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THE URINARY EXCRETION OF 5β-PREGNANE-3α: 20α-DIOL AND GESTATIONAL FAILURE IN ANGORA GOATS

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The serious proportions which habitual abortion in Angora Goats have assumed have been outlined by van Heerden (1961a, b). As the incidence in some flocks is as high as 70 per cent, the problem is the cause of severe limitations to the expansion of the mohair industry in South Africa. Recently abortions, apparently resulting from the same cause, have been reported from Texas (van Heerden, personal communication).

Regressive changes of the corpus luteum verum, at or following abortion, were described by van Rensburg & van Rensburg (1961). During the course of his studies on the histopathology of the corpora lutea from these cases, van Heerden (1961a, b) described regressive changes which he associated with a decrease in the alpha cell activity of the adenohypophysis. The theory was advanced of an inherent weakness of the hypophysealgonadal axis, characterised by premature regression of the corpus luteum verum consequent to inadequate secretion of luteotrophic hormone.

The above findings were based upon instances where abortion either had occurred or was suspected to be imminent. The extent to which these changes may have been secondary to foetal death, possibly even to unrelated causes, is not clear.

In the present study the urinary excretion of 5β-Pregnane-3α:20α-diol (pregnanediol), which is assumed to be an approximate reflection of the concentration of progesterone in peripheral blood and hence of corpus luteum function, was followed from three to four weeks after conception to the termination of pregnancy.

Experimental Procedure

The Angora ewes used in this study were obtained from three affected flocks in the Cape Midlands during 1958 and were kept under close observation at this laboratory. Fertile, raddled Angora rams were introduced during the autumn of 1960 and any marking of the ewes was noted daily. During the following six months the paddock was inspected each day for signs of aborted foetuses and the ewes were examined for evidence of the termination of pregnancy. In the event of a foetus or foetal membranes being found, all the ewes were examined *per vaginam*. If any ewe was suspected to have aborted laparotomy was performed for confirmatory examination of the uterus and ovaries.

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A total of twenty marked ewes was transferred in rotation to metabolism cages for urine collection. Each ewe was subjected to an average of eight urine collection periods each of three days duration at approximately 18 day intervals during the 150 days following service.

Urine was collected over a 72 hour period into a 3 litre storage bottle, which contained 10 ml toluene, placed in a box in dry ice. The urine in each bottle was transferred daily to larger storage bottles also containing about 10 ml of toluene. The pooled urine samples from each goat were kept in a cold room at 4°C until the end of the collection period, when they were treated as detailed below. The exact period of collection, and the total volume of urine voided was recorded for each animal.

Analytical Methods

All analyses were performed in duplicate, half the total volume of urine passed with a maximum of 500 ml being used for each determination. The method of Klopper, Michie & Brown (1955), with modifications, was used for the estimation of pregnanediol. The method depends upon isolation of pregnanediol from urine by preliminary hydrolysis of its conjugates with acid (e.g. hydrochloric acid) and subsequent extraction of the free steroid with toluene. Since other steroids are extractable by this method, the pregnanediol is separated chromatographically on alumina columns prior to colour development with sulphuric acid.

Since goat urine is highly alkaline due to the presence of considerable amounts of bicarbonate, the procedure of Klopper *et al.* (1955) was modified as follows. To 500 ml of urine 100 ml of concentrated hydrochloric acid (assay 31·8 per cent, S.G. 1·16) were added slowly while stirring. When effervescence due to the decomposition of bicarbonates ceased, 170 ml of toluene were added to the mixture. This mixture was placed in a round-bottomed flask connected to a reflux condenser, a few glass beads being added to prevent bumping. For samples of urine less than 500 ml, the proportional amounts of acid and toluene were added. Thus the quantities of acid used were in all instances sufficient to render the mixture strongly acid and to ensure complete hydrolysis of pregnanediol conjugates.

