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J. H. LONGWELL, *Director*

Spectrophotofluorometric Determination of Estrogens in the Urine and Feces of Cows During Different Stages of Pregnancy

TAWFIK EL-ATTAR AND C. W. TURNER

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SUMMARY

1. A spectrophotofluorometric method has been developed for determining the total amount of estrogen excreted daily in the urine and feces of cattle.

2. Twenty-four hour urine and feces samples were collected. Out of these, 100 ml. of urine and one kilogram of fresh feces were processed for estrogen determination.

3. To evaluate the new technique developed, known amounts of estradiol and estrone were added to urine and feces collected from a sterile cow. The average recovery percentage was 98 percent in urine and 94 percent in the feces.

4. The urine and feces samples were collected simultaneously from 20 cows of different breeds, ages, body weight and stages of pregnancy.

5. The fecal and urinary estrogen were low until about 170 days of pregnancy, then markedly greater.

6. The total estrogen excretion varied greatly among individual cows at almost identical stages of pregnancy. This individual variation increased with advanced stages of pregnancy.

7. The average total (urine and feces) estrogen excretion per 24 hours was 4.49 mg. (calculated as estradiol) in four cows pregnant 91 to 109 days, 5.56 mg. in five cows pregnant 120 to 170 days, 19.11 mg. in six cows pregnant 185 to 234 days, and 20.13 mg. in five cows pregnant 248 to 273 days.

8. The average daily estrogen excretion per 100 pounds of body weight was 417 ug. in the first group of cows, 537 ug. in the second group, 1380 ug. in the third group and 1427 ug. in the fourth group.

9. In 14 cows the fecal estrogen excretion was at higher levels than urinary estrogen, while in six cows, pregnant more than six months, the urinary estrogen exceeded the fecal estrogen.

10. The major estrogen in cows' urine and feces was the non-ketonic phenolic steroid (calculated as estradiol).

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INTRODUCTION

It has been shown in experimental animals, goats and cows that mammary gland growth and initiation of lactation can be induced by the ovarian hormones, estrogen and progesterone. An estimate of the amount of progesterone required for mammary gland growth in cows was made by removing the corpus luteum of pregnancy and substituting for it graded doses of progesterone sufficient to maintain pregnancy (McDonald *et al.,* 1952). Investigators have had to base the ratio of estrogen to progesterone given to cows to stimulate udder growth on the optimal ratio of these two hormones stimulating mammary gland growth in certain small experimental animals, since the total amount of estrogen secreted by cows during pregnancy had not been determined previously (Turner *et al.,* 1956). Estimation of the total excretion of estrogen during the first half to two-thirds of pregnancy would show the approximate amounts of these hormones required for mammary gland growth.

One means of estimating the secretory rate of estrogen is to determine the rate at which estrogens are eliminated in the excreta. Other methods involve determining the amount of estrogen in the ovaries, in blood or in the placenta of pregnancy. Estimation of estrogen secretion by attempting to determine the total estrogen excretion in the urine and feces seemed the most feasible.

Many biological and chemical methods have been used to make quantitative determinations of the estrogen excreted. In dealing with the bioassay of impure extracts unknown substances occur that may augment or inhibit the activity of contained estrogen (Pincus, 1943; 1950). Another consideration in urine and fecal extracts is the presence of at least two different estrogens of different biological activity.

Estrogens are eliminated from the body in biologically active and inactive forms thus necessitating chemical methods for their determination since bioassay methods permit determination of active forms only. When the bioassay and chemical analysis of estrogens excreted by the human female were compared, it

was stated; "Considering all points and counter points, the balance is strongly in favor of chemical estimation" (Borth and Watteville, 1952).

Since no reports were found in the literature in which the total estrogenic excretion of the cow had been determined, the present study was undertaken to develop a physicochemical method to determine quantitatively and simultaneously the total amounts of estrogens excreted by cows in the urine and feces during different stages of pregnancy. Such data will aid in determining the amounts of estrogen which when given with progesterone, in optimal ratios, to non-pregnant heifers, will stimulate mammary gland growth equal to that of normal pregnancy.

REVIEW OF LITERATURE

The Isolation of Estrogens

The ovary, like the testis, has both an endocrine and a gametogenic function. The removal of the ovaries of young animals was shown to change their nature and to cause involution of the reproductive tract and secondary sex characters. Transplantation of ovarian tissue into ovariectomized animals caused a restoration of the secondary sex characters.

Many attempts were made to extract ovaries and secure the biologically active substance. Iscovesco (1912) and Frank (1915) were the first to demonstrate that the active extracts of ovarian tissue were lipid-soluble rather than water-soluble. Studies of the changes in the reproductive tract during the various stages of the estrus cycle in the guinea pig by Stockard and Papanicolaou (1917), in the rat by Long and Evans (1922) and in the mouse by Allen (1922) set the stage for the rapid progress in the study of the ovarian hormones.

These workers observed, in rats and mice, that a striking growth of the vaginal epithelium occurred during proestrus and estrus, followed by sloughing of the new growth following ovulation. It was shown, further, that the vaginal and ovarian changes were reflected in the cell content of the vaginal smears, which was the basis of the Allen-Doisy (1923) assay for estrogens and the second important contribution after that of Iscovesco (1912) and Frank (1915).

Finally, there was Aschheim and Zondek's (1927) discovery that the urine of pregnant women, especially in late pregnancy, was an extremely rich source of estrogenic substances. This culminated, within a year and a half, in the almost simultaneous annoucement by Butenandt (1929) and Doisy *et al.* (1930) of the isolation of crystalline estrone. This was soon followed by the isolation of estriol and finally estradiol-17β. In addition to their presence in human pregnancy urine, estrogens have been found in pregnant cow's and mare's urine, stallion's urine, placental tissue, ovarian follicular fluid, adrenal tissue, and testicular tissue. Equilin and equilenin have been isolated from mare's urine. The isolation of these natural estrogens from various sources is indicated in Table 1.

TABLE 1. ISOLATION OF CRYSTALLINE NATURAL ESTROGENS

Compound	Source	Reference
17B - Estradiol	Ovary - sow	MacCorquodale et al. (1935)
($C_{18}H_{24}O_2$)	Testis - stallion	Beall (1940)
	Testis - human	Goldzieher & Roberts (1952)
	Placenta - human	Huffman et al. (1940)
	Urine - human - preg.	Smith et al. (1939)
	Urine - human - normal	Engle et al. (1952)
	Urine - mare - preg.	Wintersteiner et al. (1935)
	Urine - stallion	Levin (1945)
	Semen - human	Diczfalusy (1954)
17$^{\alpha}$ - Estradiol	Urine - mare - preg.	Hirschmann and Wintersteiner (1937)
Estrone	Ovary - sow	Westerfeld et al. (1938)
($C_{18}H_{22}O_2$)	Testis - stallion	Beall (1940)
	Placenta - human	Westerfeld et al. (1938)
	Adrenal - bovine	Beall (1939)
	Bile - cow - preg.	Pearlman et al. (1947)
	Urine - human - normal	Engel et al. (1952)
	Urine - human - preg.	Doisy et al. (1929)
		Butenandt (1929)
	Urine - mare - preg.	DeJongh et al. (1931)
	Urine - cow - preg.	Pearlman et al. (1947)
	Urine - human - male	Dingemanse et al. (1938)
	Urine - stallion	Deulofeu et al. (1934)
		Haussler (1934)
	Urine - bull	Marker (1939)
	Urine - steer	Marker (1939)
	Semen - human	Diczfalusy (1954)
Estrone sulfate	Urine - mare - preg.	Schachter & Marrian (1938)
16 - Ketoestrone	Urine - human - normal	Serchi (1953)
Estriol	Placenta - human	Brown & Collip (1931)
($C_{18}H_{24}O_3$)	Urine - human - preg.	Marrian (1930)
		Doisy et al. (1930)
	Urine - human - normal	Engel et al. (1952)
	Semen - human	Diczfalusy (1954)
Estriol glucuronide	Urine - human - preg.	Cohen & Marrian (1935)
Equilen		
($C_{18}H_{20}O_2$)	Urine - mare - preg.	Girard et al. (1932)
Hippulin		
($C_{18}H_{20}O_2$)	Urine - mare - preg.	Girard et al. (1932)
Equilenin		
($C_{18}H_{18}O_2$)	Urine - mare - preg.	Girard et al. (1932)
17B - Dihydro		
equilenin	Urine - mare - preg.	Wintersteiner et al. (1936)
3 - Desoxyequilenin	Urine - mare - preg.	Prelog & Fuhrer (1945)
Δ - 5,7,9 - Estra		
trienon - 3 - one - 17		
($C_{18}H_{22}O_2$)	Urine - mare - preg.	Heard & Hoffman (1941)
Equol (iso - flavan -	Urine - mare - preg.	Marrian & Haselwood (1932)
7:4'diol)	Urine - cow - preg.	Klyne & Wright (1956)
($C_{15}H_{12}O(OH)_2$	Urine - goat - preg.	Klyne & Wright (1956)

Likely Sites of Estrogen Synthesis

The mere fact that estrogens can be isolated from particular organs does not necessarily mean that the organ is capable of synthesizing the estrogens, but it does establish evidence for such a hypothesis. To add to this evidence, investigations have been made of estrogen excretion under various normal and pathological conditions (Doisy *et al.,* 1942; Pearlman and Pearlman, 1944). The results of these investigations have furnished some evidence as to the extent of elaboration of estrogens from the following endocrine glands.

(a) *Ovaries.*—The mature functioning ovary is the primary source of estrogen in the non-pregnant animal. It is stimulated to secretion by the proper proportions of circulating FSH and LH. In 1935, McCorquodale *et al.* isolated estradiol-17β from the liquor folliculi. It was suggested that this estrogen was the primary ovarian hormone. However, in 1938 Westerfeld *et al.* isolated a small amount of estrone. The concentration of estrone was 0.01 mg. and that of estradiol-17β was 0.014 mg./Kg. of sows' ovaries. Since estrone and estradiol are subject to interconversion in the tissue, the question of estradiol-17β as the primary ovarian hormone is less certain.

The ovarian cells secreting estrogen have been shown by histo-chemical studies to be the *theca interna* (rat) (Dempsey and Bassett, 1943) although some believe the granulosa cells participate. Estrogens are also found in the corpus luteum and, indeed, more estrogen seems to be produced during the corpus luteum phase of the cycle than during the follicular phase. It has been suggested that even the interstitial cells of the ovary may be capable of producing estrogens (Hertig, 1944).

It was observed in cattle that more estrogen was present in the follicular fluid of animals in heat than during the luteal stage. There was no definite increase of estrogenic hormone in the fluid from cystic ovaries.

The rate of estrogen secretion by the ovaries of normal animals has been estimated in various ways. In the adult Rhesus monkey (4-5 Kg. body weight) it has been estimated that they secrete the equivalent of 20 mg. of estrone per day. Comparable estimates for women were 300 mg. of estrone daily (Corner, 1940).

To produce estrous behavior in ovariectomized ewes, it was found necessary to administer 64 mg. of estradiol benzoate alone for two days or 22 mg. after pretreatment with 25 mg. of progesterone for 3 days (Robinson, 1955).

(b) *Testes.*—Very small amounts of estrone have been obtained from the urine of men (Dingemanse *et al.,* 1938). Urinary excretion of estrogens is lowered, but not completely absent, in the hypogonadal males according to Koch (1938).

The question of the source of the estrogen in the testes, whether arising from Leydig, Sertoli or germinal cells has not been determined definitely. On

the basis of testicular neoplasms in dogs and man with a diagnosis of Sertoli cell tumor, it was suggested that the Sertoli cells produced the estrogen (Pearlman, 1950). Investigators using chorionic gonadotrophin have stimulated Leydig cell activity and increased estrogen secretion. They have concluded that the Leydig cells secrete both androgen and estrogen (Maddock and Nelson, 1952).

Beall (1940) isolated 0.12 mg./Kg. of 17-β estradiol and 1.36 mg./Kg. of estrone from stallion testes. Ruzicka (1943) estimated a bioassay equivalent of 0.4 mg./Kg. of estrone in hog testes while Haines *et al.* (1948) obtained an estrone equivalent of 0.67 mg./Kg.

(c) *The Placenta.*—Present evidence suggests that the placental estrogens are probably produced by the syncytium (Wislocki and Dempsey, 1948). Birefringent sudanophilic lipid droplets are abundant in the syncytium throughout gestation. These droplets are acetone-soluble, react with phenylhydrazine, give a positive Schiff reaction, and exhibit yellowish-green autofluorescence, thus fulfilling available criteria for ketosteroids.

The human placenta has been shown to contain estriol, estrone, and estradiol-17β. The first of these compounds to be isolated was estriol (Butenandt and Brown, 1933); later estrone was isolated (Westerfeld *et al.,* 1938) and finally estradiol-17β was isolated by Huffman *et al.* (1940). They recovered the following amounts of these estrogens: 140 mg./Kg. of estriol, 35 ug./Kg. of estrone, and 38 ug./Kg. of estradiol-17β.

In addition to "free" estrogens in the placenta, there are "conjugated" estrogens. "Protein-bound" estrogens have been observed in the blood and placenta. A study of these various forms of estrogens was reported by Diczfalusy (1953) in early human and full term placentas.

