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I am submitting herewith a thesis written by Tony Alan Fitz entitled "Identification of Short Chain Fatty Acids in Cervical Mucus from Estrous Cows." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

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THESIS

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IDENTIFICATION OF SHORT CHAIN FATTY ACIDS IN
CERVICAL MUCUS FROM ESTROUS COWS

A Thesis

Presented for the

Master of Science

Degree

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Tony Alan Fitz

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ABSTRACT

Cervical mucus was obtained from estrus dairy cows by massage, per rectum, of the reproductive tract to expel mucus into a plastic bag. Mucus was collected from ovariectomized beef heifers, brought into estrus with progesterone and estrogen, by insertion of tampons into the vagina.

Initially, extraction of fatty acids from mucus with diethyl ether and with methanol was attempted. However, with one exception, the values reported in this thesis were obtained using isopropanol. Fatty acids were extracted in their sodium salt form with isopropanol, then concentrated to dryness before uptake in a diethyl ether-formic acid solvent. Aliquots were injected onto a gas chromatograph, and fatty acid peaks were quantitated with the aid of standard solutions of the appropriate acids.

Isobutyric, butyric, isovaleric, and valeric acids were found in extracts of most samples analyzed, each usually at a concentration of less than one $\mu\text{g/gm}$ sample. Propionic acid was found in all samples, usually at a concentration of several $\mu\text{g/gm}$ sample. Acetic acid was found in highest concentration, in amounts usually 10-15 times greater than the concentration of propionic acid. Differences in methods of mucus collection and extraction techniques, and the limited data gathered, made statistical analysis inappropriate.

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CHAPTER I

INTRODUCTION

Pheromones are substances produced by an individual which affect other members of the same species, and have been shown to play an important role in the social order, communication pathways, and reproductive patterns of many insect species. Numerous pheromones of insect origin have been identified and synthesized, and are being used by man as a means of exerting control over insect behavior and reproduction.

In contrast to the large amount of information available about insect sex attractants, very little is known of sex pheromonal actions in mammals. The monkey is the only mammal in which a pheromone which stimulates attempted mating by the male has been demonstrated. A mixture of short chain fatty acids has been isolated from monkey cervical mucus, which, when applied to the sex skin of a recipient female, stimulates male monkeys to attempt mating. Furthermore, mixtures of the same fatty acids, though in somewhat different proportions, have been isolated from human cervical mucus, and extracts of human cervical mucus, when applied to female monkey sex skin, increases mating attempts by male monkeys.

While cervical mucus from an estrus cow is known to stimulate the mating interest of a bull, nothing is known about the chemical basis of this stimulation. In view of the knowledge that the stimulatory factor(s) in the monkey and woman consists of a mixture of short-chain

fatty acids, it was decided to determine if similar compounds were present in the cervical secretions of the estrous cow.

CHAPTER II

LITERATURE REVIEW

Substances released by insects which have an effect upon other members of the same species are well documented. Jacobson (1965) listed references to over 150 female-produced and over 50 male-produced sex attractants found in insect species, with the earliest reference dating from 1837. However, not until Karlson and Butenandt (1959) coined the word "pheromone," from the Greek "pherein" (to carry) and "horman" (to excite or stimulate), was there an accepted nomenclature for these attractant substances.

Substances possessing pheromonal activity have more recently been identified in mammals, with the usual route of action being through olfactory pathways (Bronson, 1971). Both sexes in mammals are known to produce and be affected by pheromones. In female mice, a number of pheromonal responses have been attributed to male mouse urine, including the induction of shorter and more regular estrous cycles (Whitten, 1956), acceleration of puberty (Vandenbergh, 1969), pregnancy block (Bruce, 1960), and increase in litter size (Beilharz, 1968). Whitten (1968) showed that the estrous-synchronizing effect of male mouse urine could be airborne. Signoret (1961) demonstrated that the odor of the boar enhances the expression of estrous in the sow, and Reed et al. (1974) successfully elicited the immobilization reflex (lordosis) in estrous sows with an aerosol containing an androgen metabolite. The

breeding efficiency of anosmic sows is greatly decreased (Signoret, 1962). The presence of a ram with ewes during the transition period from the nonbreeding to the breeding seasons results in earlier onset of estrus in ewes (Coleman, 1950; Schinckel, 1954a, 1954b).

Sex pheromones produced by the female with a primary effect upon the male have been demonstrated in bovine (Hart, 1946), ovine (Kelley, 1937; Lindsay, 1965), equine (Wierzbowski, 1961), primates and other species (Michael, 1968). Other pheromones are known which are produced by both sexes of a species. Thus the gerbil produces a substance (phenylacetic acid) in the abdominal sebaceous scent gland which the male utilizes for exploration, social dominance and territoriality, and which the female produces in association with exploration and maternal responsibilities (Thiessen et al., 1974). Both sexes of the black-tailed deer produce a substance in the tarsal gland which serve as attractants for other members of that species (Müller-Schwarze, 1969), and the pronghorn produces a scent from the subauricular gland which is used in territoriality marking (Müller-Schwarz, 1974).