The mixture as prepared above was heated to boiling and then refluxed for twenty minutes. After cooling, the toluene layer was removed by means of a separating funnel, placed in a separate container and stored in the refrigerator. A further 170 ml of toluene were added to the treated urine, the mixture vigorously shaken and allowed to stand overnight. The separated toluene layer was drawn off and added to the first extract together with half of the toluene overlays accumulated from the initial collection procedures. The other half of this toluene was added to the duplicate sample extracts.

The combined toluene extracts were washed with alkaline sodium chloride, permanganate and water as outlined by Klopper *et al.* (1955) and the toluene distilled off under vacuum until a small volume remained. This latter stage of the procedure is best carried out in a rotary evaporator. The concentrated extract was either processed further at once or stored in the refrigerator for a day or two until it could be taken further.

Preliminary chromatography, acetylation and the final separation of pregnanediol diacetate were performed exactly as described in the original method. Colour was developed, as recommended, with concentrated sulphuric acid and sodium sulphite, but, in all samples, colour was allowed to develop by placing the sample tubes in an incubator at 25 °C for 17 hours.

Standards were prepared using accurately weighed amounts of chromatographically pure 5β-Pregnane-3α:20α-diol* approximating the expected 72 hour excretion of this steroid by a pregnant ewe at the different collection periods. The standard amounts of pregnanediol were then dissolved in 10 ml of toluene, added to a mixture of 500 ml of water and 100 ml of concentrated hydrochloric acid and taken through the entire range of isolation and chromatographic purification procedures.

All photometric estimations were performed using the " E.E.L." Portable Model A photoelectric colorimeter, readings being made at 430 mμ (Ilford OBIO filter) against a concentrated sulphuric acid sodium sulphite blank. At intervals throughout this work the presence of possible interfering subtances present in the solvents used was tested for by reading sulphuric acid solutions of the residues from a complete " blank " run of the entire procedure against the sulphuric acid reference blank as mentioned above. Interference from the reagents used was at all times found to be negligible.

In the final calculations the total volume of urine passed in each 72 hour test period was taken into account and all the figures obtained are expressed as mg of pregnanediol excreted per 24 hours.

All reagents used were of " Analytical Reagent " grade, the solvents and acetylchloride being redistilled before use. The alumina used throughout was " aluminium oxide standardised for chromatography ".† It was deactivated to activity 4 on the Brockman scale as described by Klopper *et al.* (1955), the standard pregnanediol samples serving as a check upon its activity and upon the procedure in general. It is most important to check the activity of the alumina repeatedly with pure pregnanediol since, if allowed to become too moist, most of the pregnanediol may be lost in the eluates from the first chromatographic procedure before acetylation. The conditions necessary for attaining the required deactivation were found to vary considerably with ambient laboratory temperatures and should be determined for every set of environmental circumstances in any laboratory using this procedure to ensure replication of results.

The columns were contained in glass burettes of approximately the dimensions of the tubes used by the authors of the original procedure. They were supported by a plug of glasswool covered by a 0·5 cm layer of fine quartz sand, the top of the alumina column also being covered by a similar layer of quartz sand.

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* Obtained from L. Light and Co., Colnbrook, Bucks., England

† Obtained from Riedel-de-Haën, A.G. (Seelze, Hannover, Federal Republic of Germany)

The goats were maintained on a diet of lucerne hay supplemented with a small quantity of green lucerne. It was found that under these conditions large amounts of indigoid pigments appeared in the urine. These are taken up by the toluene and impart to it a deep reddish purple colour after acid hydrolysis of the urine. The identification and characterisation of these pigments will be reported on. These pigments do not interfere with the estimation of the pregnanediol under the conditions of the method, since they are destroyed apparently during the washing of the toluene extracts with alkaline permanganate and the products so formed are eliminated in the chromatographic steps. The presence of other possible interfering substances in herbivore urines, e.g. ionone compounds, has been noted by Klyne & Wright (1957), and it is possible that traces of other closely related steroids may come off the columns concurrently with the pregnanediol under the specified conditions. Since, however, the work being reported on was intended to demonstrate differences in the levels of pregnanediol excreted by groups of goats maintained under identical conditions and not the absolute amount excreted the errors inherent in the method, especially those due to non-specific chromogens, may be assumed to be constant and for the purpose of interpretation of the results may be disregarded.

