the species studied.* Gonadotrophin doses: Chicken: 30 U.I./animal/day, during 6 days Rabbit: 150 U.I./animal/day, during 5 days; Rat: 100 U.I./animal/day, during 7 days; Guinea Pig: 75 U.I./animal/day, during 6 days, 24 hours after the last injection, blood samples has been taken. Mean±S.D.

Species		Baseline values		After gonadotrophin	
		Fe µg/Fe 100 ml	Fox µM Fe (II)/min/ml	Fe µg/Fe 100 ml	Fox µM Fe (II)/min/ml
Chicken	8 ♀	124±21	29± 13	170±30 [b]	54± 19 [a]
Rabbit	4 ♀	287±10	385± 17	120±20 [c]	625± 78 [a]
Rat	6 ♀	214±32	263± 53	298±46 [a]	318± 55
Guinea pig	6 ♀	283±19	789±107	215±53 [a]	870±270

a) $P<0.05$; b) $P<0.02$; c) $P<0.01$.

between birds and mammals since immature pullets or chickens showed a 10-30 fold lower values. The ferroxidase activity in rats was one third of the human one, but in rabbits and guinea-pig values were closer to human ones.

Sex differences in the ferroxidase activity were significant in guinea-pigs as has already been established in fowl. A large variability among specimens was noted in all species; except in rats and hens where the laying state produced an increase in the plasma iron, copper and ferroxidase activity (fig. 1). In specimens from laying hens where the egg production was interuppted for three days, the ferroxidase activity reached approximately immature levels but the plasma iron remained very high.

The estrogen administration (table II) increased the ferroxidase activity in all the species; the iron plasma increased in fowl and in estradiol treated rats, but decreased in rabbits, guinea-pigs and diethylstilbestrol treated rats. Administration of serum equine gonadotrophin in these species produced a clear rise in plasma in fowl and in rats but decreased in rabbits and in guinea-pigs (table III). The ferroxidase activity increased in all species but only significantly in hens and rabbits.

Table IV. *Effect of copper administration on plasma iron (Fe) and plasma ferroxidase activity (Fox) in different species.*
A simple dose is injected by day. The total doses injected are shown in brackets. The blood samples have been obtained 24 hours after the last dose. Mean ± S.D.

Species		Baseline values		Doses Cu (µg)	After Cu administration	
		Fe µg Fe/100 ml	Fox µM Fe (II)/min/ml		Fe µg Fe/100 ml	Fox µM Fe (II)/min/ml
Chicken	♂ 4	117±10	36± 16	400 (1)	156±44 [a]	100± 28 [a]
Rat	♂ 6	80±20	220± 48	500 (4)	157±21 [c]	260± 91
Rabbit						
	♀ 3	220±20	350± 60	2000 (4)	235±42	815± 65 [b]
	♀ 4	141±96	258± 53	250 (3)	192±41	480± 96 [c]
	♀ 4	131±72	300± 56	25 (3)	132±52	332± 73
Guinea pig						
	♀ 6	250±41	743±158	500 (3)	310±78	1.680±127 [d]
	♀ 5	282±23	766±115	150 (3)	277±18	626± 91

Probability of the differences: a) $P<0.05$; b) $P<0.02$; c) $P<0.01$; d) $P<0.001$.

In table IV the effect of intraperitoneal injections of copper is shown. In all groups high doses of copper increased the ferroxidase activity but a significant iron mobilization was only found in rats and chickens.

The copper-chelating effect of D-penicillamine on plasma iron and on ferroxidase activity and its influence on the estrogen response of these paramethers are shown in table V. In rats, penicillamine treatment produced a significant decrease in the ferroxidase activity and the injection of estradiol to these copper-depleted rats did not increase the plasma iron. No response was observed in the other species, where reaction to the estrogen injection was normal for each species.

