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URINARY EXCRETION OF OESTRONE AND OESTRADIOL AND OF ZIMMERMANN CHROMOGENS IN THE SOW DURING OESTRUS

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The present communication deals with the urinary excretion of oestrone and oestradiol and of Zimmermann chromogens, comprizing 17-ketosteroids, in sows during oestrus. Previously, a consistent pattern of the urinary excretion of oestrone during the oestrous cycle has been established in this species (*Velle* 1958, *Raeside* 1961, 1963 and *Lunaas* 1962 a). The pattern is characterized by a maximum associated with oestrus. In the sow, oestrone is an important urinary metabolite of oestradiol-17β (*Raeside* 1961 and *Lunaas* 1963 a) which is the major oestrogen present in the follicular apparatus of the ovary (*Lunaas* 1963 b). The fluctuations in the urinary excretion of oestrone thus presumably fairly well reflect the ovarian oestrogen production. Apparently due to inadequate methods used, earlier attempts to find variations in the urinary levels of oestradiol in the sow have been unsuccessful.

One of the objects of the present investigation has been to obtain further informations on the sequence in time of the oestrogen excretion and the events of oestrus. The study of such a relationship represents the technical difficulty of quantitative collection of the urine without interfering with the normal course of oestrus. In a previous investigation on the urinary excretion of oestrogens during the oestrous cycle (*Lunaas* 1962 a), the

animals were kept in narrow cages in which situation the psychic components of oestrus were apparently depressed or at least hard to recognize. In the present investigation the animals were allowed to move freely around on a rather large floor supplied with arrangements for collection of the urine. Recently there appeared a paper by *Raeside* (1963) in which a technique allowing for fractionation of the 24 hrs. urine was described. *Raeside* collected the urine by means of a self-retaining catheter in the bladder. This approach was evidently well suited for detailed studies on the relationship in time between oestrogen excretion and behavioural oestrus.

Behavioural oestrus may apparently be induced by parenteral administration of androgens (*Lindsay & Robinson* 1961). It can not be excluded therefore that the androgen production under physiological conditions may modify the course of oestrus. Production of androgens can be expected to result in excretion of 17-ketosteroids which are chromogenic in the Zimmermann reaction. The presence of Zimmermann chromogens in the sow's urine has been reported repeatedly (see *Lurie* 1960) but the chemical nature of the reacting substances seems to be unknown. In the present investigation some determinations of urinary excretion of Zimmermann chromogens were included with a view to possible variations which could be related to increased production of oestrogens (cf. *Herrmann et al.* 1960) or to oestrus.

MATERIAL AND METHODS

The material consisted of 4 nulliparous sows (gilts) of the Norwegian land race. A total of 7 oestrus periods were examined. During 6 of these periods the urine was collected quantitatively at intervals of 24 hrs. for at least 2 days prior to and 2 days after the first day on which behavioural oestrus was exhibited. During the additional period the urine was collected at shorter intervals and during behavioural oestrus only.

The animals were kept in a large cage the floor area of which was 2.8 m^2 (2.0 m $\times$ 1.4 m). The floor was made to retain the faeces. Water proof sails were stretched out below the floor of the cage in order to guide the urine into collection containers. Samples of the 24 hrs. urine were stored in the frozen state until processed for analysis.

The sexual receptivity was judged i. a. from the behavioural response when exerting moderate hand pressure on the loins

of the animal and in attempts by the herdsman to sit up astride its back (*Madden* 1960).