RESULTS

Five of the twenty ewes used failed to kid during the experiment and at no time was any evidence of termination of gestation observed. Although the possibility of early foetal resorption could not be excluded in these animals, for the purposes of the experiment, they were considered to have failed to conceive. Pregnancy in the other fifteen ewes terminated as indicated in Table 1.

TABLE 1.—*Result of Gestation in the Experimental Ewes*

Results of Gestation	No. of Ewes
Single kiddings, kids survived	2
Single kiddings, kids died within 3 days	4
Twin kiddings, kids survived	2
Stillborn kids, normal term	1
Premature kiddings	2
Abortions, single kids (before 125 days gestation)	3
Abortions, twins	1
Ewes that failed to kid	5
TOTAL	20

The mean pregnanediol excretion during each third of pregnancy by each individual ewe, together with outcome of gestation and the previous breeding history, is presented in Table 2. Also recorded are figures illustrating the mean pregnanediol output over the entire period and the upper and lower limits of daily excretion of this metabolite.

TABLE 2.—*Mean Pregnanediol Excretion for each third of Gestation of Individual ewes and outcome of Gestation*

Group.	Ewe No.	Previous breeding history	Mean daily pregnanediol excretion (mg per 24 hours)					Outcome of gestation
			Days after service			Upper and lower limits of individual determination	Total mean	
			0–50	51–100	101–150			
Single gestations...	1	Kidded, 1959..........	37·8	7·9	4·8	43·4– 4·2	16·8	Single surviving kid
	2	—	58·3	8·6	8·0	62·5– 6·2	25·0	Single surviving kid
	3	Maiden ewe...........	29·2	10·5	7·8	29·2– 6·3	15·8	Kid died after 2 days
	4	Maiden ewe...........	21·6	6·4	5·9	24·5– 4·2	11·3	Kid died after 3 days
	5	Maiden ewe...........	13·2	3·9	8·6	13·2– 3·6	8·6	Kid died when 1 day old
	6	Maiden ewe...........	13·0	6·1	4·8	19·2– 2·7	8·0	Kid died when 1 day old.
	7	Maiden ewe...........	5·8	4·9	4·8	6·4– 4·1	5·2	Kid stillborn.
	8	Aborted, 1958, 1959....	60·8	9·7	4·0	80·5– 3·3	24·8	Premature 19 days, stillborn
	9	Aborted, 1958..........	51·4	12·1	6·3	51·4– 5·8	23·3	Premature 12 days, ewe emaciated
	10	Maiden ewe...........	4·4	3·8	—	6·2– 1·9	5·5	Aborted 76 days after service
	11	Small weak kid, 1959...	5·5	5·4	7·8	7·8– 4·2	4·6	Aborted 111 days after service
	12	Aborted, 1958, 1959....	5·1	8·4	4·1	10·6– 1·1	3·9	Aborted 120 days after service.
Multiple gestations	13	Kidded, 1959..........	25·4	18·6	22·2	28·6–14·1	22·1	Normal monozygous twins
	14	Kidded, 1959..........	51·1	10·1	5·6	58·7– 3·6	22·3	Normal dizygous twins
	15	Aborted, 1958, weak kid, 1959	16·1	11·0	7·4	18·3– 6·1	11·5	Aborted dizygous twins at 125 days
Ewes which failed to kid	16	Kidded, 1959..........	41·2	12·4	10·2	50·1– 4·2	21·3	Failed to kid
	17	Aborted, 1958, kidded, 1959	6·6	8·5	5·1	10·7– 4·4	6·7	Failed to kid
	18	Maiden ewe...........	13·1	4·4	2·1	23·1– 1·8	6·5	Failed to kid
	19	Maiden ewe...........	16·7	2·1	3·0	30·4– 1·8	7·3	Failed to kid
	20	Maiden ewe...........	7·3	2·5	2·7	11·0– 0·9	4·2	Failed to kid

