Free Estrogens in Dog Plasma During the Estrous Cycle and Pregnancy.*† (30782)

F. METZLER, JR., B. E. ELEFTHERIOU AND M. FOX‡ (Introduced by M. X. Zarrow)
Department of Zoology, Kansas State University, Manhattan and The Jackson Laboratory, Bar Harbor, Maine

The concentrations of estrogens in plasma throughout a gestational period and menstrual or estrual cycle have been determined only in the human(1-5). Similar data for other species are totally lacking. Due to the common usage of the dog in the laboratory and the meager amount of information available concerning its reproductive physiology, this animal was chosen as the subject of study. The "free" estrogens in the plasma of both pregnant and cycling bitches were identified and quantitated fluorometrically.

Materials and methods. The study group consisted of purebred adult bitches: 3 pregnant basenjis, 1 pregnant cocker spaniel, 1 pregnant beagle and 1 cycling basenji and 1 cycling beagle from the colony maintained at the Hamilton Station of The Jackson Laboratory, Bar Harbor, Maine. Blood was collected usually at weekly intervals and centrifuged, and plasma samples of about 5 ml each immediately frozen. Prior to extraction amounts of estrone-16-C^{14} and estradiol-17β-4-C^{14} equivalent to about 22,200 and 11,100 dpm, respectively, were added to each sample for recovery purposes.

Six cocker spaniel samples were pooled (32.3 ml), extracted according to the procedure of Veenhuizen *et al*(6) except that diethyl ether was used instead of ethyl acetate and chromatographed in a Skelly B:benzene (1:1) system saturated with formamide (6). A strip counter (Atomic Accessories, Inc.) was employed to locate the labeled estrone and estradiol-17β. In addition a 6 mm strip was cut off and developed with Barton's reagent [1% $FeCl_3$: 1% $K_2Cr_4O_7$ (1:1)] followed by successive washes of 5% HCl and water. A standard strip was likewise developed. Appropriate areas were eluted with 3 washes of 5 ml absolute methanol each. The estrone fraction, which had run off the strip, then was rechromatographed in the same system for 6 hours. The estriol fraction was chromatographed on paper for 5 hours in an ethyl acetate:toluene (15:85) system saturated with 50% methanol(7). Elution after chromatography was as above.

Portions of each fraction were methylated and acetylated according to Bush(8). The derivatives, in addition to standards, were chromatographed two-dimensionally on thin-layer plates of activated silica gel G (Merck). Activation was for 30 minutes at 120°C. The first direction was run in a chloroform:ethyl acetate (2:1) system and the second in ethyl acetate:n-hexane (1:1). The plates were sprayed with 10% phosphomolybdic acid in absolute ethanol and heated for 10 minutes at 100°C. The "blue" spots were traced on paper and the standards and derivatives compared.

Plasma samples to be quantitated were likewise extracted and chromatographed on thin-layer. Areas corresponding to the standard plate run concurrently were scraped off the sample plate and eluted once with 5 ml chloroform:methanol (1:1) and once with 5 ml methanol. The sample was centrifuged after each wash at 1000 $\times$ *g* for 15 minutes and then decanted into a test tube. The solvent was evaporated under vacuum, the tube rinsed with chloroform:methanol (1:1) and then reevaporated. To each tube was added 1.0 ml absolute ethanol and 0.1 ml aliquots removed from the estrone, estradiol-17α and estradiol-17β fractions. These aliquots were placed in counting vials and counted in a Packard Tricarb scintillation spectrometer. The estradiol-17α fraction was counted to check for contamination by estra-

* Contribution No. 356, Dept. of Zoology, Kansas Agri. Exp. Station.

† Supported in part by grant 1R01-HD-00013 from National Advisory Child Health and Human Development Council, Nat. Inst. Health.

‡ Present address: Galesburg Research Hospital, Galesburg, Ill.

diol-17β, since the two compounds were not greatly separated. All tubes then were dried again.

