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Effects of Steroid and Steroid-Like Compounds on Digestion of Rations by Ruminants

IN VIVO AND *IN VITRO* STUDIES USING DIETHYLSTILBESTROL, CHOLESTEROL, ESTRONE, TESTOSTERONE, CORTISONE AND HEXESTROL.

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SUMMARY

Studies using the artificial rumen technique indicated that digestion of cellulose was improved by the addition of diethylstilbestrol (2-20 ppm.), cholesterol (20 ppm.), estrone (20 ppm.), testosterone (10 ppm.), and cortisone (25 ppm.). Hexestrol (1.5 ppm.), depressed cellulose digestion slightly.

When 10 or 20 ppm. of stilbestrol were added to a cottonseed hull-casein ration, the digestibility of cellulose and protein was increased, but sheep could not tolerate the dosages used.

When 10 mg. testosterone, 1.5 mg. hexestrol or 20 mg. of cortisone per sheep were added to a ration of 800 gm. alfalfa hay and 40 gm. corn, the T.D.N. of the ration increased from 64.7 to 67 percent; the increases were about equally distributed among the fat, fiber and N.F.E. fractions. There was no change in digestible protein.

Seven and one-half mg. hexestrol had no effect on the digestibility of silage rations by steer calves.

Probable sites of the hormone actions are discussed.

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Experiments conducted during the past three years at this Station indicate that certain steroids and steroid-like compounds play important roles in the utilization of ruminant rations. A preliminary report has appeared (Brooks *et al.,* 1954) and much of the work is available in thesis form (Brooks, 1954, and Kelley, 1956).

The studies investigated effects of these compounds on the digestion of ration components by ovine rumen organisms *in vitro* and by mature wethers and wintering calves. This bulletin summarizes the completed series of studies.

Ruminants were known to respond considerably more than swine to implanted diethylstilbestrol (stilbestrol). Investigators had shown that a number of other compounds caused similar activity in other species. Thayer *et al.* ('45) gave a comparison of the response of poultry to various estrogen-like compounds.

Many reports are available regarding the efficacy of oral stilbestrol in the rations of cattle. Good examples of responses are found in the work of Burroughs ('54) and Perry ('55). In most of the work that has been reported, the daily addition of 10 mg. stilbestrol or hexestrol to the fattening rations of steers has caused a 15 to 20 percent increase in rate of gain and a 10 to 15 percent improvement in feed efficiency.

Certain plants have also been shown to contain estrogen-like activity. Cheng *et al.* ('53) and Pieterse and Andrews ('56) have summarized much of the important work.