Pregnancies terminating in normal parturition were characterised by a very high excretion rate of pregnanediol during the first third of the gestation period, followed by a marked decline to a low level of excretion during the second and final thirds. In the group which aborted a marked deviation from this pattern was apparent in that, during the first third of gestation, the rate of excretion of pregnanediol was exceptionally low.

In the case of the four ewes (No. 3, 4, 5, 6) whose lambs died within three days of birth after a normal term of gestation, pregnanediol excretion for the first third of gestation was intermediate between that of the normal and abortion groups. These findings are readily appreciated by inspection of Figure 1.

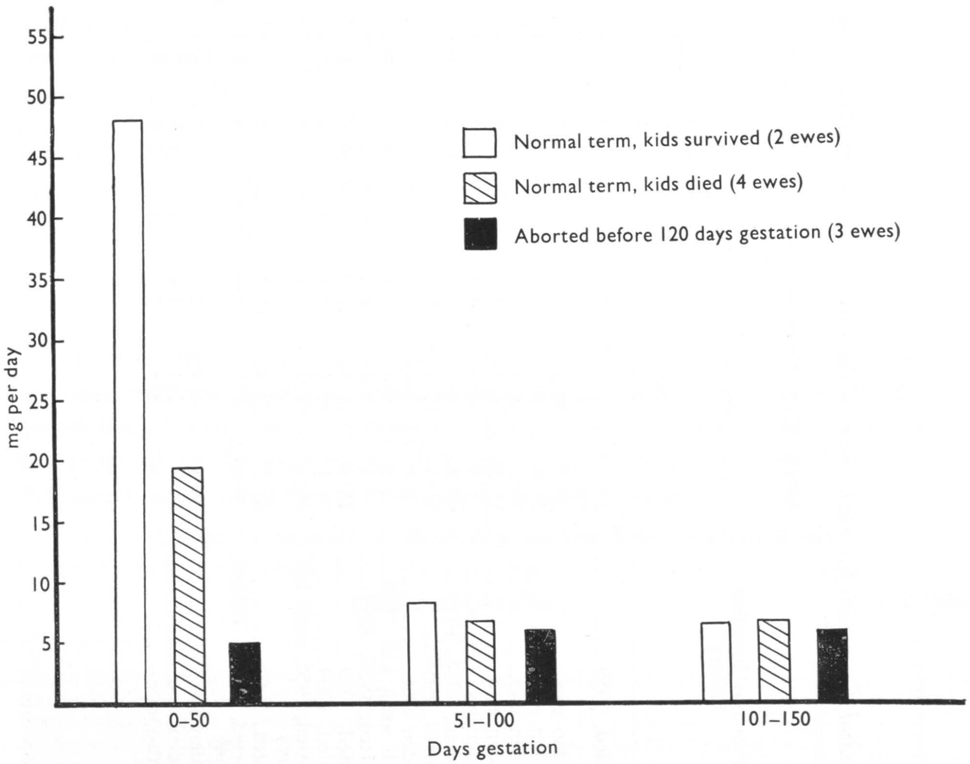

FIG. 1.—Mean daily pregnanediol excretion for each group

The pattern of pregnanediol excretion by an individual animal from each group is illustrated in Figure 2 (Ewes No. 1, 4 and 11, Table 2). The ewe (No. 7) which gave birth to a stillborn kid at normal term, complicated by apparent uterine inertia, showed the exceptionally low excretion rates pertaining to aborting ewes. In ewe No. 8, in which pregnancy terminated 19 days before term, the initial excretion values were similar to those seen in the normal group. Further, ewe No. 9, which gave birth 12 days prematurely when in an emaciated condition, showed essentially the “ normal ” excretion pattern.