The chemical composition of a large number of insect pheromones has been determined (Jacobson, 1965). Many are intermediate length unsaturated alcohols, esters, and ring compounds. Much practical application has been made utilizing sex pheromones as a means of insect population control (Wood et al., 1970; Beroza, 1970).

Several pheromonal compounds have been identified in mammals. Müller-Schwarze (1969) isolated, from tarsal gland extracts of black-tailed deer, several component fractions which showed activity when

recombined. The major active component was *cis*-4-hydroxydodec-6-enoic acid lactone. Reed (1974) was able to stimulate lordosis in sows with the steroid 5 α -androst-16-en-3-one. Müller-Schwarze (1974) recovered a mixture of organic acid and alcohol compounds from extracts of subauricular glands in pronghorn; major activity was due to isovaleric acid. Thiessen et al. (1975) isolated phenylacetic acid as the marking pheromone in gerbils while Michael et al. (1971) demonstrated that mixtures of short (c1-c5) chain fatty acids from monkey cervical mucus stimulated copulatory behavior by the male. Michael et al. (1974) recovered, from human vaginal secretions, mixtures of short chain fatty acids which stimulated male monkeys when applied to the sex skin of female monkeys. Most known mammalian pheromones are small volatile compounds. Vandenberg et al. (1975), however, found strong evidence that a protein fraction of male mouse urine accelerated the onset of puberty in immature females.

Hart et al. (1946) found that cervical mucus from an estrus cow, when applied to cows not in estrus, stimulated mating interest by a bull. Little is known of the attractant substance present in bovine cervical mucus, though analyses have demonstrated a cyclic variation in volume (Scott Blair et al., 1942b; Herman and Horton, 1948; Herrick, 1951), viscosity (Scott Blair, 1942a; Herman and Horton, 1948), percent dry matter (Scott Blair, 1942b), total nitrogen (Scott Blair, 1942b), cellular debris (Herrick, 1951), and crystallization patterns (Alliston et al., 1958).

An ovariectomized cow may be brought into physiological estrus

by means of appropriate hormonal treatment. Asdell et al. (1945) induced estrus in ovariectomized cows using estrogen. Melampy et al. (1957, 1958) claimed a synergistic effect of progesterone with estrogen on the intensity of estrus in cattle. Robinson (1955) found, in ewes, that progesterone pretreatment markedly increased the ovariectomized ewe's sensitivity to estrogen, while Carrick and Shelton (1969) determined the desirability of pretreatment with progesterone to induce estrus with estrogen in ovariectomized cows. Carrick and Shelton (1969) obtained a consistent estrus response with subcutaneous injection of 10 mg progesterone daily for five days, followed five days later with a single injection of 400 μ g estradiol benzoate. In the ovariectomized rhesus monkey, Michael and Keverne (1969) found that estrogen administration resulted in the production of a male attractant pheromone and could increase the female's sexual activity.

CHAPTER III

EXPERIMENTAL PROCEDURE

Bovine cervical mucus was collected from estrous Jersey and Holstein cows and from ovariectomized beef heifers, subsequent to treatment with progesterone and estrogen. All animals used in this study were in herds maintained by the University of Tennessee Agricultural Experiment Station.

Cows in the dairy herd are normally stanchioned when they exhibit signs of estrus. Regular trips were made to the dairy barn during the spring of 1975 to collect mucus from estrous cows. To collect mucus from a stanchioned cow, a gloved hand was inserted into the rectum and the reproductive tract was grasped at the level of the cervix. With a gentle posteriorly directed massaging action, mucus was expelled into a plastic bag. Upon collection, the bag was immediately sealed and labeled with cow number and date of collection. Bags containing mucus samples were placed in a freezer within 15 minutes of collection and held at a temperature of -12°C until analysis. Mucus, in widely varying quantities, was recovered from 21 cows by this method.

Two 18 month old heifers (one Angus and one Hereford) were ovariectomized by staff veterinarians on June 18, 1975. An ovary of each heifer contained a corpus luteum indicating that they were cycling at the time of surgery. Both were injected, on the eighth day following

the operation, with a single dose of 500 μ g estrogen to induce estrus. In subsequent tests with these heifers, each was pretreated with progesterone before estrogen administration.

Progesterone and estradiol 17 β solutions were prepared by dissolving weighed portions of hormone (enough for one treatment period) in 0.5 ml benzyl alcohol. Sufficient corn oil was added to give concentrations of 10 mg progesterone or 500 μ g estrogen per ml of oil. The corn oil-benzyl alcohol-hormone mixtures were thoroughly mixed, heated to 110°C to bring all hormone into solution, then tightly stoppered with a rubber plug.