Estriol was isolated from the meconium, the contents of the intestinal tract of the fetus and the new born. Because of the character of the meconium, the most likely source of the estriol which it contains is the bile. Therefore, the isolation of estriol from the meconium affords evidence for the biliary excretion of this estrogen in the human (Kinsella *et al.,* 1956). The finding that estriol is the principal estrogen present in meconium is similar to the findings indicating the relative predominance of estriol in pregnancy urine (Kinsella *et al.,* 1956).

The fetal membranes of horses, cattle and sheep (Beck, 1950) are reported to contain estrogen. This is true in both the embryonic and maternal portions.

(d) *The Adrenal Cortex.*—Estrone has been isolated from the adrenal cortex. It is probable that the small amounts of estrogens excreted by ovariectomized women originate in this organ. In rats spayed early in life the vagina opens later than in normal animals. In adrenalectomized spayed rats, the vagina opens much later or not at all (Parkes, 1945). In certain inbred strains of mice, early ovariectomy is followed by adrenal hyperplasia and tumors. Production of esrogen by these adrenal tumors is evidenced by the development of estrus changes in the

vagina and by stimulation of growth of mammary cancer (Parkes, 1945).

The ovaries and placenta are generally regarded as the chief sources of estrogen in the organism. The testes and adrenals appear to produce much smaller quantities, although stallion testicular tissue appears to be a prodigious producer of estrogen (Pearlman, 1950).

Estrogens were also found in the semen. Diczfalusy (1954) was the first to determine the nature of the estrogens. In 1000 ml. of human semen he calculated that there were 60 ug. of estrone, 10 ug. of estradiol-17β, and 30 ug. of estriol. The estrogens were predominantly in the free form.

Another possible source of estrogens is the metabolic transformation of other steroid hormones, especially androgen, into estrogens (Pearlman, 1950). Paschkis and Rakoff (1950) attempted to demonstrate a biological transformation of androgens into estrogens. They detected the excretion of estrogenic material in the bile and urine of female dogs following the administration of androgens.

When testosterone propionate was administered to two oophorectomized adrenalectomized women with metastatic breast cancer, estrone and estradiol-17β were identified in the urine. Neither could be identified in the control urines when the patients received no testosterone (West *et al.*, 1956).

Baggett and Engel (1956) isolated C^{14}-labeled estradiol-17β following the incubation of testosterone-3-C^{14} with human ovarian slices.

Blood Estrogens

The estrogens of the blood may come from the ovary or testes, from the adrenal cortex, and from the placenta in pregnant animals. The estrogens may be, in part, in a very labile ketonic form which is readily destroyed by ordinary methods of extraction. It is also known that a portion of the estrogen in the blood stream is in the free form, while 50 to 75 percent is closely bound in some way to plasma protein, probably beta globulin ("estroprotein"). The liver is suggested as the site of combination of the estroprotein.

It is suggested that estroprotein, the natural circulating estrogen, is constituted of estrogen linked to serum protein in an esterified or glucuronide form. The liver plays an important role in its formation. Since estroprotein is in a hydrophilic conjugated form, readily dissociable, it is available for action at the target tissue.

Blood from the ovarian veins of dogs following stimulation with gonadotropin contains little estrogen. On the other hand, the spermatic vein blood of the stallion has an estrogen concentration ten to twenty times as high as that in the general circulation (Paschkis and Rakoff, 1950).

The estrogens appear to be present in human and cattle blood in extremely small concentration, of the order of 3 to 8 ug. per liter of whole blood (Szego and Roberts, 1946). Intravenously injected estrogen disappears rapidly from the

blood stream (Paschkis and Rakoff, 1950; Pearlman and Pearlman, 1944; Pincus and Pearlman, 1943). Whatever the explanation for these phenomena, it is evident that the concentration of estrogen arriving at the target organs must be at a low level.

Urinary Estrogens

Estrogens are excreted in the human urine chiefly in conjugated form, as glucuronides and sulfates of estriol, estrone, and estradiol. Other estrogenic compounds are also excreted in certain species, e.g. hippulin, equilin, equilenin, dihydroequilenin-17α, and estradiol-17α in the urine of the pregnant mare. Equol (iso-flavan-7:4'-diol) was isolated from cow's urine (Klyne and Wright, 1956).

Urinary Estrogens in Women.—During the reproductive years, urinary estrogen follows a cyclic pattern. In the early follicular phase, the amount is low, rising progressively to an initial peak at about the time of ovulation. The estrogen then drops moderately for a few days and increases again as the corpus luteum becomes well established, after which there is a rather abrupt drop during the premenstrual period. The first peak is usually somewhat higher than the second. It has been estimated that the ovary secretes about 10 mg. of estrogen, in terms of estrone, during a normal cycle (Paschkis and Rakoff, 1950).

The increase in urinary estrogen during the early weeks of pregnancy is probably of ovarian origin. It has been suggested that the placenta does not contribute to the estrogen level until after the 60th day. However, after the 100th day, the slowly rising secretion of estrogen is largely of placental origin. After the 140th day the rise in estrogen is progressive until near term when 50.00 mg. /day may be present in the urine. In some cases, estrogens may fall slightly before parturition, whereas in other cases they continue to rise until parturition. After removal of the placenta, estrogen decreases rapidly to non-pregnant levels.

Estrogens in Cattle Urine.—The amount of urinary estrogens in mature cyclic cattle is very low (Cole, 1950). It might be expected to increase slightly at estrus. Ascheim and Zondek (1927) were the first to report the presence of estrogen in the urine of cows. Hisaw and Meyer (1929) reported the presence of estrogen in the urine of pregnant cows.

By extracting urine with olive oil without previous hydrolysis, Turner *et al.* (1930) found 11 rat units per liter in non-pregnant cow urine and Nibler and Turner (1929) found none in one sample of urine. During the first 100 days of pregnancy, the estrogenic activity of cow urine is very low; a steady increase from this time to term has been reported by all studying the problem (Anderson, 1934; Hisaw and Meyer, 1929; Nibler and Turner, 1929; Turner *et al.,* 1930).

The daily excretion values near the end of pregnancy reported by these workers varied from as low as 500 rat units (Nibler and Turner, 1929) to 6,000 rat units (Hisaw and Meyer, 1929) when urine was extracted without hydrolysis.

The peak excretion reported by hydrolyzed urine was 17,000 mouse units per liter (Barrie *et al.,* 1935). Turner *et al.* (1930) reported that Holstein-Friesian cows excreted more hormone than Jerseys and dairy breeds more than the beef breeds.

Smith *et al.* (1956) determined the urinary estrogens excreted by the cow during heat and different stages of pregnancy by a chemical method. The average total urinary estrogen excretion per 100 pounds of live weight produced a fluorescence equal to that produced by 162 micrograms of estrone on the day of heat, 248 micrograms on the seventh day after heat, and 302 micrograms on the fourteenth day after heat. The total daily urinary estrogen excretion per 100 pounds of live weight on the fiftieth and 275th day of gestation produced a fluorescence equal to that produced by 46 and 580 micrograms of estrone, respectively; on the eleventh day after parturition, estrogen excretion produced a fluorescence equal to that produced by 94 micrograms of estrone.

The primary follicular estrogen in the cow has not been positively identified (Woods, 1950); Westerfeld and Doisy (1936) stated that the estrogen in cow ovaries was entirely non-ketonic. Estrone has been isolated from cow adrenals (Beall, 1939), the bile of pregnant cows (Pearlman *et al.,* 1947), and the urine of the steer (Marker, 1939) and the bull (Marker, 1939).

Klyne and Wright (1956) isolated estrone, estradiol 17α and equol (isoflavan-7:4' diol) $C_{15}H_{12}O$ $(OH)_2$ from pregnant cows' urine. Marrian and Haselwood (1932) isolated equol from the ketohydroxyestrin fraction of pregnant mares' urine and reported that equol has no estrogenic activity. Equol is probably a metabolite of the flavonoid compounds that occur as pigments in plants. The estrogenic effect of these compounds have been demonstrated by Cheng *et al.* (1955).

The flavonoids have a skeleton consisting essentially of a benzenoid ring fused to a α-pyrone ring (Figure 1), which may be completely or partially reduced, and to which is attached another benzenoid ring at the carbon atom adjacent to the ring oxygen. Isoflavones differ from flavones in that the benzenoid ring is attached at carbon 3 instead of carbon 2.

It has been proven (Pope and Wright, 1954) that both subterranean and red clover contain three isoflavones; genistein, biochanin A and formononetin. Genistin (a glycoside of genistein) and daidzin (a glycoside of daidzein) have been isolated from soybean oil meal.

Genistin and genistein are about equal in activity. Genistein is estimated as equivalent to $^1/_{5000}$ the activity of diethylstilbestrol. Daidzein, a synthetic compound, is the most active; genistein and biochanin A have equal activity; and formononetin has the least. Biochanin A isolated from red clover and genistein had about equal estrogenic activity (Cheng *et al.,* 1953).

Asdell *et al.* (1945) attempted to assay the urine of heifers in heat by a bio-

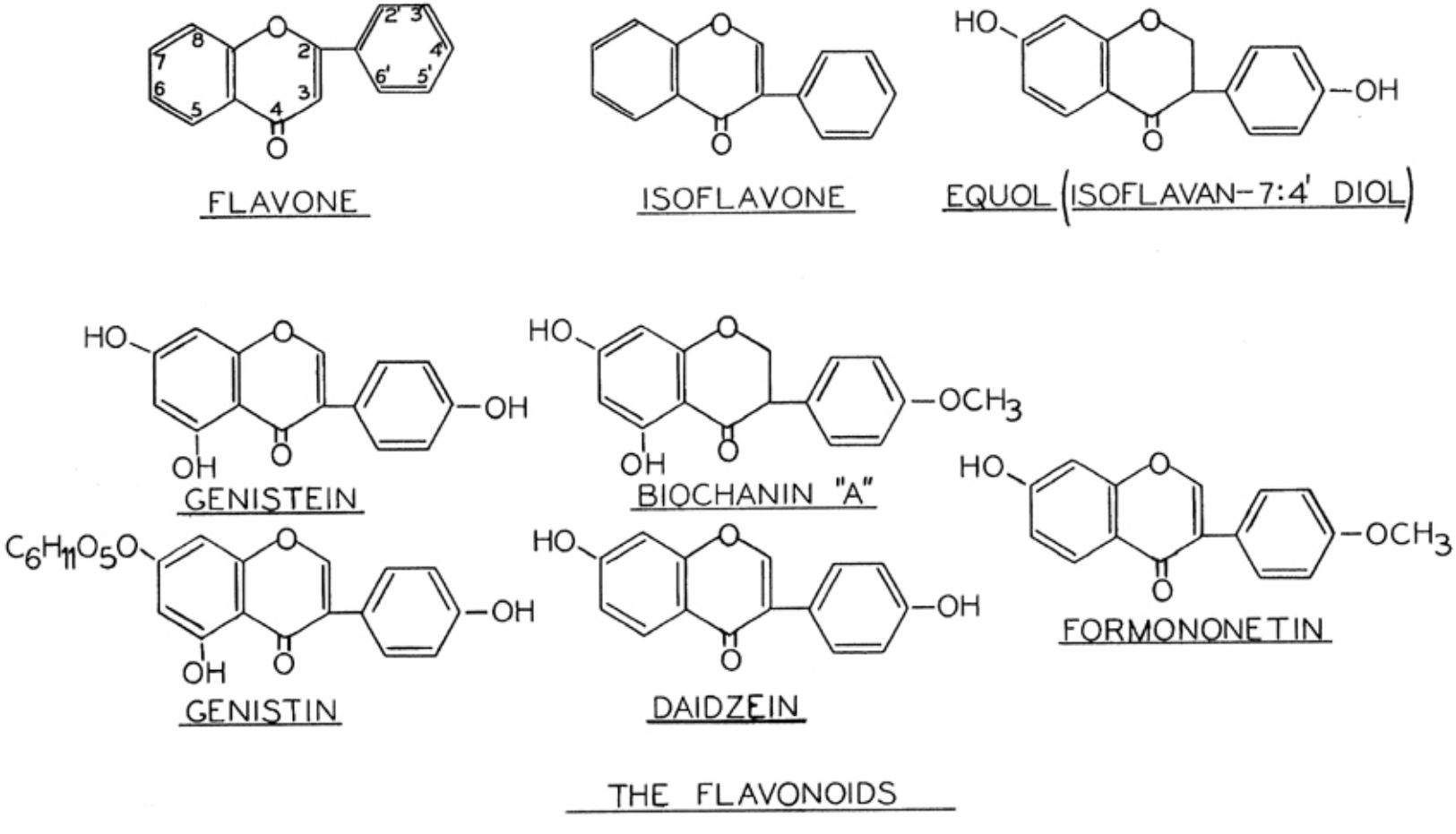


Fig. 1—The molecular structure of equol (isoflavin-7:4' diol) which was isolated from pregnant mare's, goat's and cow's urine resembles greatly that of plant estrogens (flavonoids). It is suggested that plant estrogens are metabolized within the animal's body and excreted as equol.

assay method sensitive to 18 rat units per liter. The results were negative in all cases. The urine of heifers which were injected with 116.6 ug. of estradiol benzoate daily was also negative. The average heat-producing dose of estrogen, when administered to dairy heifers two to three years old, was 100 ± 6 ug. estradiol benzoate per day for three days. This is in comparison to 50 ug. of estradiol benzoate for the induction of heat in the anestrus ewe (Beall, 1940; Cole and Miller, 1935).