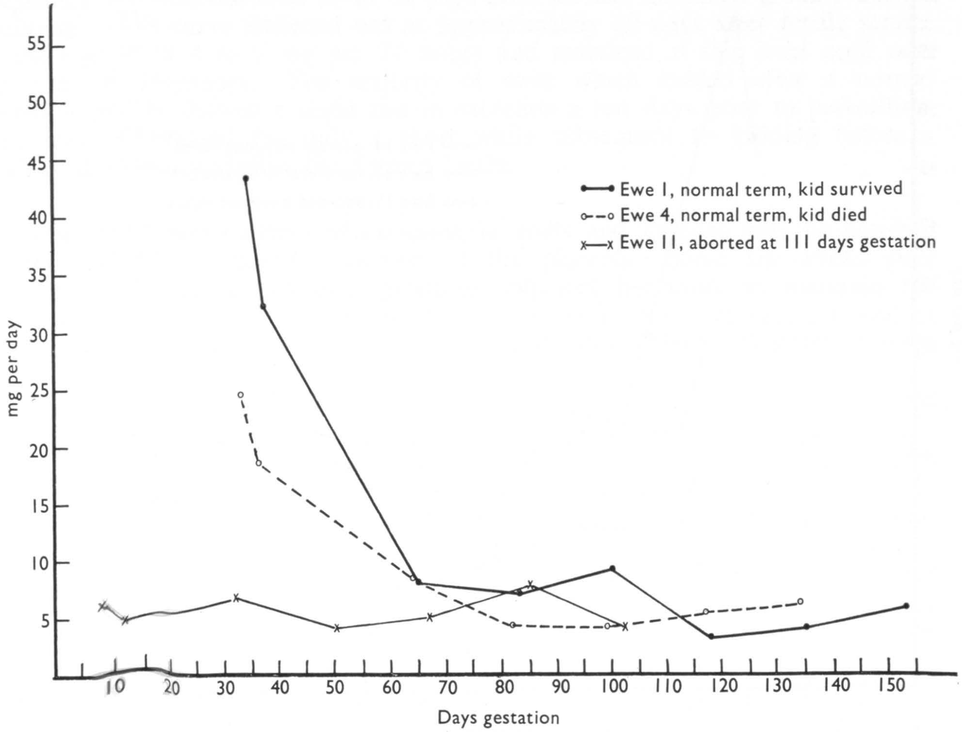

FIG. 2.—Pregnanediol excretion in single pregnancies

The two sets of normal twins were found to be monozygous (No. 13) and dizygous (No. 14) twins as judged by the number of corpora lutea seen at laparotomy. The ewe having two corpora lutea (No. 14) excreted twice as much pregnanediol during the first third of pregnancy as did the mother of the monozygous twins (No. 13). The excretion rate of this latter animal, however, was maintained, during the last 100 days of gestation, at a level sufficient to present an overall mean excretion figure similar to that of ewe No. 14.

Ewe No. 15, which aborted dizygous twins 125 days after service, showed values for pregnanediol output higher than its normal counterpart during the last two-thirds of term, but the mean value during the initial fifty days of gestation was one-third that of the normal twin-bearing goat.

Examples of pregnanediol excretion in multiple pregnancies are illustrated in Figure 3.