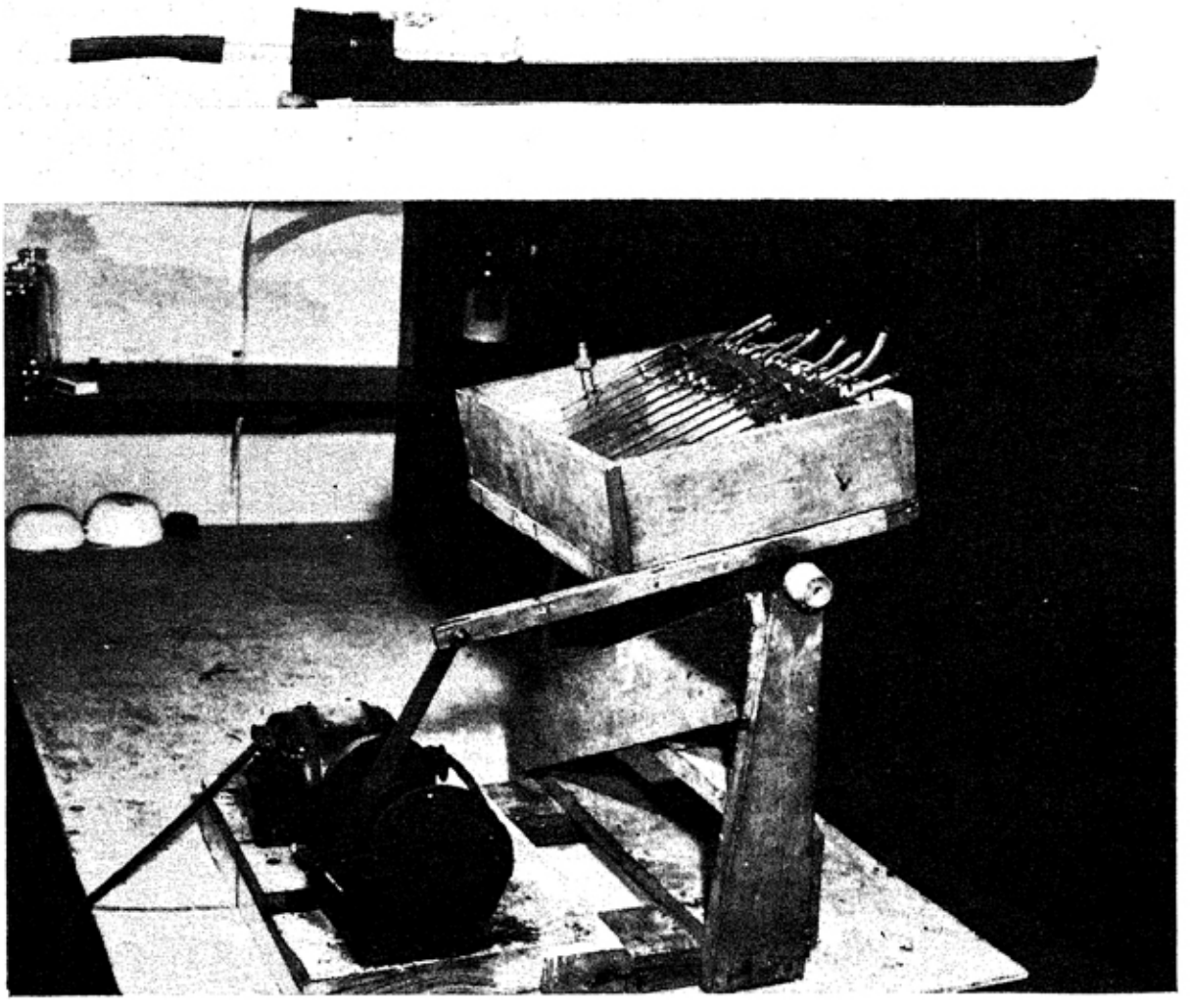

Fig. 1—"Artificial rumen" and rocking device containing "rumens" at start of a test in series I.

MATERIALS AND METHODS

Artificial Rumen Studies.

The artificial rumen was adopted as a screening device to determine the influence of various compounds on the digestion of cellulose. If responses were obtained in the artificial rumen, additional studies were to be initiated with ruminants.

The artificial rumen used in the initial study is shown in Figure 1. This was a test tube fitted with a one hole rubber stopper. An inverted glass tube was placed through the stopper and connected to rubber tubing containing a quarter inch slit in the side. The other end of the tubing was closed with a short glass rod. Groups of these tubes were placed in the rocking agitator shown in Figure 1. The stoppered end of each tube was raised 3 inches above the horizontal and on the down stroke of the agitator, the tube came to the horizontal. Speed of the agitator was con-

trolled by a drive which made 20 complete turns per minute. The entire apparatus was placed in an incubator maintained at 40° C for 40 hours.

Sheep with fistulae closed with the screw-type cannula described by Phillipson and Innes ('39) were donors of rumen organisms. Rumen fluid was removed from donor animals through glass or plastic tubing. It was shaken to remove celluloytic organisms from fiber and strained through four layers of cheese cloth. Ten mg. of the strained fluid were used as inoculum. Each artificial rumen also contained 10 ml. of McDougall's ('48) artificial saliva brought to PH 7 by bubbling carbon dioxide through it and 5 ml. of distilled water.

Several "basal rations" were used in the artificial rumen. In the first trial in series I, 1 gm. of finely ground timothy hay, containing 34 percent cellulose, 25 mg. of urea, and 5 mg. of ammonium carbonate constituted the basal ration. Two trials were conducted with 500 mg. of finely ground filter paper, 70 mg. of casein, 10 mg. of urea, and 10 mg. of ammonium carbonate. A final trial used 920 mg. of finely ground corn cobs and 80 mg. of casein.