Full estrus was induced in ovariectomized cows weighing 1000 to 1200 pounds with a single injection of 0.3 to 0.4 mg. of estradiol benzoate. A Guernsey weighing 750 pounds responded to 0.2 mg. Estrous behavior was blocked in cows receiving 30 mg. or more of progesterone simultaneously with 0.4 mg. of estrogen. Ovariectomized cows (1000 to 1200-lb.) were brought into full estrus when injected with a priming dose of estrogen (0.2 mg.) and 1 mg. of progesterone. Three cows were given 0.4, 0.8, and 1.6 mg. of estradiol benzoate daily for three successive periods of five days each. The reaction of the vulva and the secretion of mucus were often independent of the degree of estrus (Melampy *et al.*, 1956).

It is of interest to note that for both the follicular stimulating hormone content of the pituitary and the length of the preovulatory or heat periods, the descending order of the excretion of estrogens per liter of urine at ovulation appears to be horse, man, hog, and cow. Thus, there may be a general relationship between the FSH content of the pituitary, the estrogen requirement and excretion, and the length of heat period (Woods, 1950.)

El-Attar (1955) studied the urinary estrogen excretion in the Egyptian buffalo by a physico-chemical method. Estradiol and estrone were found and determined as estrone, while estriol was absent. Estrone was first detected during the third month of gestation at an average value of 37.5 ug. per liter of urine. Maximum estrogen excretion was 340.6 ug. per liter of urine at the eighth month of pregnancy.

Estrogens in Urine of Sheep and Goats.—Estrogen has been observed in the urine of ewes during the mid-cycle as well as the estrous period. The equivalent of 1.75 I.U. of estrone in 24-hour urine samples have been extracted (Bassett and Sewell, 1951). No estrogens were found in the urine from anestrous ewes (Bassett *et al.*, 1955).

In pregnant ewes little or no estrogen is excreted in the urine until the last three or four weeks of pregnancy (Whitten, 1943). The range of values excreted per 24 hours at that time was: estradiol $<$0.2-3 ug., estriol 2.0 ug. (Bassett *et al.*, 1955). About ½ of the estrogen was in the free state.

Klyne and Wright (1956) isolated estrone (0.4 mg./1.), estradiol-17α (0.1 mg./1.), and equol (10 mg./1.) from pregnant goats' urine.

Estrogens in Sows' Urine.—In the sow, the estrogen is present in the urine for

a short period in early pregnancy, approximately the twentieth to the thirtieth day (Roth, Mayer and Bogart, 1949). Bredeck (1956) showed that the estrogenic steroids found in the urine of the sow during pregnancy were estrone and estriol. Estrone may or may not be excreted early in gestation (during the third to the fifth week). Estrone is excreted during the last fourth of the gestation period and reaches a maximum around the fifteenth week. Estriol excretion, though possibly reaching a minor peak around the third week of pregnancy, reaches its maximum concentration in the urine around the eleventh week.

Urinary Estrogens in Horses.—In the non-pregnant mare there are two peaks of urinary estrogen excretion, the first during estrus and the second between the tenth and fifteenth day of the cycle (Mayer *et al.*, 1940). Glud *et al.* (1933) estimated the presence of 5000 m.u./1. in urine of non-pregnant mares.

The urine of pregnant mares is a rich source of estrogen (Zondek, 1934). This finding is confirmed by Hart and Cole (1934), Kober (1935), Cole and Saunders (1935), Beall and Edson (1936), Edson and Heard (1939) and Lozinski *et al.* (1942).

Dow and Allen (1949) determined by a chemical method the urinary estrone excretion throughout the entire gestation period of a single mare. Comparisons have been made between the estrone values for this mare and for mares at the same stage of pregnancy. The concentration of urinary estrone for a single pregnant mare rose rapidly from the low value of 2.2 mg./1. at the eighty-fifth day to an average maximum value of 170 mg./1. between the 192nd and 235th days. Thereafter it declined steadily to a value of 24 mg./1. on the day of parturition. Great variations exist in the concentration of estrone excreted by different mares at identical stages of pregnancy. The maximum production of estrone apparently does not always occur at the same time in different mares.

In the mare, the estrogen level does not increase appreciably until the third or fourth month of pregnancy. Hence the estrogen content of urine is of no diagnostic value for pregnancy until some two months after the jump of gonadotrophins in the blood. The estrogens reach a peak of 50 to 200 mg./1. of urine about the seventh month of pregnancy and then gradually fall off at term (Bates, 1953; Lozinski, 1953).

Zondek (1934) reported the excretion of large amounts of estrogens in the urine of the stallion. Deulofeu and Ferrari (1934) obtained 1.5 mg. of estrone per liter of urine of the stallion.

In addition to 17α and β-estradiol, estrone and estrone sulfate, the urine of the pregnant mare contains equilen, hippulin, equilenin, 17β-dihydroequilenin, 3-desoxyequilenin, and Δ5,7,9 estratrienol-3-one-17 (Table 1). Levin (1945) isolated estrone and 17α-estradiol from stallion urine. There appears to be great variation in the amounts excreted by individual animals; one sample contained 150,000 r.u./1., of which estradiol accounted for 90 percent of the activity. A

second sample assayed 57,000 r.u./1. and another had only 4,400 r.u./1.

Estrogens in Bile

A very high excretion of estrogen in the bile of dogs and humans of both endogenous and exogenous origin has been observed in some cases (Paschkis and Rakoff, 1950; Pearlman and Pearlman, 1944; Pincus and Pearlman, 1943). For example, as much as 90 to 95 percent of the biological activity was recovered in the bile of dogs following the intravenous injection of either estrone or α-estradiol (Pearlman and Pearlman, 1944). On the other hand, the excretion of large amounts of estrogen in the bile was not observed by Longwell and McKee (1942) who recovered only 1.3 to 8.0 percent of the biological activity in the bile following subcutaneous injection of estrone in dogs.

Pearlman *et al.* (1947) examined the bile from pregnant cows for estrogen content. They concluded from their work that the major estrogen in bile was estrone and that most of the estrogenic material of the bile was present in a free or uncombined form.

Beck (1950) observed estrogenic activity in the bile of ewes in late pregnancy. The estrogen was largely conjugated.

Fecal Excretion of Estrogens

The apparent importance of the biliary excretion of estrogens suggests that fecal as well as urinary excretion of estrogens should take place. The fecal excretion of estrogen, like the urinary excretion, is markedly increased during pregnancy (Heard and Saffran, 1949; Levin, 1945). Unfortunately, variation in techniques of extraction and bioassay makes it very difficult to estimate the actual quantities excreted.

Since the early work was carried out, the study of the fecal excretion of estrogen has been largely neglected and urinary excretion has been studied almost exclusively. In 1945, however, Levin made a systematic study of the quantity and chemical nature of the estrogens in pregnant cow feces. He observed that during the last two weeks of pregnancy, the cow excreted (apparently in unconjugated form) 5,000-10,000 I.U. of estrogen per kilogram of fecal solids. Partition studies concentrated the major portion (73-96 percent) of the activity in the alpha-estradiol fraction, where this activity is equivalent to 0.9-1.4 mg. of alpha-estradiol per kilogram of fecal solids. This is surprising because the greater portion of the estrogenic activity in cows' bile is apparently present as estrone (Pearlman *et al.,* 1947). The predominance of alpha-estradiol in the feces has been attributed by Levin (1945) to reduction by intestinal bacteria. Levin also noticed that as a result of drying the fecal specimens in air, at least a portion of the estradiol was converted to a ketonic estrogen, presumably estrone. Coincidentally, a considerable proportion of the extractable active material is lost.

The work of Pearlman *et al.* (1948) has confirmed Levin's observation that fecal estrogen is present mainly in "free" form.

After the administration of estrogen to humans or to experimental animals, negligible estrogenic activity was recovered from the feces. Dingemanse *et al.* (1938) injected 2.5 mg. of estrone into rats and recovered approximately 3 percent of the administered activity in the feces, with recovery in the urine being somewhat lower.

Heard and Saffran (1949) found that ten hours after the subcutaneous injection of I^{131}-labeled iodoestradiol in mice, approximately 35 percent of the injected radio activity was found in the feces. These authors accounted for the low recovery of estrogenic activity from feces as possibly due to incomplete extraction, and estrogen metabolites in the feces void of biological activity. Many investigators have postulated the degradation of estrone and alpha-estradiol to ring D acids. All recoveries reported in the literature have been calculated from the results of bioassay and few attempts have been made to isolate metabolites from feces (Heard and Saffran, 1949; Paschkis and Rakoff, 1950; Pincus and Pearlman, 1943).

To determine, in part, the chemical nature of the I^{131} in feces, Heard and Saffran (1949) acidified the sodium hydroxide extract of feces and partitioned between ether and water. Approximately 60 percent of the radioactive iodine was found to be ether-soluble. The ether-soluble fraction was further extracted with 10 percent sodium carbonate and then with 1N sodium hydroxide solution. Model experiments with radioactive iodoestradiol had indicated the 1N sodium hydroxide removed approximately 80 percent of iodoestradiol from ether solution, but that no appreciable amount (about 5 percent) passed from ether into 10 percent sodium carbonate solution. The ether-soluble I^{131} in the feces passed quantitatively from ether into alkaline solution, the distribution being 60 percent in sodium carbonate and 40 percent in sodium hydroxide solution. These results suggest the presence, in feces, of acidic metabolites of iodoestradiol. These workers postulated that they may be estriol-like substances, or more probably, carboxyl-containing substances.

The nature of the water-soluble I^{131} has not been determined. This may consist of inorganic iodide, water-soluble iodophenols, or of water soluble conjugates of more complex organic iodo compounds. The results of acid hydrolysis of feces do not support the possibility that water soluble conjugates are present.

Beer and Gallagher (1955) administered tracer doses of estrone-16-C^{14} and estradiol-17β 16-C^{14} in six human females. The urine contained the major portion of the metabolites (65%); the fecal excretion was minor in amount. The pattern of radioactivity excretion was similar after either intramuscular or intravenous injection of estradiol-17β-16-C^{14}. The same investigators measured

estrone and estriol by isotopic dilution in the urine of the first day after intravenous administration of estrone-16-C^{14} and estradiol-17β-C^{14} to four of the patients. Estrone accounted for from 8 to 10 percent and estriol from 11 to 31 percent of the total urinary radioactivity. The amount of these metabolites appeared to be similar for either precursor. Recently, Story *et al.* (1957) fed lambs two different levels of stilbesterol. At the 1 mg. level per lamb per day, 51 percent of the stilbesterol appeared in the feces and 25 percent in the urine. At the 2 mg. level, 45 percent appeared in the feces and 39 percent in the urine. Combining both levels of feeding, 80 percent of the fed stilbesterol was recovered in the urine and feces.

Nevertheless, as with the neutral steroid hormones testosterone and hydrocortisone, the contrast between humans and rodents is emphasized in that the fecal route of elimination for all the metabolites of these hormones is the major one in the rodent (Barry *et al.,* 1952; Bradlow *et al.,* 1954). The species difference in the route of elimination very likely reflects different metabolic alterations in the hormones and the relation of these changes to the fundamentals of hormone action remains an important area for investigation.

Estrogens in Milk

Courrier (1930) observed that if daily doses of 80 or 100 r.u. of estrogen were given to a lactating guinea pig, after 3 or 4 days its nurslings showed opening of the vagina and mammary development.

Lacassagne and Nyka (1934) reported that human colostrum in amounts of 1.5 ml. subcutaneously, caused vaginal cornification in spayed mice.

Pope and Roy (1953) extracted the estrogens of the colostrum and normal milk of cows. In one sample it was estimated that 5.2 ug. of estradiol-17β per liter was present, the amount found in pregnancy in the blood of cattle and humans. The estrogens occur mainly as conjugates in the aqueous phase of colostrum and are partly ketonic.

The estrogen in colostrum declines rapidly for no activity could be found in normal milk. Munch (1954) reported the presence of estrogens in milk during the estrus cycle and pregnancy.

To determine the estrogenic activity of milk from pregnant cows Turner (1957) showed that when ovariectomized mice were fed dried milk from cows pregnant less than 100 days their uterine weights were stimulated to an average of 11.35 mg; those fed milk from cows pregnant 100 to 200 days had uterine weights averaging 13.71 mg., and those fed milk from cows pregnant more than 200 days had uterine weights averageing 16.45 mg. From these data it appears that a slight increase occurs in the estrogen content of milk with advancing pregnancy.

Biogenesis

The ovaries of sows have been perfused for 13 to 44 hours with pigs blood containing C^{14}-labeled acetate and gonadotrophin. At the end of the experiment, the ovaries were extracted and estrone, 17β-estradiol, and cholesterol were isolated. These three compounds contained C^{14}, indicating their synthesis from the C^{14}-acetate (Werthessen *et al.,* 1950). Heard *et al.* (1954) administered C^{14}-labeled acetate and cholesterol to the pregnant mare followed by the isolation of estrone, equilin, and equilenin from the urine. After administration of acetate-1-C^{14} definitive evidence for the incorporation of C^{14} into the estrone molecule was found, whereas the administration of cholesterol-4-C^{14} resulted in no C^{14} labeling of the estrone that was synthesized during the experiment. The specific activities of the isolated estrone, equilin and equilenin were 641, 304 and 328, respectively. This suggests that the phenolic ring A of the highly unsaturated estrogens is not synthesized from acetate but rather that the aromatic portion of the nucleus originates from some other source in the body.