Quantitative determinations of oestrone and oestradiol were performed essentially according to *Brown* (1955) as described previously (*Lunaas* 1962 a) except that the method of *Ittrich* (1958) was used for the development of the Kober colour. The Kober colour complex was extracted into methylene chloride (*Ittrich* 1960) for spectrofluorimetry (*Lunaas* 1962 b). Single analyses were applied except in 15 specimens from one of the sows on which duplicate analyses were carried out in order to estimate the precision of the method. As calculated from the formula $\sqrt{\Sigma d^2/2n}$ (d = differences between duplicates) the standard deviation of the result of a single analysis corresponded to 0.76 μg oestrone and 0.07 μg oestradiol per 24 hrs. It should be noted that these estimates were based on a series of analyses of urine specimens the oestrogen contents of which were mostly rather low, the ranges being 2—8 μg (n = 13) and 35—45 μg (n = 2) oestrone and 0.2—1.5 μg oestradiol per 24 hours. It appeared, however, that the methodological errors were small in comparison with the relatively large variations in the amounts of oestrogens excreted during the period of oestrus.

For the determination of Zimmermann chromogens 30 ml of urine were mixed with 3 ml of conc. HCl and kept on a seething water bath for 20 min. The hydrolysate was shaken with 30 ml of ether and the extract washed with 10 ml 2N NaOH and with 10 ml water. The washed extract was divided into equal portions and the ether evaporated. One of the residues was developed with m-dinitrobenzene according to *Peterson & Pierce* (1960) and the other by addition of alkali only, the m-dinitrobenzene being omitted. The reaction mixtures were diluted with 50 % ethanol and extracted with methylene chloride. The extracts were dried by shaking with a little sodium sulphate. Known amounts of dehydroepiandrosterone were developed as standards. The optical density was read at 480, 520 and 560 mμ and the values inserted into the formula of *Allen* (1950). The corrected optical density of the colour produced in the absence of m-dinitrobenzene varied considerably and amounted to 31.8 $\pm$ 27.1 (s, n = 26) per cent of the corrected optical density of the colour produced in the presence of m-dinitrobenzene. The colour produced in the absence of m-dinitrobenzene was considered to be due to unspecific chromogens. In a series of 12

duplicates, the standard deviation of the net corrected optical densities in single analyses corresponded to 0.77 mg dehydroepiandrosterone per 24 hrs.

RESULTS

The individual patterns of the excretion of oestrogens during oestrus are recorded in Fig. 1—4. It may be seen that the urinary concentrations as well as the total amounts of oestrogens ex-

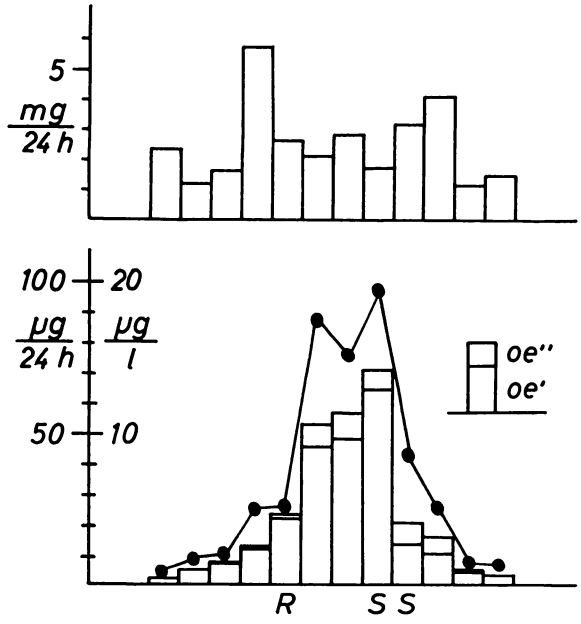
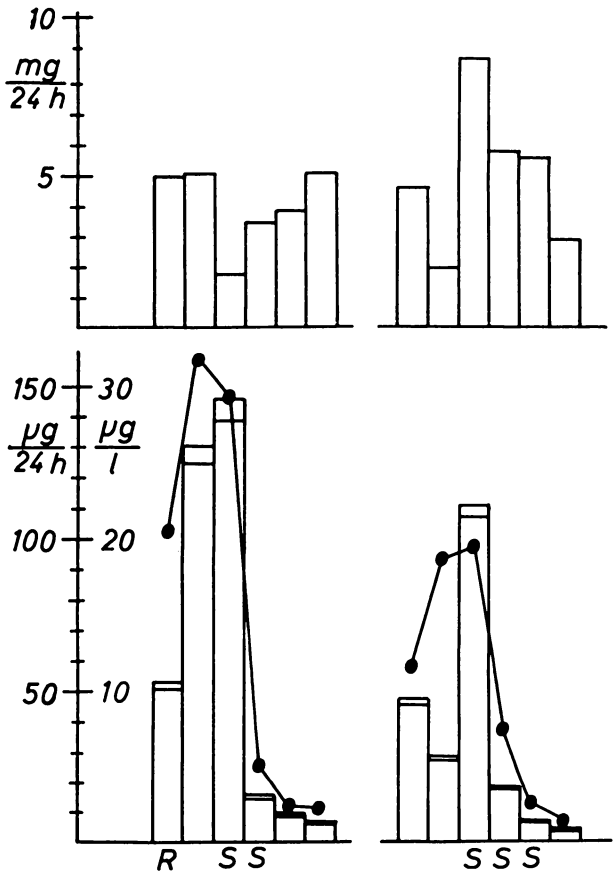