Following the incubation, residual cellulose was determined by the anthrone method of Viles ('49).

Stilbestrol was tested at 2, 4, 8, 10, 16, and 20 μg. per tube. In one test, estrone and cholesterol were tested at a level of 20 μg. per tube. The test substances were dissolved in ether and mixed with the cellulose in the "basal ration." The ether was evaporated by placing the tubes on a water bath before starting the trial.

The second series of tests incorporated modifications of the basic ideas of the artificial rumen design used in the first series. The test tube was replaced by a 50-ml. Rockefeller centrifuge tube equipped with a one hole rubber stopper through which was placed a piece of glass tubing.

Glass "T" tubes were connected with rubber tubing to the artificial rumen at the bottom of the "T". The tops of the "T's" were connected in series. The entire series was attached to a CO_2 cylinder so that CO_2 could be bubbled through the solution as desired.

The nutrient solution used in all tests in the second series contained 400 mg. of urea, 400 mg. of ammonium carbonate, 400 mg. of dextrose, 8 ml. of 10 percent solution of $CoCl_2 \cdot 6H_2O$, and 92 ml. of water. One ml. of this solution was added to each artificial rumen. The cellulose source in all tests was Solka-floc obtained from the Brown Corporation.

Digestion Trials.

Sheep:

Series I. The *in vivo* effect of stilbestrol on cellulose and protein digestion was studied in three lots of five cross-bred yearling wethers. The basal

ration provided 908 gm. of cottonseed hulls, 95 gm. of casein, 6 gm. of chromic oxide, and 2,500 I. U. of vitamin A per sheep daily. Wethers had free access to salt and a mineral mixture of equal parts dicalcium phosphate, calcium carbonate, and sodium chloride containing 2 oz. of cobalt sulfate per 100 lb. of mixture. Five sheep received the basal ration, five received 10 mg. stilbestrol, and five received 20 mg. stilbestrol per day. Fourteen-day preliminary periods were followed by four-day collection periods. The daily collections consisted of two-hour samples obtained morning and evening while the wethers were confined in the stalls. These "grab samples" were placed in plastic freezer bags and frozen at -20° F. At the end of the collection period, collections were thawed to room temperature and all collections from each sheep were thoroughly mixed and placed in a forced draft oven at 55° C. for 48 hours. They were then mixed and ground through a 20 mesh screen on a Bird Impact mill, mixed a third time and sealed in a screw-top sample jar until chemical analyses were run.

Series II. In the second series of tests, total collection was substituted for the chromic oxide reference method. Eight hundred gm of number 3 alfalfa hay and 400 gm. of shelled corn were divided into two equal parts and fed at 7:00 a.m. and 4:00 p.m. The experimental design is shown in Table 1. At the end of the digestion trials, samples of rumen fluid were obtained on three consecutive days for analysis for volatile fatty acids.

TABLE 1. EXPERIMENTAL DESIGN FOR TESTING THE EFFECTS OF HEXESTROL, TESTOSTERONE AND CORTISONE ON THE DIGESTIBILITY OF AN ALFALFA-HAY-CORN RATION.

	Treatment			
Trial	Basal Ration	+Testosterone 20 mg.	+ Hexestrol 1.5 mg.	+Cortisone 10 mg.
	Sheep number			
1	10	1	4	7
	11	2	5	8
	12	3	6	9
2	10	1	4	7
	11	5	2	6
	12	8	9	3

Steers:

Steer calves that were being wintered on drought corn, sudan grass and wheat silages were divided into two equal groups. One lot received the silage ration plus 1 lb. of cottonseed meal containing 2.38 percent chromic oxide. The other groups received the same ration but the cottonseed meal contained 7.5 mg. hexestrol per calf per day.

The details of the digestion trials have been described by Pfander, *et al.* ('57).

Chemical Procedures.

