The delayed treatment experiments were designed to test the hypothesis that the inhibitor must enter the test segment via an open wound surface. Since Sayles(1) has shown that new tissue appears at the wounded surface in *Clymenella* about 24 to 48 hours after cutting, it seems reasonable to assume that by 18 hours after cutting, wound closure would be essentially complete. The sudden increase in regeneration frequency between the 12- and 18-hour delay groups may thus be explained on the basis of a closing wound which becomes impermeable to the inhibitor.

Summary. These data suggest that one may extract from the anal collars of *Clymenella torquata* an inhibitor which acts within 3 hours, and whose effect lasts at least 8 days. The data furthermore suggest that the inhibitor is not easily metabolized, and that it blocks an initial step in regeneration. The data from the delayed treatment tests suggest that the inhibitor must enter an open wound surface, and that a closed wound is impermeable to inhibitor.

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Received January 29, 1964. P.S.E.B.M., 1964, v116.

Effect of Intravenous Estrogen on an Inhibitor of Hyaluronidase and on Clotting Factors in Blood.* (29167)

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(Introduced by Richard W. Vilter)
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Intravenous estrogen has been used extensively in attempts to control bleeding(1-5). Well controlled, double-blind studies and laboratory data have, however, often been lacking. The present study deals with the effect of i.v. estrogen† on the clotting system and the ground substance. We have not attempted here to assess the clinical efficacy of the hormone.

Materials and methods. Subjects were healthy medical students, nurses and patients with no known bleeding disorders on the wards of a large general hospital. The double-blind method was used to determine bleeding and clotting times, blotter weights, one-stage prothrombin times, factors V, VII-X complex, antithrombin, and spread of intracutaneous hyaluronidase. Ampoules containing 40 mg of estrogen or placebo were coded and paired. If the subject received both preparations, a 48- to 72-hour interval elapsed between injections. If a single ampoule was used, its contents were unknown to both subject and investigator. Results are related to a single dose of either preparation. No evidence of toxicity or side effects was noted. Double-blind techniques were omitted for determination of prothrombin by the synthetic substrate (TAMe) method, and for serum hyaluronidase assay. Unless otherwise stated, all studies were performed 1½ hours after injection of either hormone or placebo, since Johnson(6) has shown this to be the period of optimum activity. Bleeding time was measured by the method of Ivy(7). Clotting time was determined by the three tube Lee-White method(8). Prothrombin time was measured by the method of Quick(9). Prothrombin content, employing the synthetic substrate TAMe, was assayed by the method of Glueck(10). Factor V was measured by the method of Stefanini and Dameshek(11). Factor VII-X complex was determined as follows: substrate plasma with a prothrombin time greater than 25 seconds was obtained

* Supported by grants from U.S.P.H.S. and from Ayerst Laboratories, Inc.

† Premarin®.

from patients during the first week of Coumadin® therapy(12). Normal plasma was added to the Coumadin® plasma in concentrations of 10, 5, and 2% and prothrombin times of the mixtures determined. Test plasma was similarly added to the Coumadin® plasma, and compared to the normal. Antithrombin was measured by the method of Johnson and Seegers(13).

Whatman filter paper #40 was used to measure the amount of blood lost during the determination of bleeding time. The papers were stored in a desiccator. On removal they were allowed to equilibrate at room temperature and weighed. Following determination of the bleeding time, they remained at room temperature, and were weighed repetitively until a constant value was obtained. This often required 6 to 8 hours.

The spread of testicular hyaluronidase‡ was determined as follows: 1½ hours after intravenous injection of estrogen or placebo, 0.5 ml of hyaluronidase in saline was injected intracutaneously. The wheals were examined by palpation until they had completely disappeared; and the so-called "disappearance time" recorded. Reproducible results were obtained only on the skin of the back. The diameter of the wheals was variable and could not be used for analysis.