Fig. 1. Urinary excretion of oestrone (oe') and oestradiol (oe'') as µg per l (curve) and as µg per 24 hrs. and of Zimmermann chromogens as mg per 24 hrs. on consecutive days through oestrus. Sow no. 1. The 24 hrs. urine was collected in the late afternoon. R indicates the day on which hyperemia of the vulva became evident. Behavioural oestrus was exhibited on the days indicated by S.

creted increased to usually well defined maxima and then decreased rapidly. A vaginal oestrus, as indicated by hyperemia and edema of the vulva, was evident in all of the animals during the period examined. Behavioural oestrus could be demonstrated to commence 2—3 days after the onset of the vaginal oestrus except in one animal (Fig. 4). In this sow the vulva gradually turned pale and wrinkled in the usual way about the time at



F i g. 2. Urinary excretion of oestrogens and of Zimmermann chromogens on consecutive days through two periods of oestrus. Sow no. 2. Sympols as in Fig. 1.

which behavioural oestrus would be expected. The amounts of oestrone excreted in the case of apparent silent oestrus were approximately as in one of the animals (Fig. 3) exhibiting distinct symptoms of heat, but the urine was found to be virtually devoid of oestradiol.

In the animals exhibiting behavioural oestrus, the largest amounts of oestrogens were excreted during the day on which the heat symptoms became evident. The mean values of the amounts of oestrone excreted in these animals on consecutive days through oestrus are recorded in Table 1. The amounts excreted of oestradiol were relatively small, rarely constituting

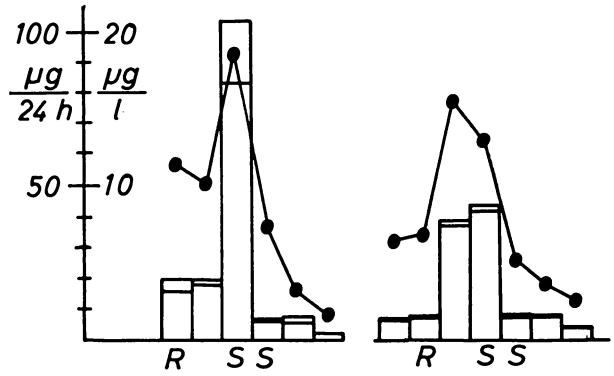


Fig. 3. Urinary excretion of oestrogens on consecutive days through two periods of oestrus. Sow no. 3. Symbols as in Fig. 1.