Fecal samples in series I tests were analyzed for cellulose by the Crampton-Maynard method and in series II, by both the Crampton-Maynard and the Matrone-Ellis-Maynard techniques. Chromic oxide was measured by the method described by Gehrke *et al.* ('50) with the oxidation procedure modified according to Bolin *et al.* ('52). Nitrogen was determined by the AOAC method, modified in series II by using boric acid in the receiving flasks. Crude fiber and ether extract were determined by AOAC methods. Moisture was determined as the loss following drying in an oven at 65° C for 16 hours. Residual ash was determined following ignition at 550° C in an electric furnace overnight. Nitrogen free extract and organic matter were calculated by difference. Volatile fatty acids were determined according to the methods of Pfander and Phillipson ('53).

Analyses of data obtained in *in vitro* tests were made by Fisher's or "Student's" "T" tests. Digestion trial data were analyzed by analysis of variance as described by Snedecor ('46) or by the "T" test of Fisher ('34).

RESULTS

Artificial Rumen.

Effects of certain steroid compounds on cellulose digestion *in vitro* as determined in the first series of experiments are shown in Table 2. At the lower levels of stilbestrol, there was a slight increase in the average cellulose digestion. The addition of 10, 16, or 20 μg. caused a significant increase in cellulose digestion. A signfiicant increase in cellulose digestion also resulted from the addition of 20 μg. of estrone or cholesterol to the "basal ration."

TABLE 2. THE EFFECT OF SOME STEROID OR STEROID-LIKE COMPOUNDS ON CELLULOSE DIGESTION BY OVINE RUMEN MICROORGANISMS IN VITRO

Trial	Mixture fermented	No. of Rumina	Avg. Cellulose Digestion (%)
1	Basal ration	6	35.1
1	Basal ration + 2μg. stilbestrol	6	36.6
1	Basal ration + 8μg. Stilbestrol	6	37.6
1	Basal ration + 16μg. stilbestrol	6	49.1*
2	Basal ration	10	36.7
2	Basal ration + 10μg. stilbestrol	10	44.7**
2	Basal ration + 20μg. stilbestrol	5	51.4*
2	Basal ration + 20μg. estrone	5	57.3**
2	Basal ration + 20μg. cholesterol	5	48.4*

*Difference in cellulose digestion (experimental vs. basal significant ($P<0.05$).

**Difference in cellulose digestion (experimental vs. basal highly significant ($P<0.01$).

Results of trials involving the addition of 2 μg. of testosterone, 0.3 μg. of hexestrol and 5 μg. of cortisone are shown in Table 3. Testosterone and

TABLE 3. AVERAGE PERCENT CELLULOSE DIGESTION IN THE ARTIFICIAL RUMEN

Trial	Testosterone	Hexestrol	Cortisone	Basal
1	22.0±1.0*	15.4±1.1	27.5±1.2	21.1±1.2
2	24.2±1.6	20.2±2.5	30.0±3.8	36.0±0.7
3	26.6±1.9	25.7±1.2	25.8±2.7	25.4±0.4
4	60.6±1.2	45.4±2.1	53.4±2.6	47.7±3.6
5	26.4±3.3	17.7±4.3	24.0±6.2	14.0±4.8
Avg.	31.9	24.9	32.2	28.8

*Each figure represents an average for the trial, and the standard error of the mean.

cortisone increased cellulose digestion in the artifical rumen 10.7 and 11.5 percent, respectively. The hexestrol decreased cellulose digestion 15.9 percent. In an additional trial, hormone levels were doubled or halved in three artificial rumens and the results did not indicate significant change. From these limited observations, there was an indication that rumen organisms obtained from sheep fed a better quality ration would give better

cellulose digestion than when the ration consisted of low quality roughage such as timothy hay.

There was a significant difference in cellulose digestion when carbon dioxide was provided for the fermentation. O'Tagaki *et al.* ('55) found that rumen microorganisms of cattle fixed carbon from CO_2 in both essential and non-essential amino acids. In their investigations and from the trial where CO_2 was utilized by rumen microorganisms, it seemed that the addition of CO_2 was indicated for artificial rumen studies.

Digestion Trials.

