Antithrombic Activity of Chicken Plasma Induced by Estrogenic Substances. (19207)

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This report deals with observations which indicate that the blood plasma of chickens under estrogenic stimulation exhibits heparin-like antithrombic activity, and that this phenomenon can be demonstrated readily under naturally occurring and experimental conditions. During the course of blood coagulation studies employing chicken plasma, it was noted that plasma from pullets approaching maturity clotted very slowly with amounts of thrombin ordinarily used to defibrinate oxalated plasma. It was further observed that some of the slow-clotting plasmas were lactescent or lipemic in appearance, and that the observed antithrombic effect was exhibited only during the stage of preparation for egg-laying and the actual period of egg production. The plasma of immature fowls of either sex, males or capons of all ages, and moulting or broody hens showed normal reactivity with thrombin solutions.

Efforts were then directed toward experimental production of this state of increased antithrombic activity. It was found that the condition could be induced consistently in chickens of any age and of either sex by the parenteral administration of synthetic estrogenic compounds. Several commercially available preparations were used with similar results, although the drugs employed showed somewhat different potencies. The dosage necessary to cause the change in blood clotting behavior also caused lipemia, oviduct growth, and other changes associated with estrogenic stimulation.

Materials and methods. The chickens were obtained from a local commercial hatchery, and were maintained on a standard control laboratory diet(1). Most of the chickens were White Leghorns, but limited numbers of White Rocks and New Hampshire Reds were also used. The estrogenic drugs employed included diethylstilbestrol, hexestrol, and dienestrol.* The given dosage of the drug was dissolved in 1.0 ml of sesame oil or corn oil (Mazola), and injected subcutaneously. Clotting activity was measured in several ways, including whole venous blood clotting time, with and without the addition of thromboplastin, and measurements of the clotting activity of blood and plasma upon the addition of measured amounts of thrombin. Venous blood clotting time was determined as the time necessary for formation of a firm clot in a clean glass test tube at room temperature. The thromboplastin was prepared by saline extraction of chick brain(2). To measure the clotting time with added thromboplastin, 1.0 ml of blood was added to 0.1 ml of thromboplastin. Thrombin from several sources was tested, including commercially prepared bovine thrombin (Parke-Davis), and preparations from chicken, dog, and bovine plasma using modifications of Sternberger's method (3). The essential modifications of Sternberger's technic employed were the substitution of oxalated plasma for whole citrated blood, and the use of beef lung or chick brain in place of human milk as a source of thromboplastin. The final dilution of thrombin used in most determinations contained 5% calcium-free acacia, which served to stabilize thrombin activity. Clotting activity of thrombin was determined by adding 2 volumes of dilute thrombin solution to one volume of oxalated plasma at a room temperature of approximately 25°C. A control clotting time of between 12 and 15 seconds was obtained by appropriate dilution of the thrombin. Blood obtained by jugular venepuncture was drawn into one-seventh its volume of 1.85% potassium oxalate, and chilled with cracked ice until used. The oxalated plasma was separated by centrifugation of blood in an angle centrifuge in a refrigerated (5°C) room. In some instances comparative studies were done employing sodium citrate or purified amberlite resin as anticoagulants(4).†

* Dienestrol was supplied by White Laboratories, Newark, N. J.

TABLE I. Response to Single Injection of Dienestrol.

Days after inj.	Clotting time (% of control) A	B	Visible lipemia A	B
0	100	100	0	0
1	128	227	1+	1+
2	152	272	2+	3+
3	136	680	2+	4+
4	125	267	0	3+
5	102	346	0	3+
6	98	910	0	3+
7	—	247	0	3+
8	100	247	0	2+
9	103	181	0	2+
10	91	167	0	1+
11	114	128	0	Trace
12	80	102	0	0

A single 10 mg inj. of dienestrol was given to each of 2 White Rock cockerels 75 days of age, designated A and B. Clotting times express the ratio of values obtained with oxalated plasma of inj. and control cockerels of the same hatch, using a thrombin solution which clotted control plasma in approximately 12 sec. Degree of lipemia was estimated from the appearance of the plasma samples.

Results and discussion. Increased resistance to the clotting action of added thrombin usually was observed in blood or plasma of the treated chickens beginning 24 to 36 hours after injection of the estrogenic drug. A maximum effect was attained consistently within 72 hours after the administration of adequate dosages. Definite effects were noted in newly hatched chicks given single doses of as little as 2 mg of dienestrol or hexestrol, or 5 mg of diethylstilbestrol. Dosages many times the minimal effective dose did not shorten the time interval necessary for demonstration of the antithrombic effect. The changes in blood clotting behavior followed the same general course as the observed lipemia, but did not exactly parallel this condition. Thus, some of the animals showed altered clotting activity a day before lipemia was observed, while others showed the reverse order of effects. As a rule, the disappearance of visible lipemia coincided with the loss of increased antithrombic activity after estrogens were discontinued.