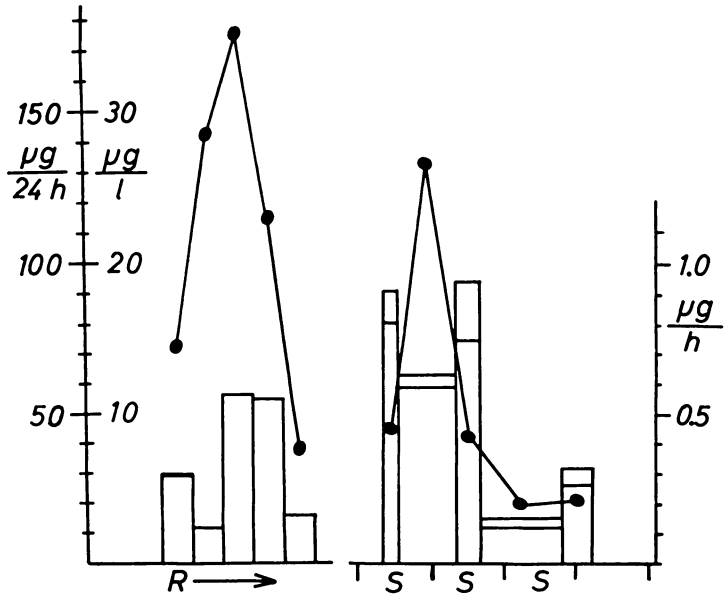


Fig. 4. Left: Urinary excretion of oestrogens on consecutive days in a sow (no. 4) failing to come into behavioural oestrus despite development of distinct vaginal oestrus commencing on the day indicated by R. Apparent regression of vaginal oestrus after three days. Right: Urinary excretion of oestrogens as µg per hr. in a sow (no. 3) exhibiting behavioural oestrus for three days. Collection of urine soon after voiding. Regression of the vaginal oestrus occurred during the second day of behavioural oestrus. Symbols as in Fig. 1.

Table 1. Urinary excretion of oestrone in sows during oestrus. Mean values ± s (n = 5) on consecutive days.
The day of behavioural oestrus commencement is designated Day 0. The individual patterns are recorded in Fig. 1, 2 and 3.

Day	Urine, l per 24 hrs.	Oestrone, µg per l	Oestrone, µg per 24 hrs.
— 2	2.5 ± 1.1	12.2 ± 5.0	32.5 ± 19.8
— 1	2.7 ± 1.1	17.4 ± 8.2	51.1 ± 42.2
0	4.5 ± 1.4*)	19.5 ± 6.0	87.3 ± 37.6
+ 1	2.0 ± 0.8	5.5 ± 0.9	11.3 ± 4.8
+ 2	2.9 ± 1.0	2.6 ± 0.6	7.4 ± 2.4

*) Significantly different (t-test) from the volumes on Day — 1 ($P = 0.05$) and on Day + 1 ($P < 0.01$).

more than 1/10 of the sum total oestrone and oestradiol. The sums total of oestrone and oestradiol excreted on the first day of behavioural oestrus amounted to 44 — 71 — 105 — 111 and 146 µg respectively and the mean value was 95.4 µg. On the following day the corresponding amounts excreted were 8 — 21 — 7 — 18 and 15 µg, the mean value being 13.8 µg. The mean values of the concentrations of these days were 20.1 µg and 6.8 µg per 1 respectively. The mean of the ratios between the amounts excreted on the two days (10/1.3 = 7.7) differed appreciably from the mean of the ratios between the corresponding concentrations (10/3.2 = 3.1). This was due to the voiding of relatively large volumes of urine on the day of the highest oestrogen excretion (Table 1).

Expressed as colour equivalents of dehydroepiandrosterone, the amounts excreted of Zimmermann chromogens (Fig. 1—3) varied between 1.2 and 8.8 mg per 24 hrs. The variations in the excretion of these substances appeared to occur independently of the variations in the excretion of oestrogens.

DISCUSSION

The first recognizable signs of oestrus in the sow are the alterations taking place in the external genitalia, i. e. hyperemia and edema of the vulva. These alterations are factors of the vaginal oestrus. The vaginal oestrus evidently coinsides in time with the growth of the follicles which are to ovulate and seems to be induced by an increase in the ovarian production of oestrogens. The data presented indicate that the resulting oestrone

excretion decreases rather abruptly at about the middle of the period of behavioural oestrus. This is the time at which regression of the vaginal oestrus occurs (*Burger* 1952), the symptoms being fading of the bright red colour and decreased edema of the vulva with incipient wrinkling of the vulvar skin. It appears therefore that the increased urinary excretion of oestrogens coincide in time rather with the vaginal than with the behavioural oestrus.