Table 4 shows the favorable effects of adding 10 or 20 mg. of stilbestrol per sheep per day on cellulose and protein digestibility.

TABLE 4. EFFECT OF STILBESTROL ON THE COEFFICIENTS OF DIGESTIBILITY OF CELLULOSE AND PROTEIN IN SHEEP.

Lot No.	Ration	Coefficients of digestibility	
		Cellulose	Crude Protein
1	Basal	41.9	37.5
2	Basal+10 mg. stilbestrol per sheep per day	47.8	43.2
3	Basal+20 mg. stilbestrol per sheep per day	48.7	44.7

Several of the sheep were continued on the 20 mg. level of stilbestrol. After a short period of time, two of them developed anorexia and appeared listless. An edematous swelling appeared around the anus of one sheep. Contractions of the abdominal muscles accompanied by apparent pain were observed 24 hours after the first symptoms had appeared. Digital examination indicated that the muscle tonus of the lower intestine was reduced and that the urethra and the prostate had enlarged. Similar conditions have been described in Australia where sheep were pastured on swards high in subterranean clover. When the one sheep showing these symptoms in our experiment was taken off the stilbestrol ration and given a subcutaneous injection of 100 mg. of testosterone, it made a rapid recovery. Other sheep receiving stilbestrol showed mild symptoms similar to those seen in the one that was treated so the experiment was stopped.

Table 5 shows the digestion coefficients for the proximate components, cellulose, and organic matter by sheep fed corn and alfalfa hay alone or with added testosterone, cortisone or hexestrol. The additives increased the digestibilities of most of the ration components and resulted in an increased T.D.N. In no case were the increases significant at the 5 percent level of probability.

TABLE 5. COEFFICIENTS OF DIGESTIBILITY OF NUTRIENTS OF AN ALFALFA HAY-CORN RATION AS INFLUENCED BY HORMONE ADDITIONS

Substance	Controls	Hexestrol	Cortisone	Testosterone
Fat	63.2	65.8	65.6	66.1
Fiber	51.9	56.2	56.2	55.4
Cellulose (C.M.)	64.3	67.2	67.8	67.9
Cellulose (M.E.M.)	61.6	64.9	65.9	64.9
Nitrogen	75.5	75.4	76.7	76.2
Organic Matter	72.4	74.2	75.2	74.9
N.F.E.	82.3	83.9	84.4	84.9
T.D.N. of Ration:				
As determined	64.7	66.8	67.2	67.1
Calculated from Schneider's coefficients	61.0			

The volatile fatty acid concentrations found in the rumens of the sheep are shown in Table 6. There were no significant increases attributable to the hormone additives.

TABLE 6. VOLATILE FATTY ACIDS IN RUMEN FLUID (MILLI-EQUIVALENTS/100 ML.)

Trial	Basal	Testosterone	Hexestrol	Cortisone
1	9.7±0.3[a]	10.4±0.5	10.1±0.5	10.7±1.3
2	10.7±0.1	11.2±0.2	10.2±0.3	10.9±1.2
Avg. for Trials	10.2	10.8	10.2	10.8

[a]The figures are the three day averages for each lot, and the standard error of the mean.

The digestion of silage rations by steer calves fed 7.5 mg. of hexestrol per day was not significantly different from the digestion of the control animals. Results are summarized in Table 7.

TABLE 7. THE COEFFICIENTS OF DIGESTIBILITIES OF NITROGEN, CELLULOSE, AND ORGANIC MATTER OF WINTERING RATIONS OF STEER CALVES.

	Coefficients of Digestibility		
Lot	Nitrogen	Cellulose	O.M.
1 Controls	50.7	53.1	58.5
2 As 1 + 7.5 mg. Hexestrol	54.4	55.0	59.8

DISCUSSION

Since our original report that stilbestrol at relatively high levels increased cellulose digestion by sheep (Brooks, *et al.* '54), other laboratories have studied the effect of steroid or steroid-like compounds on digestibility. Highly divergent and contradictory results have been reported. Data that confirm our findings have been presented by Story *et al.* ('55). However, little or no effect or possible increased N retention, was reported by Bell *et al.* ('55) and Erwin *et al.* ('55) with cattle and by Acker *et al.* ('55) with lambs. Sykes *et al.* ('56) reported increased digestibility of fiber, a slight increase in dry matter digestibility and a decreased protein digestibility when 10 mg. of stilbestrol were fed to lactating cows. Reports of inhibition from stilbestrol were made by Richardson *et al.* ('55) for cattle and by Campbell *et al.* ('56) and Struempler and Burroughs ('56) for lambs.