When estrone-16-C^{14} was administered intravenously to a pregnant mare in the ninth month of gestation, the urinary equilin and equilenin contained no significant redioactivity. This indicates that equilin and equilenin are not metabolic products of estrone but must arise from an independent biosynthetic mechanism.

It has been shown (Rabinowitz, 1956) that testicular preparations of various species are capable of incorporating acetate-2-C^{14} into testosterone, 17β-estradiol, and cholesterol.

Localization of Estrogens

Albert *et al.* (1949) administered radioactive iodo-17β-estradiol subcutaneously to female mice. Ten to 12 hours later the animals were sacrificed and individual tissues examined for radioactivity. The gastrointestinal tract contained 40 percent of the total I^{131}, with by far the greatest amount being in the contents rather than the walls. The radioactivity increased progressively down the gastrointestinal tract, as indicated by the fact that the duodenum, jejunum, and colon-cecum contained, respectively, 0.5, 3.0, and 4.0 percent of the administered I^{131}. The feces contained about 35 percent of the radioactivity. With respect to tissue concentrations, the thyroid contained the highest amount of radioactivity per milligram, more than 100 times that for the whole body. Other tissues concentrating the radioiodine included the mammary gland, the skin, and the submaxillary glands.

Radioactive dibromoestrone, when injected into rabbits, resulted in 25 percent of the radioactivity being excreted in the feces and 16 percent in the urine. Seventy percent of the steroid appeared in a bile-fistula dog within 5 hours of administration, compared with 4 percent excreted in the urine (Twombly, 1948).

After 17α-methyl-C^{14} estradiol was injected into normal adult male rats the C^{14} metabolites were found primarily in the feces, with a small amount appearing in the urine and no $C^{14}O_2$ appearing in the respiratory air. In rats bearing bile fistulas, the bile contained the major portion of radioactivity (Bocklage *et al.,* 1953; Nicholas *et al.,* 1950).

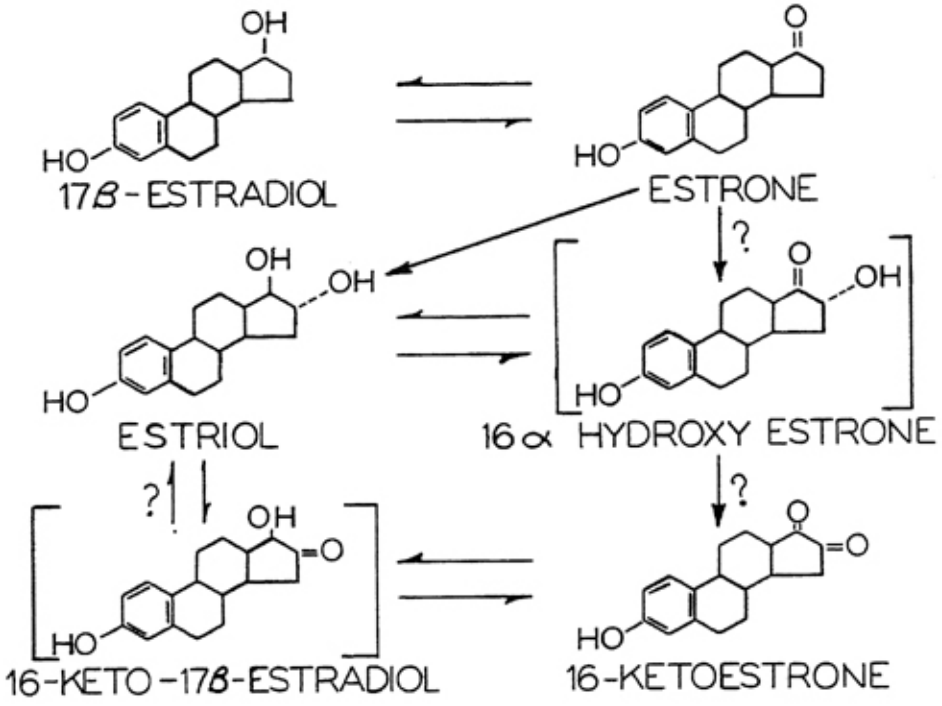
Catabolism of Estrogens

The estrogens probably leave the site of their production, the ovary, the testis, the adrenal cortex and the placenta, in free form and in small amounts, continually. A part of the circulating estrogen occurs in a very labile ketonic form, another portion in a free form, while 50 to 75 percent is bound to plasma protein.

The estrogens are removed from the systemic circulation by the liver (Rakoff *et al.,* 1944). In this again they undergo prompt degradation (Zondek, 1941). When estrogens are incubated with liver slices by liver homogenates, a large proportion of the hormone disappears, apparently being converted to non-estrogenic compounds. This inactivation of estrogens by the liver is due to an enzymatic process. The exact nature of the enzyme system involved is not known, but there is evidence which indicates the importance of dehydrogenases in this connection (DeMeio *et al.,* 1948). It is probable that the process of "inactivation" involves conversion of the original estrogen in part to biologically inactive substances and in part to estrogens of lower activity. The partial conversion of estradiol to estrone by liver slices *in vitro* has been demonstrated (Pearlman and DeMeio, 1949).

It appears more likely that conversion of estrone to 17β-estradiol and the reverse reaction is dependent upon one or two enzyme systems which appear to be present in most tissues. The significant difference among tissues is quantitative rather than qualitative. In other words, a tissue such as the liver perhaps accomplishes these transformations to the greatest extent owing to the fact that this tissue contains the highest enzyme concentrations, as well as to the fact that liver tissue is relatively abundant in the organism.

Despite the fact that estrogens cannot be recovered from the liver, a considerable degree of estrogenic activity is exhibited by bile following injection of estrogen by stimulation of estrogen production by administration of gonadotropin (Cantarow *et al.,* 1942; Longwell and McKee, 1942; Paschkis and Rakoff, 1950). This observation has given rise to the hypothesis that biliary excretion plays an important part in the removal of estrogens from the circulation. It has been suggested that they may subsequently undergo an enterohepatic circulation (Cantarow *et al.,* 1943), being gradually degraded into compounds with no, or lower, estrogenic activity. Small amounts probably escape into the systemic circulation, where they act upon the various target organs and are excreted by the kidney.



METABOLISM OF ESTROGEN IN MAN

Fig. 2—The conversion of 17β-estradiol to estrone and the reverse reaction are dependent upon one or two enzyme systems which appear to be present in most tissues. Of the six steroids listed, four, including 17β-estradiol, estrone, estriol and 16-ketoesterone, have been isolated from tissues and/or urine. The remaining two estrogens listed in the figure, enclosed in brackets, have not been isolated from either human tissue or urine. They are included in the scheme of metabolism on the basis that both steroids are possible theoretical intermediates and since one steroid, 17-keto-17β-estradiol, has been shown to be convertible to estradiol. Some reactions are listed with question marks, since evidence for these conversions is lacking (Dorfman, 1955).

The estrogen metabolism in the human being is represented in Figure 2. Of the six steroids listed, four, including 17β-estradiol, estrone, estriol, and 16-ketoestrone, have been isolated from tissues and/or urine. The remaining two estrogens have not been isolated from either human tissue or urine. They are included in the scheme of metabolism on the basis that both steroids are possible theoretical intermediates and since one steroid, 16-keto-17β-estradiol, has been shown to be convertible to estriol. Some reactions are listed in Figure 2 with question marks, since evidence for these conversions is lacking. Thus, the direct 16α-hydroxylation of either the 17β-hydroxy or 17-ketone is not known; rather, the 16α-hydroxylation and reduction of the 17-ketone is known. The re-

duction of the 16-ketone to the 16α-hydroxyl group is known, but the oxidation of the 16α-hydrozyl group to the 16-ketone is not certain. Conjugation of estrogens is also believed to take place in the liver with the formation of water-soluble glucuronidates and sulfates.

Following injection of estrogens, the hormone normally disappears rapidly from the blood; it appears in the bile promptly and continues to be excreted by this route for several days. Following subcutaneous or intramuscular injection, the highest concentrations in the blood are attained in one to five hours. In the presence of liver damage, as in acute hepatitis or cirrhosis of the liver, the ability of the liver to remove the estrogens from the blood may be impaired (Rakoff *et al.,* 1944), resulting in a prolonged persistence of estrogens in the systemic circulation and manifestations of hyperestrogenism.

Reactions with microorganisms, especially yeast, have been described in the estrogen series. These consist of oxidation of the 17β-hydroxyl group to the 17-ketone group and the reverse (Wettstein, 1939).

The term estronase has been employed by Bischoff *et al.* (1953) to designate an enzyme system which can convert estrone to a more active estrogen, most likely 17β-estradiol. This enzyme has been detected in a variety of tissues.

Assay Methods

Methods of estimation of estrogenic potency can be divided into biological, physical, biochemical, and chemical. The extraction and purification of the natural estrogens in the early days was dependent upon the development of bioassays of three types: (1) Vaginal cornification in spayed rodents (Allen and Doisy, 1923); (2) increase in uterine weight in immature or castrate rodents (Astwood, 1938); and (3) early vaginal opening in immature rodents (Hartman, Littrell and Tom, 1946).

In the reviewer's opinion it is not feasible to measure satisfactorily the daily estrogen output by the use of bioassay methods. The relative potencies of estrone, estradiol-17β and estriol depend not only on the experimental animal used (Pedersen-Bjergaard, 1939; Szego, 1950), but also on the vehicle and the number of injections (Thayer *et al.,* 1944). Furthermore, the potency of a particular estrogen can be greatly enhanced by non-specific "augmenting" substances (Emmens, 1939), or inhibited in certain types of assay (e.g. rat uterine weight method) by the presence of other estrogens (Hisaw *et al.,* 1954; Wicks and Segal, 1956). Moreover, the different estrogens exhibit significantly different potencies when assayed by different bioassay methods. Therefore, without a clean separation of urinary estrogens into at least estrone, estradiol-17β and estriol, bioassays of unfractionated extracts will have only limited significance. On the other hand, the paucity of certain estrogens in a 24-hour specimen will *a priori* exclude the possiblility of statistcally precise bioassays.

Most of the physical and chemical methods depend upon the presence of the phenolic group at position 3, the presence of a ketonic or alcoholic group at position 17. The physical assay has been based on gravimetric (Hughes, 1941), polarographic (Werthessen *et al.,* 1950), ultraviolet absorption photometry (Friedgood *et al.,* 1948), infrared spectrometry (Carol *et al.,* 1948), and radioactive tracer techniques (Leegwater, 1956). The ultraviolet and infrared spectrometry methods require highly purified extracts and are much less sensitive than chemical methods (fluorimetry and colorimetry). Polarographic methods, on the other hand, do not measure all types of estrogen. Radioactive tracer techniques and biochemical methods based on enzymic reactions are very promising, but in their present state of development they are not suitable for routine laboratory assays.

The chemical methods for estrogen determination involves fluorometry (Bates and Cohen, 1950) and colorimetry (Kober, 1931; Brown, 1955; Bauld, 1956). For the time being, chemical methods are the methods of choice.

MATERIALS AND METHODS

Experimental Animals

Five complete 24-hour samples of urine and feces were collected from one cow, No. 585, during the last month of pregnancy (June and July, 1956) for determination of total amounts of estrogen excreted. Four days after the last collection, the cow calved.

Samples of urine and feces were collected from 20 cows of various breeds, ages and stages of pregnancy for estimation of the total amounts of estrogen excreted daily (Table 2).

TABLE 2. DATA ON THE COWS

No. of Cow	Breed	Age			Body Wt.	Days of Gestation	Date of Collection
		Yr.	Mo.	Day			
800	Holstein	2	2	29	1120	91	Feb. 12, 1957
806	Holstein	1	11	10	1050	94	Feb. 11, 1957
497	Holstein	2	4	4	1195	97	Feb. 13, 1957
807	Holstein	1	10	17	945	109	Feb. 13, 1957
804	Holstein	1	12	16	972	120	Feb. 11, 1957
617	Jersey	2	2	29	854	154	Feb. 14, 1956
805	Holstein	1	11	26	1085	160	Feb. 12, 1957
463	Holstein	2	11	24	1300	167	Sept. 11, 1956
458	Holstein	3	1	16	1000	170	Sept. 11, 1956
434	Holstein	3	0	26	1277	185	Sept. 18, 1956
597	Jersey	5	1	28	1200	200	Sept. 18, 1956
435	Holstein	4	4	29	1417	211	Jan. 30, 1957
598	Jersey	5	3	9	1028	215	Jan. 30, 1957
376	Holstein	6	3	27	1614	228	Jan. 29, 1957
3	Brown Swiss	7	6	11	1775	234	Jan. 29, 1957
604	Jersey	7	11	27	1141	248	Jan. 28, 1957
453	Holstein	3	3	5	1490	250	Sept. 22, 1956
450	Holstein	3	4	9	1515	263	Sept. 22, 1956
304	Holstein	9	0	25	1462	271	Jan. 28, 1957
444	Holstein	3	7	2	1445	273	Sept. 22, 1956

Urine and Feces Collection

Urine.—A size 24, Bardex hemostatic catheter with a 75-ml. balloon was used for collecting the urine from mature pregnant cows. The urethral orifice of the cow lies on the floor of the vagina about three to four inches from the entrance of the vulva. It should not be confused with a small blind sac, the suburethral diverticulum, which is located immediately posterior to it. A clean, sterile catheter is lubricated with a mild disinfectant jelly and introduced through the urethral orifice until the balloon lies just inside the neck of the bladder. The balloon is inflated with 50 to 75 ml. of warm water by means of a steel syringe and the inflation tube is sealed with a clamp or with a special valve which is made to fit the nozzle of the syringe. The catheter is then pulled gently so that the bulb fits snugly into the neck of the bladder and prevents any loss of urine around the tube (Figure 3, and 4).