Fluorometry was run by adding 0.1 ml absolute ethanol and then 0.7 ml 90% sulfuric acid. This mixture was heated at 80°C for 20 minutes, cooled to room temperature, then diluted with 4.3 ml 65% sulfuric acid (7). The fluorescence was measured with a Turner model 110 fluorometer using a primary filter combination of Kodak Wratten filters 2A and 47B having a transmission peak at 436 mμ, and a 2A-12 secondary filter which passed wavelengths greater than 510 mμ(9). A 10% neutral density filter was placed over the secondary filter to improve linearity between estrogen concentration and fluorescence (7). Estrogen equivalents in the samples were determined from similarly run standard curves. In instances where radioactivity was detected in the estradiol-17α fraction corrections for fluorometric readings were made. Within the range of standard concentrations most often measured (about 0.4 μg) the average estradiol-17α:estradiol-17β fluorescence ratio was found to be 1.9. The following equation therefore was used to redistribute the fluorometric values correctly:

2.9X = initial estradiol-17α fluorometer reading
X = estradiol-17β contaminant reading
1.9X = actual estradiol-17α reading

Results and discussion. Chromatography of the unsubstituted forms and methyl ether and acetylated derivatives led to the conclusion that estrone, estradiol-17α, estradiol-17β and estriol were present in dog plasma in their free forms. One- and two-dimensional thin-layer chromatography of unsubstituted extract and in several instances the presence of an estrogen in the sample extracts which corresponded exactly with an oxidative product (very likely 16-hydroxyestrone) of pure 16-epiestriol gave strong indications of the presence of 16-epiestriol also. Though not all of the derivate tests were conclusive, the very fact that spots were obtained gave support of identity since the estrogens already had been purified with paper chromatography. These results are in agreement with those of Siegel *et al*(10) who found evidence of estrone, esradiol-17α, estradiol-17β, estriol and other 16-oxygenated estrogens in the dog. No differences in qualitative estrogen production between breeds was detected.

Fluorogenic material which was present in most sample extracts and visible under short UV (2537Å) was avoided wherever possible. In some cases interference with fluorometric readings was obvious and these values were discarded. Recovery of the tracers was very low: an average of 16.4% for estrone-C^{14} and 31.6% for estradiol-17β-C^{14}. The low recoveries most likely were due to impurities which affected spotting efficiency and also distorted the normal migration of the estrogens enough so that their distribution pattern did not match that of the standards. The extraction procedure efficiency was better than 90% and that of the elution procedure between 85% and 95%.

Because of the limited number of subjects used in the study and the differences in estrogen levels between individuals, the possibility of breed variations in levels could not be examined. Therefore, the quantitative data from both beagles and basenjis were pooled. Though the estrous cycle of the dog covers approximately 6 months, for all practical purposes the active period lasts for only about 3 weeks. Therefore, only the active period was examined and for convenience it was divided into three 1-week stages: 1) Early; initial slight bleeding at the vaginal orifice (early proestrus), 2) Middle; full bleeding (late proestrus) and 3) Late; cessation of bleeding (estrus).

Both the individual and combined estrogen levels showed a progressive decline from the early to middle to late part of the active period (Fig. 1). The total estrogen level decline was from 143.0 to 69.2 to 29.6 μg/100 ml. The very high level during early proestrus must be considered with caution since it represents the values from only one sample. However, the value cannot be disregarded since 2 other samples obtained within 5 days after the aforementioned sample approached levels of 130 μg/100 ml each. Due to adherence to the division of the estrous cycle, the latter samples were included in the middle part of the active period. Those values