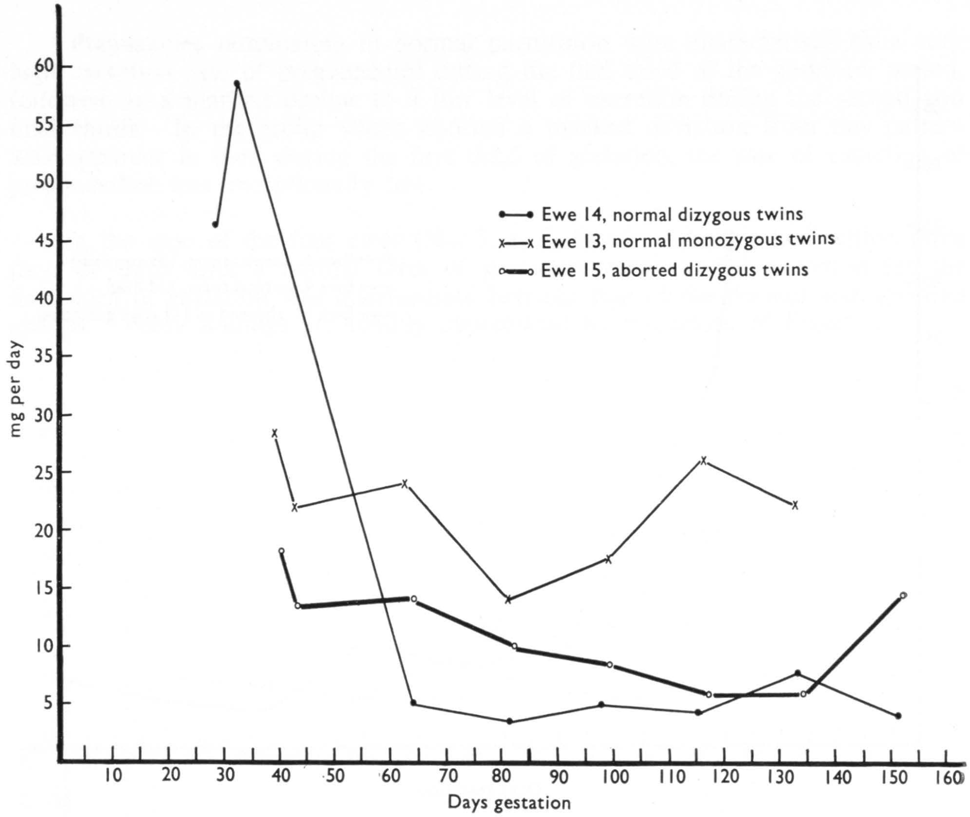

FIG. 3.—Pregnanediol excretion in multiple pregnancies

On the whole, pregnanediol output in the ewes which presumably failed to conceive was surprisingly similar to that of the pregnant animals which carried to full term. However, with the exception of ewe No. 16, the figures obtained during the first third of term were far below those observed during the same period in the two normal ewes whose kids survived (No. 1 and 2). Ewe No. 16 is interesting in that she has a history of a previous normal kidding. In this experiment she showed a pregnanediol excretion pattern very similar to that of the normal pregnant ewes. Ewe No. 17 with her consistently low excretion rate and history of a previous abortion parallels the aborting group very closely. It is possible that in these cases early resorption of the foetus may have occurred.

DISCUSSION

Previous work by Klyne & Wright (1957) on pregnanediol excretion by pregnant goats (in which the breed was not specified) revealed an excretion rate in the order of 5 mg per day. Their urine samples were taken at a late stage of pregnancy. During the course of the present investigation determinations were performed from 25 days after service until after kidding.

The human shows a curve of pregnanediol excretion that rises progressively until approximately the 37th week of gestation (Venning, 1938; Coyle, Mitchell & Russel, 1956) when values of 40 to 50 mg per 24 hours are encountered.

A strikingly different pattern of excretion by pregnant Angora goats has been found in the present work. High values, comparable to those found in late human pregnancy were encountered 25 to 45 days after service, thereafter a sharp decline occurred. The curve flattened out at approximately 60 days after fertile service to the region of 4 to 9 mg per 24 hours and remained at this level until near the end of pregnancy. The majority of ewes which kidded after a normal gestation period showed a slight rise in excretion a few days prior to parturition. This was maintained for only a short while subsequent to kidding before a gradual decrease to below the former levels.

The discrepant patterns of excretion in goats and humans may be ascribed to differences in endocrine function of the placenta. Some six weeks after conception the human placenta produces sufficient hormones to maintain the integrity of the site of implantation (Tulsky & Koff, 1957) whereas removal of corpora lutea in the pregnant goat results in abortion (Meites, Webster, Young, Thorp & Hatch, 1951).