A 3-inch length of ¼-inch diameter glass tube is used as a connection between the catheter and a 3/16-inch bore rubber tube, which drains the urine into a carboy. The tail is tied up by a piece of string around the neck of the cow to prevent its switch from jerking the tube from the carboy.

A complete 24-hour urine specimen was collected without a preservative, cooled overnight, and filtered. The urine sample to be processed was divided into two aliquots of 100 ml. each. To one aliquot was added 50 ug. of each estrogen, estrone and estradiol (Internal standard).

Feces.—Twenty-four hour feces samples were collected at the time of urine collection.

Determination of Estrogen in Cow's Urine

Hydrolysis.—The urine samples were refluxed for 30 minutes at 80° C after adding 5 percent by volume of concentrated sulfuric acid.

Separation of the Acid Fraction.—The cooled solutions were extracted three times with the same volume of ether, interfacial solid residues being discarded after vigorous shaking of the combined extracts. The ether solutions were submitted to the following procedures: (a) Washed with 100 ml. of concentrated carbonate solution (pH 10.5 approx.), which was then discarded; (b) shaken with 25 ml. of 2N-NaOH; (c) the aqueous phase, without removal from the separatory funnel, was partially neutralized to pH 9.5 with 100 ml. of M-$NaHCO_3$; the ether and the aqueous phases were again shaken and the aqueous phase was discarded; (d) washed with 25 ml. M-$NaHCO_3$, and then four times with 10 ml. of water. The ether solutions were evaporated and the residue dissolved in a mixture of 100 ml. of toluene and 25 ml. of N-sodium hydroxide. The aqueous phase was separated and the toluene was reextracted with three more 25-ml. portions of N-sodium hydroxide. The toluene was back-washed three times with 5 ml. of water, and the washings were added to the combined

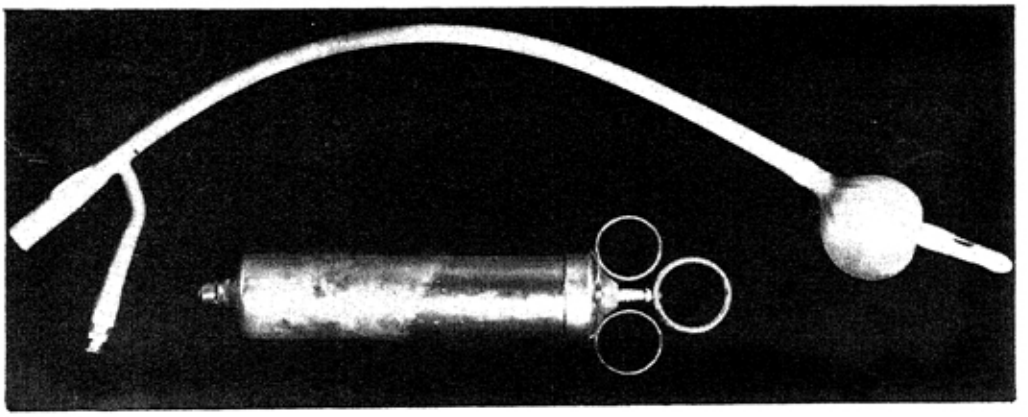


Fig. 3—A size 24 Bardex hemostatic catheter with a 75 ml. balloon (Bard catalog No. 113). To obtain clean 24-hour urine samples, quantitatively, from cows, a sterile catheter is lubricated with a mild disinfectant jelly and introduced through the urethral orifice until the balloon lies just inside the neck of the bladder. The balloon in inflated with 50 to 75 ml. of warm water by means of a hypodermic syringe and the inflation tube is sealed with a valve which is made to fit the nozzle of the syringe. The catheter is than pulled so that bulb fits into the neck of the bladder.

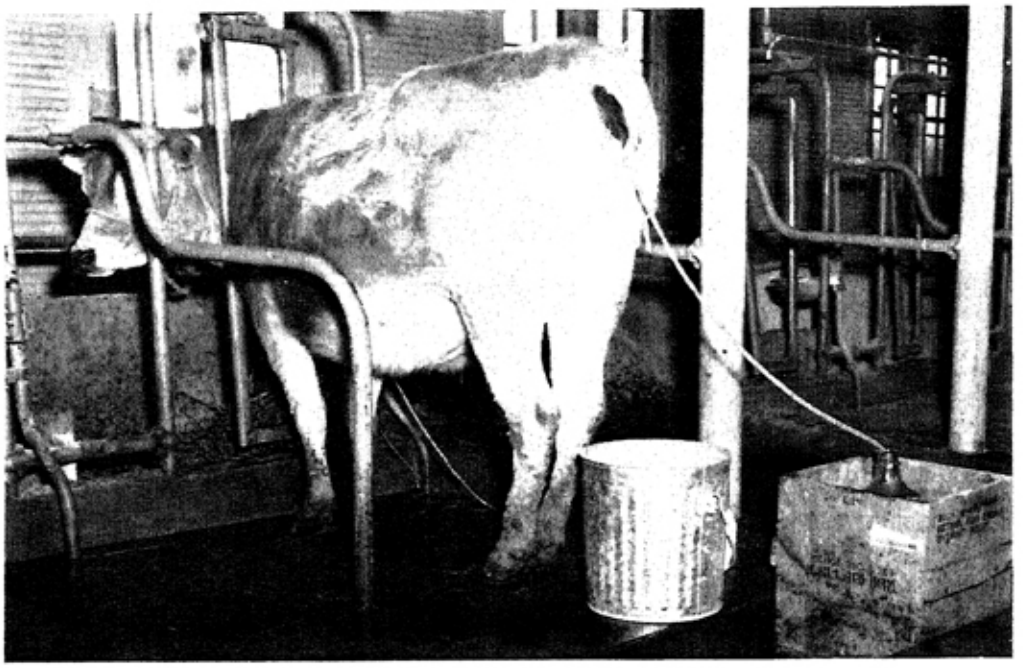


Fig. 4—A 3-inch length of ¼-inch diameter glass tubing is used as a connection between the catheter and a 3/16-inch bore rubber tube, which drains the urine in a carboy.

N-sodium hydroxide extract. The toluene solution contained the *neutral fraction.* The N-sodium hydroxide extract was brought to pH 9.3-9.5 and extracted four times with 100 ml. of ether. The extract contained the *phenolic fraction.*

Final Purification of the Phenolic Steroid Fraction.—The ether extracts were washed twice with 20 ml. of water and then four times with 20 ml. of 2.5 percent sodium carbonate solution. The combined sodium carbonate extracts were back-washed with 100 ml. of ether, and the ether wash was added to the ether phase. This was then washed three or four times with 20 ml. of distilled water.

Separation of the Phenolic Steroid Fraction into Non-Ketonic and Ketonic Components.—The ether solutions were evaporated and to the fraction dissolved in 10 ml. of ethanol was added 1 gm. of Girard reagent "T" and 1 ml. of glacial acetic acid. After refluxing for one hour, the mixture was poured into 100 ml. of iced distilled water and adjusted to neutrality with 2N NaOH. The aqueous solution was extracted three times with an equal volume of ether, and the combined ether extracts were back-washed twice with 0.1 volume of water. The ether extract contained the non-ketonic phenolic fraction. The combined portion and washings were treated with 0.1 volume of concentrated HCl, and extracted with equal volume of ether three times. This contained the ketonic phenolic fraction.

Spectrophotofluorometric Measurement of Phenolic Steroid Extract.—The ether extract of each fraction was evaporated on a steam bath to dryness and then dissolved in 20 ml. of 95 percent ethanol. Samples of 0.2 ml. were pipetted into separate fluorometer tubes. Two ml. of 90 percent sulfuric acid were added to each tube and the tubes were shaken. All tubes were placed in a water bath at 80° C for 10 to 20 minutes. The tubes were removed from the bath and 12 ml. of 65 percent sulfuric acid were added. The contents of each tube were mixed thoroughly using a flattened, spirally-tipped stirring rod. After standing for several minutes to permit the bubbles caused by agitation to disappear, the relative fluorescence of the standard and the unknown was determined. The difference in the readings given by the two aliquots was assumed to be due entirely to the added material; this difference was used as the *reference standard.*

The fluorescence was measured in a Model 14 Coleman Universal Spectrophotometer. The filters used were Corning filters #5113-#3389 (facing the light source) as the primary filter, and Corning filter #3486 as the secondary filter.

The optical density of the sample at the fluorescence wave length was determined and divided by two. This was to take into account the depth of solution through which the fluorescent light had to pass. This value was converted to percentage transmission and the observed fluorescence intensity was divided by the percentage transmission to obtain corrected value.

Determination of Estrogen in Cow's Feces

The fresh feces were thoroughly extracted with ethyl alcohol (two liters of

95 percent ethanol for every kilogram of feces) by stirring for 30 minutes. The samples were left at room temperature for 48 hours, then filtered in a Buchner funnel under reduced pressure. The alcoholic extracts were evaporated to an aqueous sludge to which was added 30 ml. of 95 percent ethanol, 250 ml. of water and acidified to Congo red with concentrated HCl, then extracted four times with the same volume of ether.

Purification, fractionation and measurement of estrogen were the same as used for urine except for one step in connection with the separation of the phenolic fraction. Instead of dissolving the ether extract in 100 ml. of toluene to separate the phenolic fraction from the neutral fraction, the crude tarry material was freed of neutral substances by being dissolved in 100 ml. of ether and extracted with 200 ml. 1 N NaOH.

In all determinations, it has been found necessary to run a blank consisting of 100 ml. of urine and 1 Kg. of feces collected from a sterile cow and extracted in the same manner. This was set at zero in the fluorometer and spectrophotometer.

Evaluation of Methodology

After modifying the chemical method until it seemed to be reasonably successful for the analysis of estrogen, ten trials were made to check recovery of estradiol and estrone added to urine and feces. Data reveal that quite a high recovery of added estrogen was accomplished by this method of analysis (Table 3). To evaluate the consistency of the chemical method, ten urine and feces samples were analyzed in duplicate; they seldom varied by more than 1 or 2 micrograms. This does not mean that the method of analysis determines all of the estrogenic substances in the urine. The acid hydrolysis may have resulted in some destruction of estrogenic substances.

TABLE 3. RECOVERIES OF KNOWN AMOUNTS OF ESTRONE AND ESTRADIOL ADDED TO URINE AND FECES OF PREGNANT COWS PRIOR TO HYDROLYSIS AND CALCULATED AS ESTRADIOL

Hormone Added		Recovery as Estradiol	
Estrone ug.	Estradiol ug.	%	
50	50	100	
50	50	97	Urine
50	50	99	
50	50	95	Samples
50	50	100	
50	50	100	
50	50	91	Feces
50	50	89	Samples
50	50	100	
50	50	94	

RESULTS

Twenty cows were divided into four groups according to their stage of pregnancy. The total amount of estrogen excreted daily and determined as estradiol varied greatly among individual cows at almost identical stages of pregnancy; it averaged 4.49 mg. ± 1.7 in the first group of four cows, pregnant 91 to 109 days; 5.56 mg. ± 2.8 in the second group of five cows, pregnant 120 to 170 days; 19.11 mg. ± 4.5 in the third group of six cows pregnant 185 to 234 days; and 20.14 mg. ± 7.4 in the fourth group of five cows pregnant 248 to 273 days (Tables 4, 5, 6, 7,) (Figures 5, 6). It was noticed that individual variation increased in advancing stages of pregnancy as shown by standard deviation.

The ratio of urinary to fecal estrogen was 0.6:1 in the first group, 0.81:1 in the second group, 0.82:1 in the third group and 1:0.87 in the fourth group (Tables 4, 5 6, 7). The fecal estrogen was higher than the urinary estrogen except in cows 458, 597, 435, 376, 453, 450 (Figure 7).

It was observed that the total estrogen excretion remained at low level until about 170 days of pregnancy, then gradually increased and maintained a high level toward the end of pregnancy (Figure 5).

The average daily estrogen excretion per 100 lb. body weight was 417 ug. in the first group with an average body weight of 1077 lb., 537 ug. in the second group with an average body weight of 1042 lb., 1380 ug. in the third group with an average body weight of 1385 lb., 1427 ug. in the fourth group with an average age body weight of 1411 lb. (Table 8, Figure 8).