In association with oestrus, an increase, followed by a sudden decrease, in the urinary concentration of oestrone has previously been observed by *Velle* (1958) and *Raeside* (1961). The findings presented here indicate that urinary concentration of oestrogens largely reflects the rate of oestrogen excretion. Serial estimations of the urinary concentration of oestrogens would thus seem adequate for the purpose of assessing variations in the ovarian activity with respect to oestrogen production. The implication is that, in clinical investigations including analyses of oestrogens, valuable information on the ovarian function may be obtained without necessarily taking efforts to collect the urine quantitatively in order to determine the total amounts of oestrogens excreted. As far as the normal oestrus is concerned, the maximal excretion of oestrogens was frequently associated with a simultanous maximum in the volume of the 24 hrs. urine. At this stage the day-to-day variations in the urinary concentration of oestrogens were therefore smaller than the corresponding variations in the total amounts excreted. Although it would be assumed that the total amount of oestrogens excreted is the better parameter of the ovarian production of oestrogens, it can not be excluded that the urinary excretion of oestrogens is to some degree dependent on the amount of urine formed. Indeed, *Savard* (1961), in a study on oestrogens in pregnant mares, noticed that the amounts excreted apparently varied with the urinary volumes. The physiological significance of the fluctuations found in the urinary volume during oestrus in the sow is obscure.

The abrupt decrease subsequent to the maximum of the urinary excretion of oestrogens during oestrus obviously indicates decreased oestrogen production in the ovaries. It seems reasonable to assume that the regression of ovarian oestrogen production is timed with the ovulation which in the sow occurs at some time during the middle of the period of behavioural oestrus (*Burger* 1952). It remains to be shown if the oestrogen produc-

tion proceeds up to the time of follicular rupture or if it is terminated at an earlier time. For the study of such a relationship other methods than those used in the present investigation would be required, for example analyses of urine collected continously by an indwelling catheter (cf. *Raeside* 1963) or estimation of blood oestrogens. It has been shown that oestradiol-17β administered intramuscularly gives rise to urinary oestrone within 3 hrs. and that most of the metabolite is eliminated within 2 days (*Lunaas* 1963 a). When administered in this way the absorption from the injection site might be considerably protracted. The time required for the elimination of oestrogens released from the ovaries may be expected to be much shorter since oestrogens seem to be very rapidly removed from the blood (*Shaver et al.* 1963). In man it has been shown that the blood levels of oestrone during the menstrual cycle are correlated with the amounts of oestrone excreted with the urine (*Roy* 1962). The present results, as well as those recently published by *Raeside* (1963), indicate that the urinary excretion of oestrogens in the sow decreases prior to the regression of the behavioural oestrus. It seems safe to conclude therefore that the sexual receptivity in this species persists for a period of time after clearance from the blood of ovarian oestrogens. This sequence of events under physiological conditions is in correspondence with the finding of *Brander & Robinson* (1962) that, in the spayed, progesterone primed ewe, one single injection of oestrogens is as effective for the induction of behavioural oestrus as is a continous infusion over a period prolonged up to or beyond the time at which heat symptoms become evident. As pointed out by these authors, the possibility exists that the ovarian production of oestrogens ceases at some time prior to ovulation and that the subsequent decrease in the blood levels of oestrogens permits a release of pituitary gonadotrophins required for the final maturation of the follicles. This hypothesis is compatible with the findings in the sow when it is considered that a lag must necessarily occur between the time at which the oestrogens are circulating and the time at which the 24 hrs. urine containing the metabolites can be secured.