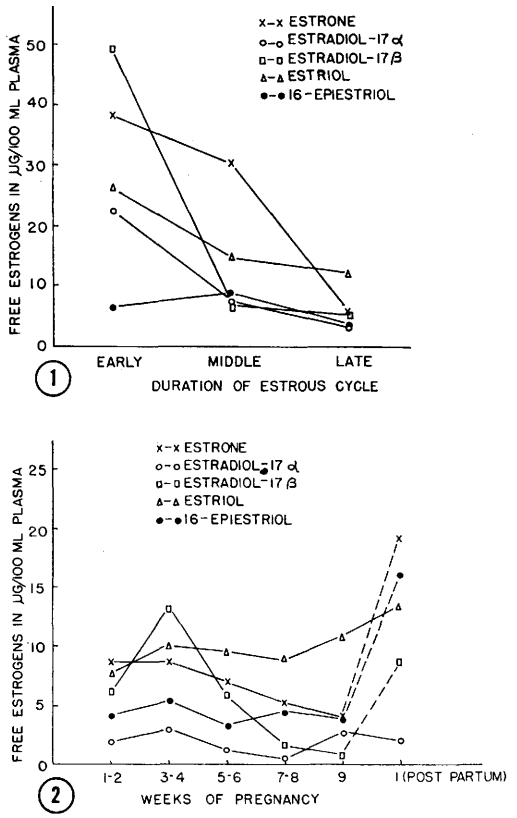

FIG. 1. Individual free estrogen levels in plasma during estrous cycle. The early period of the cycle represents one sample, whereas the middle and late periods are the means of 6 and 2 samples, respectively.

FIG. 2. Biweekly levels of individual free estrogens in plasma during pregnancy and including the 1st week post-partum. Each point represents the mean of 3-7 samples.

during late proestrus are the means of 6 samples each and those of estrus 2 samples each. The trend toward reduction in free estrogen concentration during the cycle is apparent. It is difficult to explain this decline at a time when estrogen levels are supposedly rising. Perhaps the estrogens are being bound at a more rapid rate as the cyle progresses.

Pregnancy presents a somewhat different picture (Fig. 2). From a level of 7.6 μg/100 ml during the first 2-week period of pregnancy estriol showed an overall increase of about 43% by the ninth week. The initial concentrations of 16-epiestriol and estradiol-17*a* were lower and remained fairly constant throughout pregnancy. After peaking during the 3-4-week period estradiol-17β as well as estrone both underwent a continuous decline as pregnancy progressed. This latter observation might be a result of increased metabolism since these two estrogens are by far the most active biologically of the estrogens measured.

When viewed as total free estrogens, the levels were relatively constant throughout gestation except at 3-4 weeks, when a peak nearly 43% greater than the overall concentration was reached. The basal concentration averaged 23.2 μg/100 ml while during the 3-4-week period a level of 40.5 μg/100 ml was attained. The latter period corresponds with the formation of the placenta (21-30 days of pregnancy). Therefore the substantial rise might be attributable to placental activity and an as yet unaccelerated estrogen metabolism. However, the question of whether or not the placenta is a major source of estrogen in the dog is still unanswered.

Except for estradiol-17*a* there is a dramatic increase in all of the estrogen levels during the first week post-partum. This occurrence was wholly unexpected and cannot be explained until the protein-bound and conjugated forms are also measured.

Although Pearlman *et al*(12) and Siegel *et al*(10) reported that estriol was not a major metabolite in the dog, present evidence indicates that of the free estrogens in pregnancy plasma estriol predominates and this is nearly the case also during the estrous cycle. Paschkis and Rakoff(11) have reported that the blood from ovarian and femoral veins of dogs during estrus contains very little estrogenic activity. Since they used a bioassay method to measure the estrogens it is difficult to compare results. Paschkis and Rakoff(11) also stated that the estrogens in the dog blood were for the most part in an esterified form. If this is the case, then the dog must indeed have high concentrations of estrogen in its blood.

Summary. Solvent partitioning, paper and thin-layer chromatography, and fluorometry were employed to isolate, identify and quantitate the free estrone, estradiol-17*a*, estradiol-17β, estriol and 16-epiestriol in dog plasma during the estrous cycle and pregnancy.