The total urinary and fecal estrogen excreted daily by cow No. 585 was 3.58 mg. (calculated as estradiol) at 231 days of pregnancy. This amount increased gradually to 9.96 mg. at 267 days of pregnancy. The phenolic steroid extracts were fractionated into non-ketonic (calculated as estradiol) and ketonic (calculated as estrone) components. It was found that the non-ketonic fraction in both urine and feces was the major component and that estrogen was excreted mainly by way of the feces (Table 9). Pilot experiments showed the absence of the non-ketonic strong-phenolic estrogen (estriol) in both urine and feces.

TABLE 4. SPECTROPHOTOFLUOROMETRIC CONCENTRATIONS OF ESTROGENS FOUND IN URINE AND FECES OF FOUR COWS PREGNANT 91 TO 109 DAYS

Cow No.	Days of Gestation	Body Weight in lbs.	Mg. Estradiol Equivalent/ 24 hr. Period		Total Estradiol Excretion in mg.
			Urine	Feces	
800	91	1120	2.280	3.000	5.280
806	94	1050	0.440	1.440	1.880
497	97	1195	1.848	3.024	4.872
807	109	945	2.249	3.680	5.929
Mean		1077	1.704	2.786	4.490±1.7
Ratio			0.6 = 1		

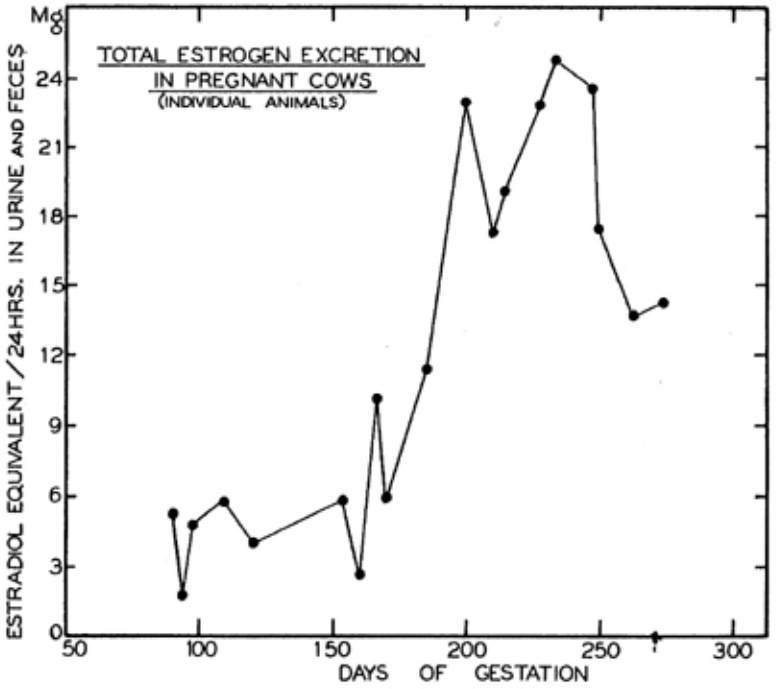


Fig. 5—Total estrogen excretion per 24 hours by 20 cows in different stages of pregnancy. Small amounts of estrogens were excreted during the first six months of gestation; then the amounts increased rapidly toward the end of pregnancy. The drop in the curve after reaching a peak may be due to individual variation. The maximum production of estrogen apparently does not always occur at the same time in different cows.

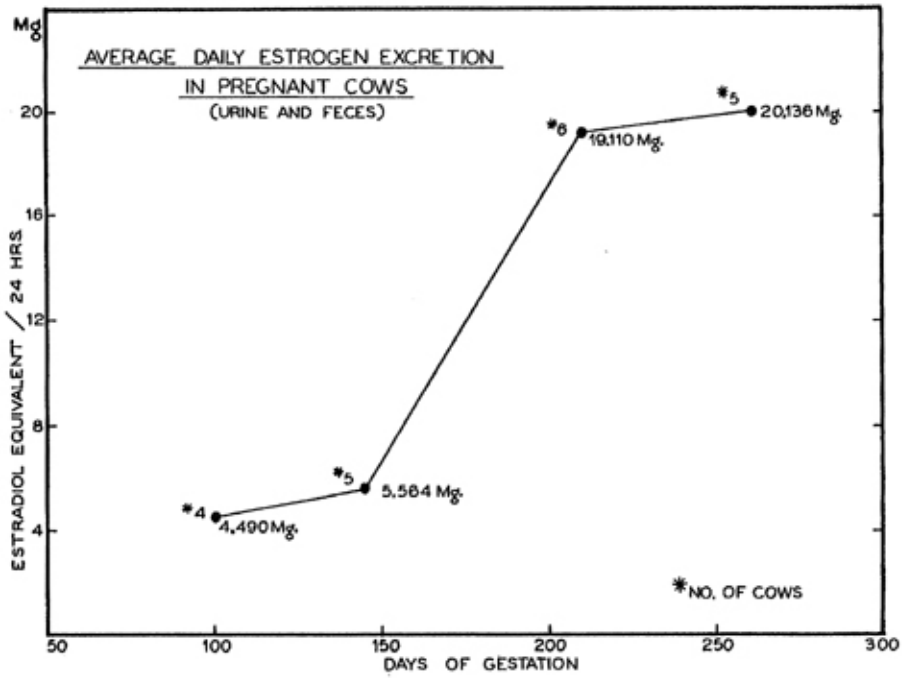


Fig. 6—The average daily total (urine and feces) estrogen excretion per 24 hours is 4.49 mg. in a group of four cows pregnant 91 to 109 days, 5.564 mg. in a group of five cows pregnant 120 to 170 days, 19.11 mg. in a group of six cows pregnant 185 to 234 days and 20.136 mg. in a group of five cows pregnant 248 to 273 days.

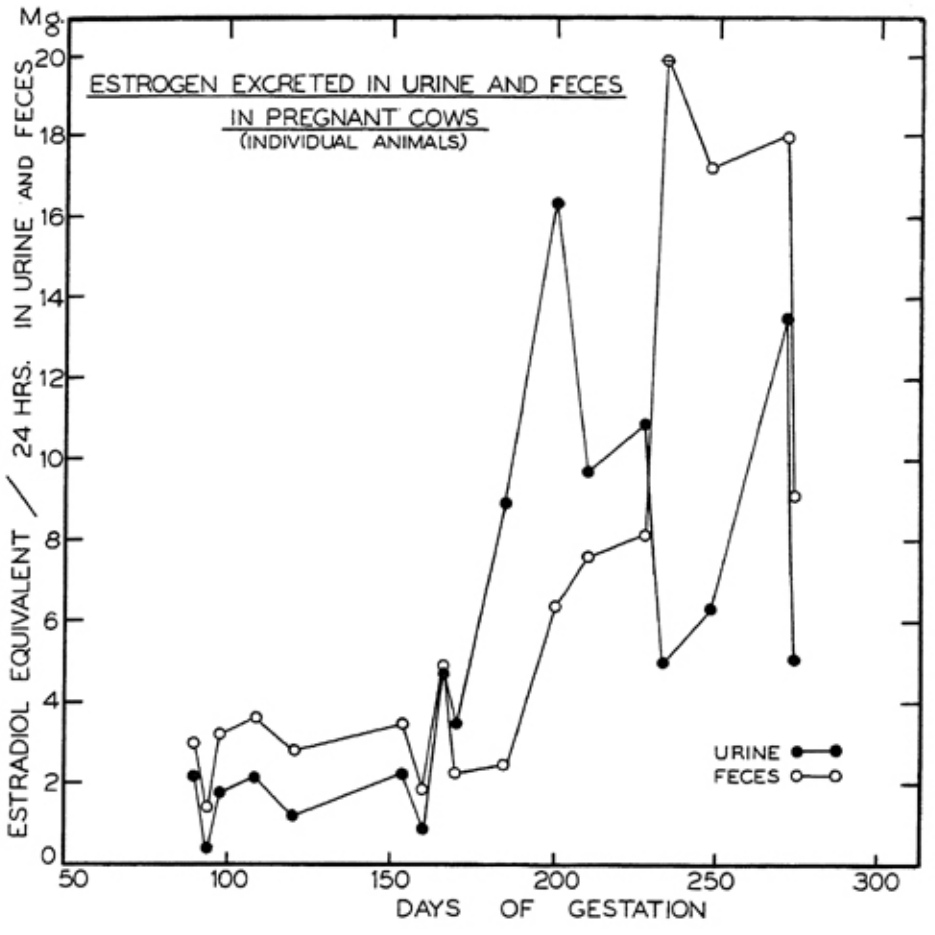


Fig. 7—Both urinary and fecal estrogens excreted by pregnant cows are at a low level during the first 6 months of pregnancy; then they increase rapidly toward the end of pregnancy. In 14 cows the fecal estrogen excretion was at higher levels than urinary estrogen, while in six cows, pregnant more than six months, urinary estrogen exceeded fecal estrogen.

TABLE 5. SPECTROPHOTOFLUOROMETRIC CONCENTRATIONS OF ESTROGENS FOUND IN URINE AND FECES OF FIVE COWS PREGNANT 120 TO 170 DAYS

Cow No.	Days of Gestation	Body Weight in lbs.	Mg. Estradiol Equivalent/ 24 hr. Period		Total Estradiol Excretion in mg.
			Urine	Feces	
804	120	972	1.200	2.880	4.080
617	154	854	2.340	3.563	5.903
805	160	1085	0.900	1.908	2.808
463	167	1300	4.960	5.190	10.150
458	170	1000	3.550	2.330	5.880
Mean		1042	2.590	3.174	5.564±2.8
Ratio			0.81 : 1		

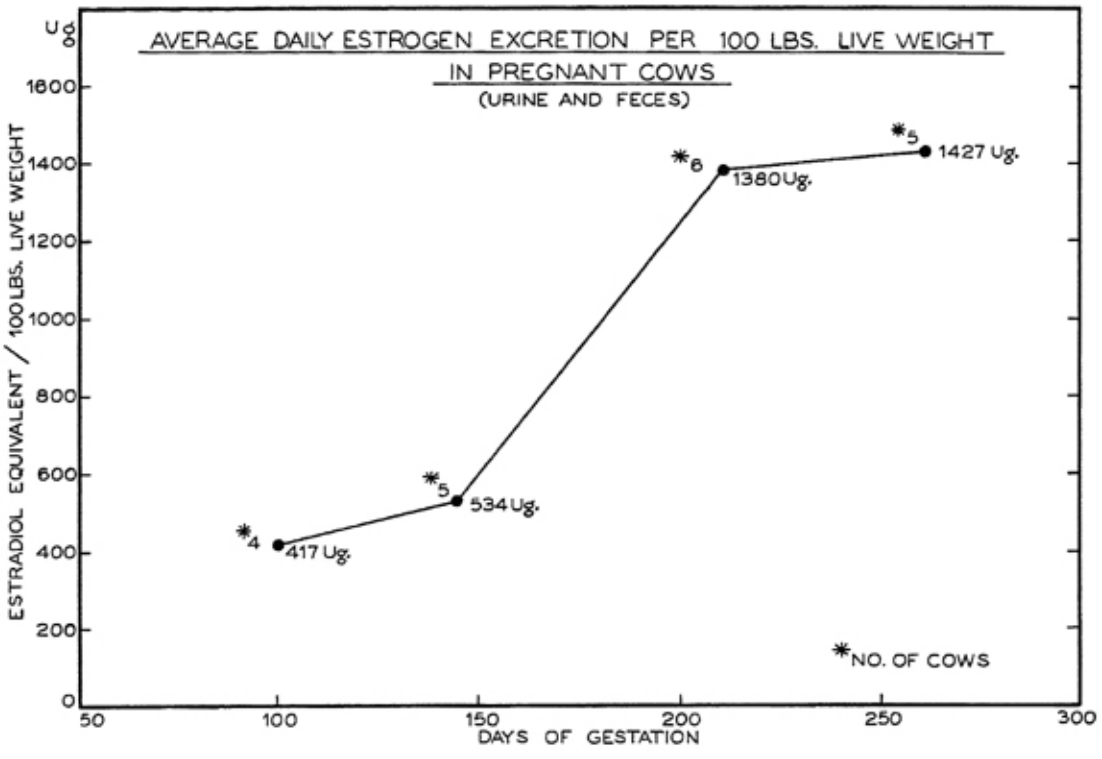


Fig. 8—The average daily estrogen excretion per 100 pounds of body weight was 417 ug. in a group of four cows with an average body weight of 1077 pounds and pregnant 91 to 109 days, 534 ug. in a group of five cows with an average body weight of 1042 pounds and pregnant 120 to 170 days, 1380 ug. in a group of six cows with an average body weight of 1385 pounds and pregnant 185 to 234 days and 1427 ug. in a group of five cows with an average body weight of 1411 lbs, and pregnant 248 to 273 days.