The results obtained in this work show little correlation between the excretion of pregnanediol in late pregnancy and the outcome of pregnancy. A striking relationship exists, however, between low initial excretion and gestational failure. Only two of the 15 ewes had high initial excretion with an unsatisfactory gestation outcome. Ewe No. 9 gave birth 12 days prematurely when in an emaciated conditon, and pregnancy terminated 19 days prematurely in No. 8, a few days after an excessively low rate of excretion was recorded. This value of 3·3 mg per 24 hours (Table 2) was lower than any figure obtained from goats that produced viable kids.

It would appear from the results presented in this paper that an initial high production of progesterone is essential for maintenance of gestation in the Angora goat. A comparison of excretion patterns of the two sets of dizygous twins further supports this conclusion. Ewe No. 15, which aborted, actually had excretion values slightly higher than No. 14 during the last two-thirds of gestation, but excretion in the first 50 days was only one-third that of the ewe which kidded normally.

The results obtained from the goats having multiple pregnancies appear to be in agreement with those of Rawlings & Krieger (1960) in the human, which suggested that higher blood progesterone values found in multiple pregnancy (Short, 1961) are essential for a favourable conclusion of such states.

Short (1957) was unable to detect any progesterone in the placenta of the goat. Since the pattern of pregnanediol excretion found in the ewes which failed to kid was similar to that seen in pregnant ewes, this suggests further evidence of an extra-trophoblastic origin of this metabolite.

It would appear from the present work that in the Angora goat, at least, adequate secretion of progesterone by the corpus luteum is the most important single factor in maintaining gestation. Pregnanediol excretion from as early as 30 days after conception would appear to be an outstanding prognostic guide as to the outcome of gestation in these animals and to the probable fate of the foetus.

In habitually aborting ewes the evidence presented indicates that luteal function is usually defective from the commencement of pregnancy. In one instance, however, a ewe (No. 8) appeared to conform to the previously held theory of premature regression of a normal corpus luteum in mid-pregnancy followed by abortion.

The four single kids which succumbed shortly after birth were all out of maiden ewes. The fact that the initial pregnanedol excretion rate of this group was intermediate between those of the normal and aborting groups would suggest that low pregnanediol excretion, and hence low progesterone secretion, is associated with the birth of non-viable kids in these animals. One should, however, interpret these findings with caution since in these maiden ewes maternal or lactational factors may also have been involved. Very poor mammary development has been noted in aborting ewes by van Rensburg & van Rensburg (1961). Brown & de Wet (1963) have demonstrated remarkably low liver iron levels in aborted foetuses as compared with normal foetuses of the same age, which may suggest that placental transfer of nutrients is seriously impaired at an early stage in aborting animals. On the whole, it seems a reasonable assumption that sub-optimal luteal function in the early stages of gestation in these animals is associated not only with prenatal foetal death but also with neonatal mortality.

The limitations of pregnanediol determinations have recently been emphasised by Ober & Kaiser (1961) and Short (1961). Nevertheless, in this group of experimental animals estimation of this compound proved an excellent prognostic guide as to the course to be taken in pregnancy. De Watteville (1951) has also emphasised the useful prognostic value of these estimations in humans.

Summary

The urinary excretion of pregnanediol has been studied over the full period of gestation in a group of twenty Angora ewes. A comparison is drawn between animals in which conception presumably did not occur, those which kidded normally producing strong healthy kids, and those which produced weak under-sized young, stillborn kids or aborted before term.

Evidence is presented suggesting that abortion, prenatal and some instances of neonatal mortality in these animals are associated with deficient luteal function and progesterone secretion during the first third of the gestational period. The urinary excretion of pregnanediol during this period appears to be a useful prognostic guide as to the outcome of gestation in pregnant Angora ewes,

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