The method of Glick and co-authors(14) using umbilical cord hyaluronate and testicular hyaluronidase was modified to measure the nonspecific hyaluronidase serum inhibitor as follows: (1) serum was substituted for the inhibiting substances in mast cells; (2) the reagents were made up in 0.1 M barbital buffer pH 6.8, in place of saline-phosphate buffer; (3) the human umbilical cord hyaluronate prepared by the method of Jeanloz and Forchielli(15) was dissolved in saline-barbital buffer containing 6 volumes of 0.1 M barbital buffer pH 6.8 and 1 volume of 2 M NaCl(16). The enzymes were commercially available. The final volume of the system was 1.0 ml. Readings were performed in a Beckman D.U. spectrophotometer adapted with Pyrocell semimicrocuvettes containing 0.5 ml. Blood collected with careful venipuncture was incubated at 37° for approximately 2 hours, until clot retraction was evident. Following centrifugation the serum was removed by aspiration. The sera were frozen at —20°C and stored until tested. Sera obtained during the control period and after the estrogen, were each tested against the same enzyme-substrate preparation, since the latter varies somewhat from day to day. The values were expressed as percent of hyaluronidase activity inhibited by the serum (hyaluronidase serum inhibitor). Erroneous readings were produced by gross lipemia, hemolysis and icterus, conditions which were accordingly avoided.

Results. The effect of estrogen on bleeding times, blotter weights and factor V is summarized in Table I. Statistically,§ the only significant change was a slight rise in factor V following the estrogen‖(17). An increase in this factor ranging from 6 to 44% above initial levels was noted in 13 of 40 subjects receiving the estrogen. We did not consider the rise important, because of the small magnitude of the increases and the wide range of normal values(18). Other clotting studies included the Lee-White clotting time, (19 estrogen, 11 placebo): the one-stage prothrombin time (28 estrogen, 24 placebo): the synthetic substrate (TAMe) assay of prothrombin (25 estrogen treated subjects): antithrombin (14 estrogen treated subjects 11 with placebo): and factor VII-X complex (17 estrogen treated subjects and 11 with placebo). None of these procedures showed any significant change with either preparation.

Our inability to detect changes in the clotting mechanism following intravenous estro-

‡ Cordase®, Testagar Co. or Wydase®, Wyeth Laboratories. When reconstituted with saline each ml of solution contained 150 turbidimetric units.

§ Preliminary studies from this laboratory indicated that I.V. estrogen shortened bleeding time. The original group included several subjects with known bleeding disorders and prolonged bleeding times. More recent studies, reported herein, excluded all such patients. Under such circumstances it appears that bleeding time is not affected by I.V. estrogen.

‖ Studen't T test(17) determined separately on values obtained before and after estrogen, and before and after placebo. This method was used for all subsequent statistical analyses.

TABLE I. Coagulation Studies in Patients Receiving Intravenous Estrogen or Placebo.

		No. of subjects	Before	After	P
Estrogen	Bleeding time, min	35 (23)*	4.6 ± 1.9†	4.2 ± 2.5†	.4
	Blotter wt, mg	29 (19)	17.1 ± 2.6	14.1 ± 1.6	.1
	Factor V, % of normal	40 (34)	106 ± 43	112 ± 44	.01
Placebo	Bleeding time, min	30 (23)	5.0 ± 2.3	5.1 ± 2.6	.7
	Blotter wt, mg	27 (19)	7.8 ± 8.3	7.3 ± 6.0	.6
	Factor V, % of normal	37 (34)	108 ± 38	108 ± 47	.7

* Numbers in parentheses indicate subjects who received both estrogen and placebo.
† Mean ± S.D.; P values for student's t test determined separately on samples obtained before and after estrogen and before and after placebo(17).

gen directed our attention toward the role of estrogens on the ground substance. Our first approach utilized the disappearance time of hyaluronidase wheals. Thirteen male and female subjects were tested, each receiving both preparations (Fig. 1). Following the estrogen, disappearance time was prolonged in 12 of the 13 males ($P = 0.001$). No such changes were observed in females, regardless of age ($P = 1.0$). The mean value for estrogen treated males was similar to that of the females, regardless of the therapy in the latter group. The wide deviation in females, in contrast to males, is indicative of the absence of a characteristic pattern in the former sex.