TABLE 6. SPECTROPHOTOFLUOROMETRIC CONCENTRATIONS OF ESTROGENS FOUND IN URINE AND FECES OF SIX COWS PREGNANT 185 TO 234 DAYS

Cow No.	Days of Gestation	Body Weight in lbs.	Mg. Estradiol Equivalent/ 24 Hr. Period		Total Estradiol Excretion in mg.
			Urine	Feces	
434	185	1277	8.900	2.550	11.450
597	200	1200	16.380	6.420	22.800
435	211	1417	9.730	7.600	17.330
598	215	1028	0.780	18.300	19.080
376	228	1614	10.920	8.210	19.130
3	234	1775	5.070	19.800	24.870
Mean		1385	8.630	10.480	19.110±4.5
Ratio			0.82	1	

TABLE 7. SPECTROPHOTOFLUOROMETRIC CONCENTRATIONS OF ESTROGENS FOUND IN URINE AND FECES OF FIVE COWS PREGNANT 248 TO 273 DAYS

Cow No.	Days of Gestation	Body Weight in lbs.	Mg. Estradiol Equivalent/ 24 hr. Period		Total Estradiol Excretion in mg.
			Urine	Feces	
604	248	1141	6.300	17.250	23.550
453	250	1490	16.920	0.520	17.440
450	263	1515	11.760	1.960	13.720
304	271	1462	13.500	18.160	31.660
444	273	1445	5.130	9.180	14.310
Mean		1410	10.720	9.414	20.136±7.4
Ratio			1	0.87	

TABLE 8. DAILY ESTROGEN EXCRETION PER 100 LBS. LIVE WEIGHT IN 20 PREGNANT COWS (URINE AND FECES)

Cow No.	Days of Gestation	Body Weight in lbs.	Total Estradiol Excretion in mg.	ug. Estradiol Equivalent/ 100 lbs. Live Weight	Mean ug.
800	91	1120	5.280	571	
806	94	1050	1.880	179	
497	97	1195	4.872	407	417
807	109	945	5.929	676	
804	120	972	4.080	419	
617	154	854	5.903	691	
805	160	1085	2.808	258	534
463	167	1300	10.150	780	
458	170	1000	5.880	588	
434	185	1277	11.450	896	
597	200	1200	22.800	1900	
435	211	1417	17.330	1223	1380
598	215	1028	19.080	1856	
376	228	1614	19.130	1185	
3	234	1775	24.870	1401	
604	248	1141	23.550	2063	
453	250	1490	17.440	1170	
450	263	1515	13.720	905	1427
304	271	1462	31.660	2165	
444	273	1445	14.310	990	

TABLE 9. SPECTROPHOTOFLUOROMETRIC CONCENTRATIONS OF ESTROGENS FOUND IN URINE AND FECES OF COW NO. 585 DURING LATE PREGNANCY

Days of Gestation	ug. Estrone Equivalent/ 24 hr. Period		ug. Estradiol Equivalent/ 24 hr. Period	
	Urine	Feces	Urine	Feces
231	47	259	444	2833
232	40	139	2204	2465
250	240	460	1120	6560
255	443	1178	2234	5840
267	438	319	3093	6110

DISCUSSION

Hydrolysis Of Conjugated Estrogens In Urine

The hydrolysis of conjugated estrogens in urine is a complex problem which is still being investigated. There is no procedure known at present which will cleave the conjugates of all steroids without any alteration of the steroid molecule Yet the hydrolytic step is necessary to free the estrogens from their conjugates (Friedgood *et al.,* 1948; Stimmel, 1946).

Since acid hydrolysis fits more easily than enzymatic hydrolysis into a routine method, the hydrolytic procedure followed in this investigation is a modification of that of Dobriner *et al.* (1948). The urine was refluxed at 80° C for 30 minutes with 5 percent (volume per volume) of concentrated sulfuric acid. Woods (1950) showed that sulfuric acid was most suitable for hydrolysis of conjugated steroids in cow's urine. The efficiency of enzyme (beef spleen B-glucuronidase, or phenol-sulfatase) over acid hydrolysis was not shown for the phenolic steroids.

Beer and Gallagher (1955) found that the use of β-glucuronidase was superior to boiling with 5 percent sulfuric acid for hydrolysis of the conjugated metabolites in human urine. Marrian and Bauld (1951) showed that the best results were obtained by boiling human urine under reflux one hour with 15 volumes of concentrated hydrochloric acid per 100 volumes of urine. Hydrolysis of cattle urine with hydrochloric acid is considered to be more destructive of the steroids than hydrolysis with sulfuric acid (Arrhenius, 1950).

After acid hydrolysis, the color of the urine changes from a bright yellow to a dark reddish brown. Dark brown flakes appear, indicating some kind of polymerization. The change of color is more marked in the urine of cattle than in that of the human, and is still more marked in the urine of the stallion (Arrhenius, 1950). The amounts of these pigments increase with pregnancy (El-Attar, 1955). This may be due to the transformation of substances like urochrome-indol derivatives, the other non-steroids, but it is most likely that some of the steroids are destroyed. Lipid extracts of urine hydrolyzed with strong acids are dark brown; the color turns lilac after removal of the phenolic steroids. The colors of the extracts are often considered to be due to derivatives of steroids formed by dehydration and oxidation (Miescher, 1946) produced by strong acids. In routine analysis it is impossible to separate the colored substances from the steroids, and it is known that the unspecific chromogenic substances will give values that are too high when measured colorimetrically.

Extraction with Ether and Removal of the Acid Fraction

Ether seems to be the most suitable solvent for extracting the three estrogens from aqueous solutions (Brown, 1955; Mather, 1942).

Many workers remove the acid fraction from the ether extract of hydrolyzed

urine by shaking with sodium bicarbonate solutions (Clayton, 1949). Cohen and Marrian (1934) passed carbon dioxide into solutions containing the acid and phenol fractions from urine until the solutions were neutral to phenolphthalein (pH 9.0) and then extracted the estrogen with ether. Engel *et al.* (1950), and Engel (1950) modified this procedure and showed that it was more efficient than any other described for removing the acid fraction. Brown (1955) found that estriol was easily extracted with ether from aqueous solutions at a pH as high as 10.5, provided the aqueous solutions were saturated with sodium carbonate or sodium bicarbonate at that pH. He also found the partition coefficient of estriol between ether and concentrated carbonate solution of pH 10.5 to be practically the same as between ether and saturated sodium bicarbonate. Apparently, even at this pH, ionic concentration rather than pH determines the solubility of estriol in aqueous soutions. Concentrated carbonate solution of pH 10.5, which is much more effective as a washing agent than sodium bicarbonate, can therefore be used for removing the acid fraction from ether extracts without any appreciable loss of estriol.

Urine extracts contain substances which change to colored products when dissolved in alkali and cannot be re-extracted with ether at a pH above 7. These substances, which are effectively removed from the estrogen fraction in the procedures of Cohen and Marrian (1934) and of Engel *et al.* (1950), are changed and removed in the present method by shaking the ether extract directly with 2N sodium hydroxide solution after first washing out the acid fraction with concentrated sodium carbonate solution at pH 10.5. The sodium hydroxide, which also extracts some of the estrogen, was then partly neutralized to pH 10, and the carbonate ion concentration increased, by adding M sodium bicarbonate solution. Some of the estrogens transferred to the aqueous phase are returned to the ether phase by shaking. The colored substances are retained in aqueous layer and discarded. The ether was washed with M sodium bicarbonate to neutralize the alkali still present, thus eliminating excessive loss of estriol in the subsequent water wash. The ether was then washed with a small amount of water to remove the bicarbonate. This is the procedure which was used in the present investigation and which is essentially that of Brown (1955).

Separation of the Phenolic Steroids from Neutral Steroids

The ether extract, after being washed with water, was evaporated to dryness on a steam bath and the residue was dissolved in toluene. Estrogen was then removed efficiently by extraction with 1N sodium hydroxide and the neutral fraction remained in the toluene phase which was discarded after washing three times with 5 ml. of water.

The sodium hydroxide solution containing the phenolic steroids was adjusted to pH 9.5 and extracted with ether again. This is of great importance since

it is thus possible to exclude some of the highly pigmented and fluorogenic materials which are extracted by ether at pH range 3 to 9 (Engel, 1950).

Final Purification of the Phenolic Steroid Extract

Washing the ether extract with water and 2.5 percent sodium carbonate following the separation of the phenolic from the neutral fraction removes almost as much pigment as was removed by the same treatment of the original ether extract. A significant amount of pigment also remains in the neutral fraction.

The fractionation of the phenolic steroid extract into non-ketonic and ketonic components by means of Girard's reagent "T" was used for further purification required in the case of feces which contains extraneous green pigments. It was also used in fractionating urine samples collected from cow No. 585 (Table 9). The non-ketonic, weak phenolic and the ketonic phenolic steroid values found in cow's urine and feces were expressed as estradiol since it is the estrogen secreted primarily by ovaries. In pilot fractionation experiments, the strong phenolic non-ketonic fraction (estriol) was not found. This was confirmed by Levin (1945), Woods (1950), and Klyne and Wright (1956).

Spectrophotofluorometric Measurement of Estrogens

Fluorometric Determination.—The urine and feces excreted by pregnant cows contain such small concentrations of estrogen (Smith *et al.,* 1956; Woods, 1950) that the colorimetric methods proposed usually lack sufficient sensitivity for its measurement. This necessitates the use of large quantities of urine and feces so that non-specific chromogens do not interfere with accurate determination.

All colorimetric (Kober) methods for measurement of estrogen in urine residues need correction equations due to interfering materials absorbing at the same wave length as estrogen. Since impurities in urine are not always the same (Allen, 1950) and increase toward the end of pregnancy (El-Attar, 1955), the non-specific color will not be the same when the phenolic steroid extract is treated with the reagent used for developing the specific color. This would necessitate a separate study of the absorption spectra of the pure compound and the contaminating substances in order to obtain a correction factor with every estrogen measurement.

Woods (1950) ran the phenolic steroids of pregnant cow's urine on activated alumina (Stimmel, 1946), then esterified them with p-phenylazobenzoyl chloride and further purified them by chromatographing on florisil (Umberger and Curtis, 1949). The phenolic steroids recovered were still contaminated, and the amounts of estrogen were too low to permit quantitative estimation by any of the common colorimetric or photometric procedures.

Since fluorometric methods permit determination of estrogen in very low concentrations, they are most suitable for measurement of low titers of estrogen

excreted by cows. In fluorometric analysis the contaminating pigments and fluorophors in urine and fecal extracts are highly diluted (Engel, 1950).

Accordingly, the method of Bates and Cohen (1950) for fluorometric determination of estrogen after heating with sulfuric acid was adapted for use with Model 14 Coleman Universal Spectrophotometer. This method, when compared with four other fluorometric procedures was found to be the best for high sensitivity (Braunsberg, 1952).

Internal standards.—Chemical methods for the determination of estrogen usually give higher values than bioassay methods. This is due, in part, to interfering substances in urine and fecal extracts. To compensate for this, an internal standard was used. The internal standard also corrects automatically for all manipulative and instrumental variables.

Spectrophotometric Absorption.—As an additional check upon the internal method, the absorption data were used only when there was appreciable loss of fluorescence intensity due to the presence of other compounds absorbing at the activation wave length. This occurred when high concentrations of estrogen were present as in the case of late pregnancy. Self quenching or concentration quenching by the sample leads to low results. These results were corrected by using spectrophotometric absorption data.

In general, the spectrophotofluorometric assay offers several distinct advantages over other physical methods utilizing light absorption. The fluorescent method, where applicable, offers a sensitivity at least two orders of magnitude greater than spectrophotometry and the additional specificity of two spectral requirements instead of one (Duggan *et al.*, 1957).

Excretion of Estrogen During Pregnancy

In experimental animals optimal amounts and ratios of estrogen and progesterone required for complete lobule-alveolar growth of the mammary gland tissue have been determined. In the dog, Trentin *et al.* (1952) showed that the ratio of estrogen to progesterone was of the order of 1:1000. Similarly, in the mouse (Yamada *et al.*, 1954; Damm and Turner, 1957)and rat (Elliott and Turner, 1953; Kirkham and Turner, 1954), this ratio was found to give optimal results.

To induce lobule-alveolar mammary gland growth in cows, Turner *et al.* (1956) injected daily a mixture of 100 mg. of progesterone and 100 ug. of estradiol benzoate for a period of 180 days. The level of progesterone used was based upon the amount required to maintain pregnancy in dairy heifers from which the corpora lutea had been removed. Observations of Raeside and Turner (1950), Uren and Raeside (1951), and McDonald *et al.* (1952) indicated that 100 mg. of progesterone daily would accomplish this objective. With 100 mg. of progesterone injected daily, the estrogen requirements on the basis of 1:1000 would be 100 ug.

The determination of DNA in the mammary gland tissue as an index of its growth (Damm and Turner, 1957) is not practical in the cow. The amount of progesterone which was used by Turner *et al.* (1956) for induction of udder growth in cattle was established on the basis of the amount of this hormone required for maintenance of pregnancy in dairy cattle.

The ratio of 1 part of estrogen to 1000 parts of progesterone given to cows (Turner *et al.,* 1956) with the purpose of the induction of udder growth was based on that ratio of estrogen to progesterone which, when given to experimental animals, induced optimal mammary gland growth.

The quantitative determination of the total estrogen excretion during pregnancy in urine and feces, simultaneously, would make it possible to estimate the actual amount of this hormone which is normally secreted by pregnant cows during the first two-thirds of pregnancy. The amount of estrogen secreted daily by pregnant dairy cattle observed in this study, in conjunction with 100 mg. progesterone, might be expected to stimulate growth of the udder experimentally comparable to the growth stimulated during normal pregnancy.