The ratio between oestradiol-17β and oestrone in the follicular fluid in the sow's ovary is about 9:1 (*Lunaas* 1963 b). Oestradiol-17β can be expected to constitute a large proportion of the total amounts of oestrone and oestradiol-17β released from the

ovaries during oestrus. The amounts of oestradiol, probably oestradiol-17β, found in the urine were small in comparison with those found of oestrone, presumably due to transformation peripherally of oestradiol-17β into oestrone prior to the excretion (*Lunaas* 1963 a). The urinary levels were, however, above the detection limit, at least during maximal oestrone excretion, except in one animal. Although the vaginal oestrus was easily recognized in this animal, any subsequent behavioural oestrus could not be demonstrated. Before any significance can be attached to the urinary levels of oestradiol regadring behavioural oestrus, further studies on the normal variations are needed.

Behavioural oestrus has been induced by parenteral administration of testosterone in the ewe (*Lindsay & Robinson* 1961). It is generally held that libido in women is influenced to a considerable degree by androgens of adrenal origin (cf. *Waxenberg et al.* 1957). The excretion of androgen metabolites appears not to vary appreciably during the menstrual cycle (*Borth et al.* 1957). The role played by androgens in the development of oestrus under physiological conditions seems to be unknown. Androgens are present in the follicular fluid of i. a. the mare (*Short* 1961) and of the cow (*Short* 1962), probably as intermediates in the biosynthesis of oestrogens. The follicular levels of androgens and of oestrogens appear to be of the same order of magnitude. Ovarian androgens could give rise to urinary 17-ketosteroids which are chromogenic in the Zimmermann reaction. In the sow it was found that the amounts of Zimmermann chromogens present in the urine, calculated as colour equivalents of dehydroepiandrosterone, exceeded the amounts of oestrone by a factor of 100 or more. If the ratio between androgens and oestrogens in the porcine ovary is comparable to those in the species mentioned, is seems likely that 17-ketosteroids originating from ovarian androgens represent only a very small fraction of the Zimmermann chromogens found in the urine and that possible fluctuations in their excretion are entirely masked by variations in the urinary levels of adrenal steroid metabolites or nonsteroidal chromogens. Adrenal production of androgens could depend on the functional state of the ovaries since oestrogens tend to accumulate selectively in, among other tissues, the adrenal cortex (*Ullberg & Bengtsson* 1963) and since oestrogens seem capable of interfering with the biosynthesis of adrenal steroids (*McKerns* 1963). In the present investigation,

however, no relationship could be demonstrated between the amounts of Zimmermann chromogens and of oestrogens excreted during oestrus. It should be noted that the Zimmermann reaction for the determination of "total urinary 17-ketosteroids" may yield results of very questionable significance even in man (*Goldzieher & Axelrod* 1962) although human urine presumably contains relatively small amounts of plant material known to give the Zimmermann colour in analysis of for example bovine urine (*Holtz* 1957). As indicated by the development of apparently specific Zimmermann colour in the absence of m-dinitrophenol, interfering substances evidently occurred in variable amounts in the sow's urine examined. Despite introduction of measures believed to improve specificity, the method used was probably adequate only for the detection of gross alterations in the urinary excretion of 17-ketosteroids.

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SUMMARY

By analyses of 24 hrs. urine, the excretion of oestrone and oestradiol and of Zimmermann chromogens, comprizing 17-ketosteroids, was estimated in sows during oestrus. Generally it was found that the excretion of oestrogens increased during vaginal oestrus. The maximal excretion of oestrogens, mean: 95 μg per 24 hrs. (n = 5), occurred at the first day of psychic oestrus. On the following day the mean value was 14 μg per 24 hrs. The decrease in the rate of excretion of oestrogens during the course of psychic oestrus was more pronaunced than the corresponding decrease in the urinary concentration of oestrogens, means: 20 μg to 7 μg per l, due to appreciable variations in the volume of the 24 hrs. urine during oestrus. The relationships in time between the oestrogen excretion and the vaginal and the psychic oestrus is discussed.