There was a continuous decline in both individual and combined estrogen concentrations from early proestrus to late estrus. Throughout pregnancy the levels of estradiol-17α and 16-epiestriol remained fairly constant. Estrone and estradiol-17β attained peak levels at 3-4 weeks and declined thereafter. Estriol was the major metabolite and by the last week of pregnancy showed a 43% increase over the initial level. All of the estrogens except estradiol-17α rose substantially during the first week post-partum.

1. Slaunwhite, W. R., Jr., Sandberg, A. A., Proc. Soc. Exp. Biol. and Med., 1959, v101, 544.

2. Roy, E. J., Brown, J. R., J. Endocrinol., 1960, v21, 9.

3. Preedy, J. R. K., Aitken, E. H., J. Biol. Chem., 1961, v236, 1300.

4. Roy, E. J., Mackay, R., J. Obstet. Gynaec. Brit. Cwlth., 1962, v69, 13.

5. Roy, E. J., Harkness, R. A., Kerr, M. G., J. Endocrinol., 1965, v31, 177.

6. Veenhuizen, E. L., Erb, R. E., Gorski, J., J. Dairy Sci., 1960, v43, 270.

7. Mellin, T., personal communication, 1964.

8. Bush, I. E., The Chromatography of Steroids, Pergamon Press, New York, 1961.

9. McAnally, J. S., Hausman, E. R., J. Lab. Clin. Med., 1954, v44, 647.

10. Siegel, E. T., Dorfman, R. I., Brodey, R. S., Friedman, M. H. F., Proc. Soc. Exp. Biol. and Med., 1962, v111, 533.

11. Paschkis, K. E., Rakoff, A. E., Recent Progr. Hormone Res., 1950, v5, 115.

12. Pearlman, W. H., Rakoff, A. E., Paschkis, K. E., Cantarow, A., Walkling, A., J. Biol. Chem., 1948, v173, 175.

Received September 9, 1965. P.S.E.B.M., 1966, v121.

Multiple Molecular Species of Interferons of Mouse and of Rabbit Origin. (30783)

G. P. LAMPSON, A. A. TYTELL, M. M. NEMES AND M. R. HILLEMAN
Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pa.

Interferon(1-4) is a term which is often applied, collectively, to a variety of viral inhibitory substances of protein composition that are commonly formed by cells in response to stimulation by viruses and by other microbes or their components. Interferons commonly appear in the blood and tissues of animals and man in the course of viral and other infections and appear to provide a mechanism for recovery from viral disease which is separate and distinct from the specific immunological mechanisms. Exogenous interferons, administered prophylactically or therapeutically, presently show little promise in clinical medicine for preventing or treating viral disease. On the contrary, stimulation of endogenous interferon in the host itself by appropriate substances may contribute significantly to enhancement of host defenses. This emphasizes the need to characterize and to classify interferons, and to study their mechanisms for and mode of induction as a background to eventual application of the interference phenomenon to clinical medicine.

Investigations in this laboratory led to the first preparation of highly purified chick embryo interferon and to characterization of chick interferon induced by both RNA and DNA viruses(5,6). Continuing studies have been made of interferons induced in cells or animals of other species. The present report describes the characterization of interferons induced in mice, in mouse cell culture, and in rabbit cell culture by Newcastle Disease Virus (NDV). These studies are of significance in that they demonstrate 2 distinct kinds of interferon in mouse serum and different molecules with interferon activity in mouse and rabbit cell cultures infected with NDV virus.

Materials and methods. In vitro *assay for interferon activity.* The method was a modification of the procedure described previously(6). Serial 2-fold dilutions of the interferon sample were diluted in Eagle's medium containing 5% agamma calf serum and antibiotics and 1 ml amounts were added in quadruplicate to roller tube cultures of mouse