Studies were next directed toward the measurement of the non-specific serum hyaluronidase inhibitor. This inhibitor, present in all mammalian serum, must be distinguished from the species-specific bacterial antibodies against hyaluronidase. Patients will collagen disorders, acute infections, or those receiving heparin were excluded since these factors are known to influence inhibitor levels(19). The value of the control serum inhibition of the hyaluronate-hyaluronidase system expressed as percent inhibition was subtracted from that obtained following estrogen. The individual differences were then averaged (Table II). Regardless of the initial values with their wide range of activity, the inhibiting effect of the serum was augmented by injec-

TABLE II. Effect of Intravenous Estrogen on Non-specific Serum Inhibitor of Hyaluronidase.

Time after inj, hr	Total No. of samples tested	Mean increase over initial value	P (♂ and ♀)
1–2	29 (19)*	19.3†	<.001
4	12 (9)	10.4	<.01
8	13 (11)	14.9	<.01
24	14 (8)	8.4	<.05

* Figures in parentheses represent number of males in each sample.
† % inhibition before estrogen subtracted from % inhibition following estrogen.

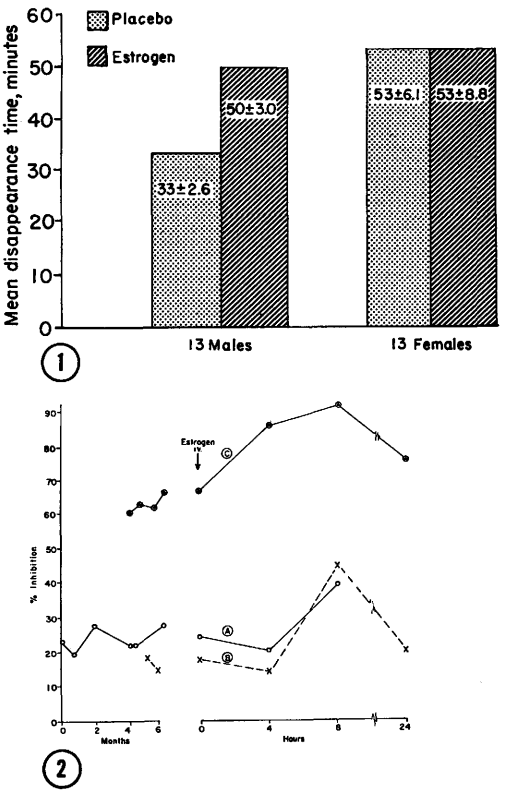

FIG. 1. Mean disappearance time in minutes of hyaluronidase wheals following I. V. estrogen or placebo in 13 male and female subjects. Figures represent standard error of mean.

FIG. 2. Serum hyaluronidase inhibitor levels in 3 subjects obtained at various time intervals. Arrow indicates administration of I. V. estrogen.

tion of the hormone.¶ Twenty-five of 29 samples tested at the 1- to 2-hour interval showed an increase in inhibitory power, 2 were unchanged, and 2 showed a slight drop. Twelve of the same subjects were retested at 4 hours. The rise persisted in 9, remained unchanged in 1, and fell in 2 instances. Some inhibitory effect still persisted in 11 of 13 subjects retested at 8 hours. At 24 hours the values remained moderately elevated in 9 of 14 subjects, had fallen to the preinjection level in 2, and were slightly less than the initial value in 3 instances. When the subjects were divided by sex, significant changes occurred in both groups at the 1- to 2-hour level (male $P = <0.001$, female $P = 0.05$). The number of samples derived from females at the 4-, 8-, and 24-hour intervals was insufficient for statistical analysis. However, in the latter group in all but one instance the serum inhibitor remained elevated above preinjection control levels.

To study the reproducibility of the method, 3 subjects were followed for weeks or months with samples drawn at various times of the day (Fig. 2). Values were fairly constant in all 3 subjects. Following I.V. estrogen, the anticipated rise in inhibitor level was again noted, although somewhat delayed when compared with the larger sampling.