The amounts of oestradiol in the urine were relatively small, rarely constituting more than $^1/_{10}$ of the sum total oestrone and oestradiol. The excretion of oestradiol was demonstrated to increase appreciably during oestrus except in one animal which, despite development of hyperemia and edema of the vulva, failed to come into psychic oestrus.

Large variations were found in the excretion of Zimmermann chromogens (expressed as equivalents of dehydroepiandrosterone: 1 to 9 mg per 24 hrs.) but these appeared to occur independently of the variations in the excretion of oestrogens.

ZUSAMMENFASSUNG

Die Ausscheidung von Östrogenen und Zimmermann-Chromogenen mit dem Harn von Säuen während des Östrus.

Bei Analysen von Harn ausgeschieden im Laufe von 24 Stunden, wurde die Ausscheidung von Östron und Östradiol und von Zimmermann-Chromogenen, welche die 17-Ketosteroiden umfassen, bei Säuen während des Östrus bestimmt. Im allgemeinen zeigte es sich, dass die Ausscheidung von Östrogenen während des vaginalen Östrus zustieg. Die maximale Östrogenausscheidung, Mittel: 95 μg pro 24 Stunden (n = 5), zeigte sich am ersten Tag des psychischen Östrus. Am fol-

genden Tag war der Mittelwert 14 μg pro 24 Stunden. Der Fall in der Totalausscheidung der Östrogene im Laufe des psychischen Östrus war grösser als der entsprechende Fall in der Östrogenkonzentration des Harns, Mittel: 20 μg bis 7 μg pro Liter, da das tägliche Harnvolumen während des Östrus grossen Variationen ausgesetzt war. Die Zeitverhältnisse in Bezug auf die Östrogenausscheidung und auf den vaginalen und psychischen Östrus werden diskutiert.

Die Östradiolmengen im Harn waren verhältnismässig gering und machten selten mehr als $^1/_{10}$ der Summe von Östron und Östradiol aus. Ein bedeutender Anstieg in der Östradiolausscheidung während des Östrus wurde bei allen Tieren festgestellt — mit einer Ausnahme, wo trotz Hyperämie und Ödem in der Vulva kein psychischer Östrus eintraf.

Grosse Variationen in der Ausscheidung von den Zimmermann-Chromogenen wurden festgestellt (ausgedrückt als Äquiavalenten von Dehydroepiandrosteron: 1 bis 9 mg pro 24 Stunden). Jedoch schienen diese unabhängig von den Variationen in der Östrogenausscheidung stattzufinden.

SAMMENDRAG

Utskillelsen av østrogener og Zimmermann-kromogener med urinen hos purke under østrus.

Ved analyser av døgnurin ble utskillelsen av østron og østradiol og av Zimmermann kromogener, omfattende 17-ketosteroider, bestemt hos purker under østrus. Stort sett ble det funnet at utskillelsen av østrogener steg under vaginal østrus. Den maksimale østrogenutskillelse, middel: 95 μg per 24 t (n = 5), fant sted ved første dag av psykisk østrus. Den påfølgende dag var middelverdien 14 μg per 24 t. Fallet i totalutskillelsen av østrogener i løpet af psykisk østrus var mer uttalt enn det tilsvarende fall i urinens konsentrasjon av østrogener, middel: 20 μg til 7 μg per l, i det urinens døgnvolum undergikk betydelige variasjoner under østrus. Forholdene i tid med hensyn på østrogenutskillelsen og på den vaginale og den psykiske østrus diskuteres.

Mengdene av østradiol i urinen var forholdsvis små og utgjorde sjelden mer enn $^1/_{10}$ av summen østron og østradiol. Det ble påvist en betydelig økning av østradiolutskillelsen under østrus unntatt hos ett dyr som, til tross for utvikling av hyperemi og ødem i vulva, ikke kom i psykisk østrus.

Det ble funnet store variasjoner i utskillelsen av Zimmermann-kromogener (uttrykt som ekvivalenter av dehydroepiondrosteron: 1 til 9 mg per 24 t), men disse syntes å finne sted uavhengig av variasjonene i østrogenutskillelsen.

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