Table V. *Penicillamine (100 mg/kg) treatment in different species analyzed with control groups and the influence of this drug on the estrogenic response in the plasma iron (Fe) and on the ferroxidase activity (Fox).*

The penicillamine lots (Pen.) show in brackets the number of days of treatment before the blood sample. During the estrogen administration the animals received simultaneously this drug. Diethylstilbestrol (DES) or estradiol benzoate (EB) was adminstered intramuscularly in a single dose each day, followed by penicillamine. The blood samples were taken 24 hours after the last estrogen injection. Mean ± S.D.

Species		Baseline values and after penicillamine		After estrogens		
		Fe µg Fe/100 ml	Fox µM Fe (II)/min/ml	Estrogen mg/kg	Fe µg Fe/100 ml	Fox µM Fe (II)/min/ml
Chicken						
Control	5 ♂	135±19	48± 7	2 DES 6 mg/kg	225± 5 d) *	56± 21
Pen. (6 days)	5 ♂	128±20	53± 5	2 DES 6 mg/kg + Pen.	234±69 c) *	69± 6 c) *
Control	4 ♂	84±20	66± 26	6 DES 6 mg/kg	185±21 c) *	60± 16
Pen. A)	4 ♂	—	—	6 DES 6 mg/kg + 6 Pen.	238±33 a) **	44± 22
Rat						
Control	7 ♂	133±30	300± 55	2 EB 4 mg/kg	336±65 c) *	330± 47
Pen. (14 days)	7 ♂	167±19	192± 40 c) **	2 EB 4 mg/kg + 2 Pen.	131±37 d) **	163± 38 d) **
Rabbit						
Control	4 ♀	231±48	455±125	2 EB 2 mg/kg	160±38 a) *	605±107
Experimental Before Pen.	4 ♀	245±47	610±158	—	—	—
After Pen. (14 days)		135±24 c) **	501± 46	2 EB 2 mg/kg + 2 Pen.	130± 6	587± 78
Guinea pig						
Control	10 ♀	271±46	776±170	—	—	—
Pen. (9 days)	5 ♀	329±39	988±290	—	—	—

a) $P<0.05$; b) $P<0.02$; c) $P<0.01$; d) $P<0.001$.
A) Penicillamine and estrogen administration for 6 days without a previous treatment the chelating agent.
* Significance between horizontal values (estrogen effect).
** Significance between vertical values (penicillamine effect).

Discussion

Ceruloplasmin promotes iron mobilization through its ferroxidase activity (13) and experimental evidence has been found in pigs (20, 22), rats (5, 12) and chickens (19). In normal mammals and fowl a positive correlation between plasma iron and ferroxidase activity was not observed, but it has been quite evident in laying hens and turkeys (18), and in estrogenized roosters (19). On the other hand it has been confirmed that estrogen produces an increase in serum copper and ceruloplasmin in rats (4, 12, 25) and man (23, 24). The induction of ceruloplasmin by estrogens seems to be a general phenomenon in mammals and it is commonly noticed during pregnancy when copper and iron are supplied to the embryo (2). The administration of gonadotrophin (table 3) produced de induction of endogenous estrogens with the same results. GREENGARD *et al.* (7) has already observed a 400 % increase of plasma iron in cockerels after a injection of pregnancy urine. Normal females have a higher level of plasma iron than males and such sex differences were evident in all the species studied but were only statistically significant in rats and guinea-pigs and in fowl when the females were in the laying period. In rats, HERSHKO and EILON (8) observed sexual differences not only in plasma iron but in the plasma iron turnover and iron stores, and they atributed these events to the stimulating effect of estrogens on iron absorption.

However, the experimental effect of the administration of estrogens on plasma iron is not uniform. MEDURI and PETRONIO (11) as in our data, have observed that in rats diethylstilbestrol caused a drop in plasma iron. A decreased effect brought about by estrogens in rabbits has alredy been observed by PALMER (17) and LEDERER and PRINZIE (10). In respect to the ferroxidase activity, ALIAS (1) has stated that estradiol failed to increase ceruloplasmin but diethylstilbestrol produced a 150 % increase. The present results disagree with this because of an increase in ceruloplasmin after an administration of estrogens or gonadotrophin, in rabbits and in guinea-pigs, a significant drop in plasma iron has been noticed. This response of the plasma iron and ferroxidase activity in rabbits and guinea-pigs was unexpected.