Discussion. Asboe-Hansen(20) has recently reviewed the effect of hormones on the ground substance. Parenteral estrogens have been shown to produce an increase in the hyaluronic acid content of connective tissues in mice, in the sex skin of the macaque monkey, and the genital tract of female rabbits. Cutaneous and vascular permeability are diminished by estrogens. Of particular relevance to our studies are the observations of Schiff and Burn(21) who utilized histochemical methods to demonstrate the effects of estrogens. They noted an increase in the acid mucopolysaccharides of connective tissues following estrogens. These changes were observed in the cheek pouch of the hamster, in monkeys, and in the oral and tonsillar mucosa of humans. Later studies in humans showed that I.V. estrogens induced not only an increase in ground substance, but also a rise in number and granularity of the mast cells, from which it is presumed that hyaluronic acid is derived. Subsequently, the mast cells underwent rapid degeneration. These changes in both mast cells and ground substance were observed as early as 30 minutes after estrogen, were most marked at 2 to 4 hours, and persisted in a lesser degree for approximately 8 hours.

Pecora, Putnam and Baum(22) have recently shown that I.V. estrogen produces a decrease in pulmonary diffusing capacity. They speculated that this change was due to an increase in the mucopolysaccharides of the alveolar capillary zone. Johnson(6) found a marked increase in factor V and prothrombin, and an increase in antithrombin in dogs receiving I.V. estrogen. In agreement with the observations of others(23,24), we found no significant changes in the coagulation mechanism in humans following I.V. estrogen. The slight increase in factor V observed in ⅓ of our subjects would have little physiological effect in accelerating coagulation. Pronounced increases in concentration of the individual clotting components are required to bring about acceleration of clotting. It would seem unusual to expect patients with defective hemostasis, yet without demonstrable deficiencies of clotting factors, to improve after I.V. estrogen by slight elevation of already normal levels of these factors. Our studies showing inhibition of intracutaneously injected hyaluronidase, and an increased inhibition of hyaluronidase activity in serum following I.V. estrogen, would indicate that estrogens favor the build-up of ground substance by inhibiting the enzyme presumed to destroy it. Any presumed hemostatic effect of estrogens would thus be indirect and related to these changes.

We have purposely avoided an evaluation of the clinical efficacy of this preparation or any attempts to compare it with steroids of known usefulness. It is clear that more careful clinical studies with large numbers of subjects are needed to clarify the therapeutic usefulness of the preparation.

Summary. The clotting mechanism in the human, as measured by conventional meth-

¶ Addition of *in vitro* Premarin® in amounts presumed to be present in plasma (0.015 mg/ml) had no effect on the serum inhibitor.

ods, appears to be unaffected by intravenous estrogen other than by a slight increase in factor V. Following estrogen, intradermal hyaluronidase wheals persist for a longer period than noted with placebos. These changes are confined to the male. Nonspecific serum hyaluronidase inhibitor increases in both sexes following I.V. estrogen. The peak effect is noted at one to two hours, but the changes persist for approximately 8 hours, usually approaching base line levels at 24 hours. These experiments indicated that I.V. estrogens affect the ground substance. We have not attempted clinical evaluation of the use of intravenous estrogens in control of hemorrhage.

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Received January 30, 1964. P.S.E.B.M., 1964, v116.

Compensatory Hypertrophy of Right Adrenal after Left Adrenalectomy: Observations in Fetal, Newborn and Week-old Rats.* (29168)

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It has been reported that the hypophysis-adrenal axis of the rat does not respond to experimental stress during the early postnatal period(1,2) but that it does respond during the prenatal period(3). It also has been reported that volume and weight of the adrenal are reduced for a few days after birth(4,5,6) but that such reduction does not occur in a fetus which is subjected to extra time *in utero* by the experimental prolongation of pregnancy(6).

The experiments to be reported support the working hypothesis that in the rat there is a reduction in activity of the hypophysis-adrenal axis during the first 3 days after

* Aided by grant from Nat. Inst. of Child Health and Human Development.

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