Table I shows representative results obtained by giving a single injection of an estrogenic compound to immature cockerels. There was considerable variation in response. Chick A showed a sub-maximum response, while the peak response of Chick B was equal to the maximum effect obtained by injecting several times the given drug dosage into other cockerels of the group. Oxalated whole blood reacted in a manner similar to oxalated plasma and comparable data were obtained using sodium citrate or amberlite resin as anticoagulants. Similar results were obtained with all types of thrombin preparations tested.

The venous blood clotting time of estrogenized chickens was greatly prolonged, as shown by Table II. The very slow clotting of chicken blood is greatly accelerated by the thromboplastic activity of minute quantities of contaminating tissue juice. Despite this source of error, the observed difference between the mean clotting times of the control group and the estrogen-stimulated group was more than 4 times the standard error of the difference between the mean values of the two groups. Variability was greatly reduced in the shortened clotting times obtained by adding large amounts of thromboplastin to the blood. The observed difference in this group was more than 6.5 times the standard error of the difference.

The clotting activity could be modified by the *in vivo* or *in vitro* addition of protamine sulfate in a fashion similar to the interaction of this drug with commercial heparin preparations.‡ Table III illustrates the effects produced by addition of protamine to normal cockerel plasma, with and without the addition of heparin, and to the plasma of an estrogen-treated cockerel or a laying hen. The addition of protamine shortened the clotting time of the control plasma. This may have been due to the presence of an appreciable amount of heparin or a heparin-like substance in normal plasma. The effect of added heparin was easily overcome by addition of small amounts of protamine. Much larger amounts were necessary to bring about comparable reductions in clotting time of plasma of the laying hen and the estrogen-treated cockerel. Addition of larger amounts of protamine length-

† Amberlite Resin IR-100, Rohm and Haas Co., Philadelphia, Pa.,

‡ Protamine sulfate was supplied by Eli Lilly & Co., Indianapolis, Ind.

TABLE II. Clotting Time of Venous Blood.

	Control	Estrogenized	Observed diff.	Stand. error of diff.
Venous blood clotting time (min)	29.7	70.6	40.9	9.38
Clotting time with added thromboplastin (sec)	35.6	44.1	8.5	1.25

Venous blood coagulation times are mean values of 31 determinations on control cockerels or capons, and 31 determinations on estrogen-injected chickens or laying hens. Clotting times with added thromboplastin are mean values for 84 determinations in each group, in controlled series employing equal numbers of immature White Leghorn chickens of both sexes in each group. The treated group received 5 mg of hexestrol daily.

TABLE III. Effect of Protamine Sulfate Added to Plasma-Thrombin Reaction Mixture.

Protamine added, mg	Clotting time (sec)			
	Normal cockerel	Normal cockerel + heparin	Laying hen	Estrogenized cockerel
0	14.3	25.2	18.9	27.4
.05	13.6	12.7	15.3	25
.10	9.9	9.2	14.6	20.3
.20	10	8.1	10.3	17.3
.30	16.8	8.6	8.2	14.3
.40	19.6	9.9	7.9	14.3
.50	20	20.5	8.2	13
.75	—	—	12.6	11.7
1	19.5	20	21	8.4
1.5	—	—	14.7	10.7
2	21	20	22	16.9

Clotting mixture consisted of .1 ml plasma, .1 ml protamine sulfate solution in normal saline, and .2 ml chicken thrombin solution. Heparin (Abbott) added to normal cockerel plasma was 1 Toronto unit per ml. The estrogenized cockerel had received 10 mg of dienestrol three days before determinations were made.

ened the clotting time of control plasma, and caused an abnormal, flocculent clot. The addition of heparin partially protected the control plasma from the deleterious effects of excess protamine. The plasma of estrogen-stimulated fowl also showed greater tolerance to excess protamine than shown by the control plasma.