Apparently the various results have all been obtained under different dosage levels, different rations, and markedly different environmental conditions. The important conclusion to be drawn from the work that has been reported is that stilbestrol and related compounds do affect the utilization of rations in the gastro-intestinal tract, probably through their effect on the microorganisms.

The action by which orally administered hormones influence the digestibility of nutrients in ruminant rations has not been established. A possible mode of action is an increased production in the extracellular enzymes of the rumen flora. Marsh ('54, '55) and Karunairatnam and Levvy ('51) have described a glucuronide decomposing enzyme produced by the bacteria in the rumen. The studies of Marsh showed that this enzyme was present only under conditions that maintained a normal bacterial population. Fishman and Fishman ('44) described a Beta-glucuronidase in uterine tissue of rats. Fishman ('47) and Leonard and Knobil ('50) established that the content of this enzyme in uterine tissue was dependent on the estrogen concentration present in the animal. When rats were ovariectomized, the Beta-glucuronidase content in uterine tissue decreased significantly. The concentration of the enzyme described by Marsh and Karunairatnam and Levvy may be increased, or its activity may be enhanced in the rumen by estrogen-like hormones.

Rakoff *et al.* ('44) point to one of the possible actions of the injected or implanted hormones on metabolic activity. As stated in their summary, "Estrogenic activity disappears rapidly from the peripheral blood of normal dogs and humans after intravenous or intramuscular injection of alpha-estradiol." In five to ten minutes there was less than 10 percent activity, and after five hours less than 2 percent activity. "The behavior of

alpha-estradiol closely resembles that of bile acids. This lends support to the hypothesis of an entro-hepatic circulation of estrogens, similar to that of bile acids." Such a circulation would make part of the orally administered compounds available to the lower gastro-intestinal tract and may be one of the reasons why oral hormone administration influenced digestibility. This circulation would also account for the continued effects of the hormone after its administration in feed has ceased.

Many investigators have studied the effects of hormones on bacteria. Such studies have limited value in interpreting what occurs in the rumen. Most of the investigations were limited in scope. For example, no synthetic medium can have all the features of the micro environment of a normal rumen. Bacteriologists generally consider only a few colonies or groups of microorganisms, rather than the entire population of the rumen; in fact, the culture techniques of the bacteriologist seldom permit a normal population to exist. For these reasons, only a few investigations will be discussed here. The following ones were chosen because they showed a possible mode of action of steroid or steroid- like compounds on ration digestibility by ruminants through the rumen flora.

Faulkner and Edin ('43) reported that stilbestrol was bactericidal to two strains of gram positive cocci studied. The bactericidal activity of stilbestrol was reduced in the presence of blood serum. No effect was found on gram-negative organisms.

Pugh ('48) studied the effects of testosterone propionate on resistance of rats of *Bracillus abortus* infection. When 2.5 mg. of testosterone propionate were given subcutaneously in oil, six hours before infection, 12.5 percent of the immature male rats and 36.5 percent of the immature female rats lived. All of the control rats died.

Gains and Totter ('50) reported that the addition of dehydroepiandrosterone to the media produced the same action as did pterylglutamic acid, and in many ways it acted in a manner similar to thymine. Their observations were made with cultures of *Streptococcus faecalis, Lactobacillus casei,* and *Escherichia coli.*

The investigations of Cohen ('54) showed that stilbestrol, in 1 mg. per ml. of media, gave total inhibition of coccidioides. At a 0.5 mg. per ml. media, very small colonies of coccidioides were present.

These observations from the literature, together with results obtained in the current investigation, strongly suggest tha steroid-like compounds influence the celluloytic activity of rumen microflora.

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