It is believed that cows which have lower estrogen secretion rates will develop smaller mammary glands than those which secrete optimal amounts. This belief is based upon two observations. First, in experimental animals, when estrogen is injected in varying amounts with a constant amount of progesterone, the mammary glands vary in extent of growth (Damm and Turner, 1957). Second, the estrogen secretion of beef animals, which have poor udder development, is lower than in dairy animals (Turner *et al.,* 1930).

The chemical determination of the total amount of estrogen excreted daily in urine and feces of 20 cows of different breeds, ages, and stages of pregnancy, showed great variability among individual cows at almost the same stage of pregnancy.

This variability or the standard deviation from the mean increased from $\pm$ 1.7 in the first group of four cows pregnant 91 to 109 days to $\pm$ 7.4 in the last group of five cows pregnant 248 to 273 days (Tables 4, 5, 6, 7).

With regard to this great variability in excretion of estrogen by cows pregnant at almost identical stages, it might be concluded that in experimental induction of maximum udder growth, the optimal dose of estrogen given to cows should be identical to the actual amount of estrogen secreted by each individual animal during the first six months of pregnancy which is the period required for growth of the udder. This is probably not true since some animals inherit poor mammary gland development due to inheritance of insufficient secretion of estrogen or an insufficient secretion of progesterone or to abnormal balance of the two hormones. Therefore, if the estrogen dose is increased and given in optimal ratio with progesterone, maximum udder growth could be accomplished.

The total amount of estrogen excreted during the first five to six months of

pregnancy was low when compared to that excreted during the last three to four months of pregnancy. The average daily estrogen excretion rate was 4.49 mg. in four cows pregnant 91 to 120 days, 5.56 mg. in five cows pregnant 120 to 170 days, 19.11 mg. in six cows pregnant 185 to 234 days and 20.14 mg. in five cows pregnant 248 to 273 days. The period through which low titers of estrogen were excreted coincided with that required for growth of the cow's udder and which was found by Turner *et al.* (1956) to be five to six months. Casein synthesis also does not start within the cow's udder before the end of six months pregnancy (Cutler and Lewis, 1933), which indicates that this period is required for complete udder growth.

In dairy heifers the growth of the udder can be stimulated by the daily injection of 100 ug. of estrogen and 100 mg. of progesterone for 180 days (Turner *et al.,* 1956). The daily excretion rate of estrogen by cows pregnant 180 days showed much higher levels than those injected by Turner *et al.* (1956). Thus it is not possible to say that 100 ug. of estrogen per day is the optimal level for maximum udder growth. It is true that good udder growth has been obtained in sterile dairy heifers.

The total amount of estrogen excreted in cows' urine was low as compared to the urine of the mare (Beall and Edson, 1936; Dow and Allen, 1949) or to that of the human female (Cohen and Marrian, 1935). This was confirmed by all investigators studying the problem (Anderson, 1934; Hisaw and Meyer, 1929; Kust, 1934; Lipschutz *et al.,* 1929; Nibler and Turner, 1929; Turner *et al.,* 1930; Woods, 1950; Smith *et al.,* 1956).

The phenolic steroids extracted from urine of cow No. 585, when fractionated into non-ketonic and ketonic components, were found to be primarily non-ketonic. This is in agreement with the results of Woods (1950) and Smith *et al.* (1956). In the ewe, the ketonic estrogen (calculated as estrone) of both urine and feces was higher than the non-ketonic estrogen (as estradiol) (Beck, 1950).

The fecal excretion of estrogen, like the urinary excretion, was markedly increased during pregnancy, and was higher than urinary estrogen. Levin (1945) has studied the excretion of estrogen in the feces of cows and found 5000 to 8000 r.u. per kilogram of solids in late pregnancy. His studies indicated that estradiol accounted for most of the estrogenic activity. This is in agreement with what was found in the present investigation. In all samples of feces collected from cows and processed for estrogen determination, the non-ketonic (calculated as estradiol) fraction was the major steroid.

Recent investigations have pointed to the bile as an important medium of excretion of estrogens. After the intravenous and subcutaneous administration of α-estradiol and estrone to bile-fistulated dogs, Cantarow *et al.* (1942) found that estrogenic activity disappeared rapidly from the circulation, but was recover-

ed almost quantitatively from the bile in 2 to 3 days after injection. The apparent importance of the biliary excretion of estrogen suggests that fecal as well as urinary excretion of estrogen takes place. The earliest investigators of estrogen metabolism detected estrogenic activity in feces as well as in urine. Siebke and Schuschania (1930) claimed that the normal female excreted almost as much estrogenically active material in the feces as in the urine. Kemp and Pedersen-Bjergaard (1933) found a higher titer (three to seven fold) of estrogen in the feces of normal men than in their urine. The fecal estrogen excretion by women increases markedly during pregnancy (Dohrn and Faure, 1928).

From the study of the bile of the pregnant cow, appreciable estrogenic activity was found and estrone was obtained in crystalline form as the major estrogen; α-estradiol may also be present (Pearlman *et al.,* 1947). The estrogen concentration in the bile considerably exceeds the concentration in blood and in urine (Pearlman *et al.,* 1947). Another rather interesting pathway of elimination of estrogen is by way of the intestinal mucosa (Pearlman *et al.,* 1948). This has been shown in feces of bile-fistulated dogs, which had been injected parenterally with estrogen, but whose bile was not permitted to reach the intestines.

That estrogen is excreted mainly by pregnant cows via the feces, as found in the present study, was confirmed by the experiments of Pearlman *et al.* (1947) on the cow's bile and Levin (1945) on the cow's feces, which were previously mentioned. The presence of estradiol as the major estrogen in cow feces while estrone is the major one in the cow bile may be due to intestinal bacterial flora rather than intermediate processes in the body (Levin, 1945).

In most instances (dog after injection of estrogen; bile of pregnant cows) almost the entire estrogen in the bile is present in free form, whereas urinary estrogens and the estrogen in the blood are largely esterified. The question may be raised whether the liver removes, and excretes in the bile, only the free moiety of plasma estrogens, or whether the liver can achieve, in the process of excretion, hydrolysis of the esterified estrogens. Human bile of pregnant women was found to contain appreciable amounts of esterified "combined" estrogen (Pearlman and Rakoff, 1949).

Furthermore, the fact should be stressed that the concentration of estrogenic activity is higher in the bile of pregnant women than in the serum (Cantarow *et al.,* 1943) and that biliary exceeds urinary excretion in the bile-fistulated dog after exogenous administration of estrogen (Pearlman *et al.,* 1948).

The liver is involved in the fate of estrogens in two ways; one, by metabolizing the compounds, and secondly, by biliary excretion (Paschkis and Rakoff, 1950). The latter is probably important for the rapid disappearance of estrogens; the hormone delivered to the intestines in the bile may, to a large extent, be reabsorbed and, by passage through the portal circulation, be returned to the liver, thus going through repeated interhepatic circulation. During the passage through

the liver the estrogenic compound is subject to metabolic changes; these are largely in the direction of compounds of lesser activity (estradiol⇄estrone→ estriol) with ultimate complete inactivation due to conjugation. Activating processes (estrone→estradiol) may occur at the same time, namely, formation of compounds with greater activity. The overall net result, however, is gradual inactivation (Paschkis and Rakoff, 1950).

It seems likely that under normal conditions the liver removes part of the estradiol from the circulation and excretes it or its metabolites, by way of the bile, into the digestive tract. If for any reason an increased production of estrogen or change in the body mechanism occurred, there would be a tendency for greater outflow of estrogen into the urine. This may explain why urinary estrogen exceeded fecal estrogen in cows No. 458, 597, 435, 376, 453 and 450.

The relatively minor excretion of estrogen metabolites in the human feces (Beer and Gallagher, 1955) suggests that biliary and intestinal elimination play but minor roles in the metabolism of the estrogens by human subjects. The species differences in route of elimination very likely reflects different metabolic alterations produced in the hormones, and the relations of these changes to the fundamentals of hormone action remains an important area for investigation.

The curve representing the estrogen secretion pattern found in the present investigation resembles that of the human (Cohen and Marrian, 1935) in shape but not in the amount of estrogen excreted. In the Egyptian buffalo, urinary estrogens were first detected during the first month of pregnancy. They continued at a low level until the sixth month, and then increased markedly toward the end of pregnancy (El-Attar, 1955). Estrone is excreted by the sow during the last fourth of the gestation period and reaches a maximum around the fifteenth week (Bredeck, 1956). In pregnant ewes little or no estrogen is excreted in the urine until the last three or four weeks of pregnancy, then it starts to appear at very low levels (Bassett *et al.,* 1955).

Since the amount of estrogen excreted increases during gestation and drops markedly with the termination of pregnancy, there must be an active secreting source during pregnancy. It has been suggested that the placenta is the chief source of estrogen secretion. The low secretion of estrogens in early pregnancy is related to the small placenta at that time. With increasing size or weight of the placenta, the secretion of estrogen increases. At the sixth to seventh month of pregnancy, both placental weight (Hammond, 1927) and estrogen secretion increase markedly. Allan and Dodds (1935), Pincus and Pearlman (1943), and Diczfalusy (1953) have shown that the placenta secretes estrogenic compounds.

Estrogens play an important and complex role during pregnancy, parturition, and initiation of lactation. The primary role of estrogen is undoubtedly that of stimulating and maintaining the secondary organs of reproduction. There is also good evidence which indicates that estrogens act synergestically with progesterone

in the maintenance of pregnancy. In the rabbit, the quantities which act synergistically have been accurately determined. The ratio of progesterone to estradiol was found to be 325:1 or less when gestation was interrupted, but pregnancy was maintained when the ratio of progesterone to estrogen was 750:1 or larger (Courrier and Kehl, 1938).

In the cow it is difficult to determine directly the ratio of estrogen to progesterone required for maintenance of pregnancy for two reasons: (1) No pregnanediol has been detected in the urine (Stevenson, 1947; Hill *et al.,* 1954). (2) Miller and Turner (1955) have shown that progesterone is converted to androgens in the feces. McDonald (1952) found that 100 mg. of progesterone was required for the maintenance of pregnancy after removing the corpus luteum, but he made no estrogen determinations to establish a ratio between these hormones.

In the blood of pregnant guinea pigs (Zarrow, 1947), rabbits (Marder and Money, 1944), dairy cattle (Wada and Yuhara, 1955), ewe and goats (Wada and Yuhara, 1956) it was found that a steady increase of blood relaxin occurred during the first two-thirds of pregnancy, then plateaus until parturition. It disappears rapidly after parturition. As a result of a series of experiments Frieden and Hisaw (1953), suggested that inhibition of uterine automoticity in the guinea pigs and rats may be a physiological property of relaxin. Thus estrogen and progesterone result in the formation of relaxin by the tissues of the reproductive tract. The effect of progesterone upon uterine motility may thus be indirectly due to the action of relaxin.

Wada and Yuhara (1956) studied the effect of relaxin upon the uterine motility of the rat and guinea pig. At concentrations of 0.025 GPU/ml., the motility of the uterus was reduced and at 0.05 GPU/ml. was greatly reduced. Relaxin had no effect upon the motility of the intestines.

In the rabbit (Hall, 1955), the injection of progesterone induced the formation of relaxin in the blood with an intact uterus but not when it was removed. Relaxin has been found in the blood serum of castrated guinea pigs and castrated rabbits following injection of desoxycorticosterone. These results are not surprising since it has been shown that this compound can be converted in the body to progesterone.

Steintz *et al.,* (1956), working on mice, found that induction of parturition with oxytocin was dependent upon a progesterone-relaxin balance. Exogenous estrogens appear not to be needed for maintenance of pregnancy, pelvic changes, or effective parturition in spayed mice. Relaxin therapy restored the normal development of the interpubic ligaments in totally spayed pregnant mice.

From the review which has been presented it can be seen that the combination of estrogen and progesterone during pregnancy stimulates the ovary or other tissues to the production of relaxin. In pregnancy or when these hormones are injected in proper synergism, the endogenous production of relaxin is adequate

for maintenance of pregnancy. It is concluded that relaxin, rather than progesterone, may be the "hormone of pregnancy."

The mechanism of the onset of lactation is believed to be as follows: During the first two-thirds of pregnancy, progesterone neutralizes the stimulating effect of estrogen on pituitary lactogen secretion, but during the last third of pregnancy, estrogens gradually become predominant and are able to evoke a gradual rise in the lactogen content of the pituitary and thus initiate milk secretion. The amount of estrogen excreted by cows in the present study is a further proof for the fundamentals of the theory of mammary gland growth and onset of lactation presented. A number of reports have appeared which are in substantial agreement with the basic concepts of this theory (Hammond and Day, 1944; Selye, 1940; Mixner and Turner, 1943).

Estrogens act directly upon the mammary gland causing an increased vascularity and permeability of the blood vessels. There is an increase of blood plasma which nourishes the growing mammary gland. It also increases the amount of circulating hormones bathing the mammary gland. Estrogen also directly stimulates the growth of the teats (Mixner and Turner, 1942; 1943).

It has been demonstrated that estrogen given in physiological dosages will induce lactation in certain species, particularly the goat (Folley and Malpress, 1948) and the cow (Turner *et al.,* 1956). Most observers agree that some degree of suppression will occur if the dosage is greatly increased. It seems probable that small dosages of estrogen stimulate the lactogenic function of the anterior pituitary, while large dosages are inhibitory (Meites and Turner, 1948).

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