No abnormality of other blood clotting factors of the estrogenized chickens was detected. Prothrombin levels were normal by the two-stage method(1). Fibrinogen was present in amounts sufficient to form a firm clot, and the addition of prepared fibrinogen (Armour) did not correct the clotting defect. The formed clot was stable at room temperature over night with no indication of abnormal fibrinolysis. The plasma antithrombin levels were determined by a method similar to that of Klein and Seegers(5). Chicken plasma was incubated for two hours at room temperature with an equal volume of bovine thrombin preparation (Parke-Davis) containing 1500 units per ml. One ml of either the control or estrogenized plasma inactivated approximately 700 units of thrombin under these conditions. The plasma of the estrogen-treated chickens thus was shown to have essentially normal total capacity to inactivate thrombin. This is in contrast to its heparin-like ability to interfere with the thrombin and fibrinogen reaction.

The heparin-like effect produced in the experimental fowl was prevented by agents which interfere with normal metabolism of the injected estrogen. This was demonstrated by the simultaneous administration of estrogenic drugs and known estrogenic antagonists. The folic acid antagonist, 4-amino pteroylglutamic acid (Aminopterin), and the purine antagonist 2, 6-diaminopurine were used in a manner similar to that reported by Hertz(6), and Hertz and Tullner§(7). The effects of

§ Aminopterin was supplied by Dr. J. M. Ruegsegger, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., and the 2,6- diaminopurine was supplied by Dr. G. H. Hitchings, Wellcome Research Laboratories, Tuckahoe, N. Y.

a single 5 mg injection of hexestrol or dienestrol were partially or completely prevented by daily subcutaneous injections of 5 mg of Aminopterin or 50 mg of 2, 6- diaminopurine. Results were somewhat variable and the antagonistic drugs were extremely toxic in the amounts given, resulting in death of most of the test chicks between the third and seventh day of drug administration.

Lipemia produced in chickens by oral administration of 2 ml of lard or corn oil per 100 g body weight was not accompanied by significant changes in clotting. Emulsions of plasma and oil were prepared by repeated forcible ejection of the material through a 25 gauge needle attached to a glass syringe. Preparations containing 10% oil and 1 mg of hexestrol or dienestrol per ml clotted normally.

Plasma from estrogenized chickens retained the heparin-like effect after extraction with 15 to 20 volumes of ether, and the fatty residue remaining after evaporation of the ether at room temperature had no antithrombic properties. Transfusions of blood from an estrogen-treated fowl to a normal fowl caused the transfused animal to exhibit lipemia and the typical heparin-like effect when 50% to 75% of its calculated blood volume had been replaced. The inhibition of the thrombin-fibrinogen reaction disappeared within a few hours, whereas the lipemia still was visible 24 hours later.

Attempts to isolate or further identify the substance responsible for the described effects have not been successful to date. Heparin assays by the methods of Monkhouse and Jaques(8) or Gibson and co-workers(9) have yielded negative results. It was found, however, that added heparin was recovered incompletely from control chicken plasma by these methods, and not at all when added to the lipemic plasma of estrogenized chickens in the usual amounts.‖ Recently reported investigation indicates that native heparin in mast cells exists as a lipoprotein complex(10). The above observations suggest that the active principle in estrogenized chicken plasma may be heparin in a combined form from which the heparin moiety is not readily isolated.

Summary. The plasma of laying hens exhibits an inhibitory effect on the interaction of thrombin and fibrinogen. Similar inhibitory activity can be induced in the plasma of chickens of any age and of either sex by the administration of synthetic estrogenic compounds. This effect can be prevented by agents which interfere with normal metabolism of the estrogenic material in the experimental animal. The observed effect is similar to that produced by heparin, but plasma assays have been negative for heparin, and the substance responsible for the heparin-like activity has not been isolated or identified.

‖ We wish to thank Dr. R. B. Gibson and Mr. Saul Green for assistance with heparin assays of plasma.

1. Tidrick, R. T., Joyce, F. T., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, v42, 853.

2. Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1939, v125, 296.

3. Sternberger, L. A., *Brit. J. Exp. Path.*, 1947, v28, 168.

4. Stefanini, M., PROC. SOC. EXP. BIOL. AND MED., 1948, v67, 22.

5. Klein, P. D., and Seegers, W. H., *Blood*, 1950, v5, 742.

6. Hertz, R., *Science*, 1948, v107, 300.

7. Hertz, R., and Tullner, W. W., *Science*, 1949, v109, 539.

8. Monkhouse, F. C., and Jaques, L. B., *J. Lab. and Clin. Med.*, 1950, v36, 782.

9. Gibson, R. B., Carr, T. L., Green, S., and Fowler, W. M., *Fed. Proc.*, 1951, v10, 49.

10. Julen, C., Snellman, O., and Sylven, B., *Acta Physiol., Scand.*, 1950, v19, 289.

Received August 27, 1951. P.S.E.B.M., 1951, v78.