The importance of the copper supply or of a depletion was analyzed in order to establish the real role of the ceruloplasmin in the different species studied. It is known that copper injections induce a biosynthesis of ceruloplasmin (3) and normalize the iron metabolism in copper-defficient pigs (22) and rats (5). In our data, in accord with this result, when high copper doses were adminstered, a significative increase in ferroxidase activity was observed in all the species; but only in rats and fowl, was iron mobilization evident through out the plasma iron increase.

The D-penicillamine produced a significant drop in the ferroxidase activity, only in rats, treated during 14 days, at which time copper depletion suppressed the estradiol increase effect on plasma iron. OWEN *et al.* (16) has observed that in rats on a penicillamine diet for a 47 days period, the serum copper decreased and the plasma ceruloplasmin levels fell slightly; but during a 10-20 weeks treatment, the plasma copper and the ceruloplasmin increased, while urinary copper increased and tissue copper decreased.

WILLIAMS *et al.* (29) pointed out that the ceruloplasmin is essential for the flow of iron from reticuloendotelial cells to the transferrin. However, it has been recently demostrated that the ferroxidase activity in rat plasma has an azide-resisten component, the ferroxidase II (27) which is equivalent to a similar enzyme described in human plasma by TOPHAM and FRIEDEN (26), and also that the plasma citrate could possibly play some role in the ferroxidase activity (30). From the recent paper

from TOPHAM *et al.* (27) the ferroxidase system in rats has been increased by this new enzymic component, the ferroxidase II, which is also copper dependant as copper atoms are essential for its catalytic activity, and the association of protein, phospholipids and copper componets are indispensables for such an activity.

On the other hand, in chickens, the copper-chelating drug (penicillamine) did not disturb the ferroxidase activity and the iron mobilization produced by estrogens. It was observed (19) that after 40 days of a copper deficient diet, the ferroxidase activity was eliminated, but a later administration of estrogen increased plasma iron if stores were normal. OSAKI *et al.* (15) has concluded recently that phosvitin, present in the plasma of estrogenized chickens, with a primary role in iron transport, also has a catalytic activity as does ferroxidase. As at now, it seems to be evident that the ferroxidase system in fowl could be formed by ceruloplasmin (ferroxidase I) and also by the phosvitin, a estrogen-dependant phosphoprotein present in all oviparous vertebrates.

For the moment, we heve no explanation for the special behaviour of rabbits and guinea-pigs to the estrogen administration and the plasma iron response. But the possible existence of other componets in the ferroxidase system in such species could be the clue. Futher works must be done in this direction.

Resumen

Se estudia el efecto de los estrógenos sobre el hierro y la actividad ferroxidasa en el plasma de rata, conejo, cobayo y gallina.

Los estrógenos provocan la movilización del hierro con el consiguiente ascenso de la sideremia en la rata y en la gallina; la administración de gonadotrofinas confirman este resultado. Un efecto contrario se aprecia en el conejo y en el cobayo.

La actividad ferroxidasa del plasma se muestra dependiente del cobre en la rata, pues la inyección del metal provoca el incremento de la misma y la eliminación del mismo por el tratamiento con D-penicillamina, desciende esta actividad y deja sin efecto la inyección de estrógenos. La presencia de dos ferroxidasa (I y II) en el plasma de rata, conteniendo ambas cobre en su molécula, pueden explicar estos resultados.

Las gallinas responden en igual sentido al tratamiento con el cobre pero se muestran sin efecto al quelante. La presencia en el plasma de la ceruloplasmina (ferroxidasa I) juntamente con una fosfoproteína (fosvitina), inducida también por los estrógenos pero independiente del cobre por la cual posee actividad ferroxidasa, podrían explicar la respuesta obtenida.

No se encuentra una explicación plausible a la respuesta apreciada en conejo y cobayo.

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