Starting on the twelfth day following ovariectomy, both heifers were injected with 10 mg progesterone daily for five days followed by a single subcutaneous injection of 500 μ g estrogen on the third day following the last progesterone injection. Subsequently, both heifers received an additional 1.0 mg estradiol (two 0.5 mg doses at 12 hour intervals) on the sixteenth day after the first progesterone injection. Following this treatment, both heifers were allowed one week of rest before the second series of progesterone injections were initiated.

In the second period, both heifers received 10 mg progesterone daily for five days, followed by four doses of 500 μ g estradiol (at eight hour intervals) on days 13 and 14 of the treatment period. They were then allowed 10 days of rest prior to initiation of the third treatment.

In the third and final stimulation period, both heifers were injected on three consecutive days with 25 mg progesterone, but the

dose on the fourth day was reduced to 10 mg. On day seven, both received three 500 µg doses of estrogen at six hour intervals.

Approximately six hours after the initial estrogen administration in each stimulation period, tampons were inserted into the vagina of each heifer to collect cervical mucus and tampons were replaced at intervals of six to 12 hours over the next 36 hours. Tampons were prepared by tying together four Tampax Super tampons with white cotton thread, extending the drawstring approximately six inches with twine, refluxing each bundle in hot diethyl ether or hot methanol for two hours, then drying overnight in an oven at 110°C. After drying, tampons were placed in clean screw-top glass jars, and jars plus contents were labeled and weighed. Tampons were inserted in the vagina of each heifer through a two inch diameter speculum which was lubricated with K-Y Jelly. Upon collection, each tampon bundle was placed in its original jar, and jars plus contents were again weighed. The approximate weight of each mucus sample was assumed to be the difference between the initial and final weights of each jar. Jars containing used tampons were tightly stoppered and refrigerated at 5°C within 10 minutes of collection until time of extraction.

Several different techniques of mucus extraction were used. Mucus samples from dairy cows were pooled and aliquots weighed before each extraction; mucus samples from beef heifers were individually analysed. Extraction procedures used were as follows:

Extraction procedure no. 1. Portions of samples from three or four animals were pooled and weighed. While the samples were still

cool, sufficient 0.1 N NaOH was added to raise the pH to approximately 10. Two ml of diethyl ether were shaken with each sample in a stoppered separatory funnel and the phases allowed to separate. If an emulsion persisted, then sufficient ether was added to induce phase separation when the mixture was re-agitated. After phase separation, the mucus fraction was discarded and the ether was evaporated to dryness. The residue was taken up in 1 ml H_2O , reacidified with 0.01 N HCl, and again extracted with 2 ml ether. Finally, 2 ml of 0.1 N NaOH was added and the ether was evaporated. The resultant samples were divided in half; one portion was used for behavioral testing after reacidification, while the other half was analyzed by gas chromatography.

Extraction procedure no. 2. Mucus samples were pooled, weighed, and buffered to pH 4.5 with NaH_2PO_4 before extraction with 5 ml of diethyl ether. The ether extract was evaporated to dryness, taken up in 3 ml H_2O , and reacidified with 25 percent metaphosphoric acid before gas chromatographic analysis and behavioral testing.

Extraction procedure no. 3. Pooled mucus samples with 1 ml H_2O added were extracted with 5 ml diethyl ether. One ml 0.01 N NaOH was added, contents were shaken in a separatory funnel, then the entire mixture was evaporated to dryness. The residue was taken up in 2 ml H_2O and reacidified with 25 percent metaphosphoric acid before behavioral testing and gas chromatographic analysis.

Extraction procedure no. 4. Pooled, weighed mucus samples were washed with 5 ml methanol. Mucus was removed from the mixture by straining through glass wool. The eluate was mixed with 1 ml 0.1 N NaOH

and evaporated to dryness. The residue was taken up in 2 ml H_2O and reacidified before behavioral testing and analysis by gas chromatography.

To determine if extracts of mucus did have stimulatory effect on a bull, extracts prepared by Procedures 1, 2, 3, and 4 were tested on a small group of cows and a bull. The extracts were acidified, mixed with mineral oil, then applied to the rump area of individual cows. The bull in the herd was then observed for a minimum of 10 minutes per application for any changes in behavior.

Extraction procedure no. 5. Individual mucus samples were weighed, then washed two times with 10 ml diethyl ether. Both ether extracts were pooled and washed three times with 10 ml 0.1 N NaOH. The three alkaline washes were combined and evaporated to dryness in a water bath, with transfer to small test tubes before evaporation of the last few ml of solution. When dried, the test tubes were sealed with a layer of parafilm, then injected with 200 μ l of a freshly mixed 9:1 diethyl ether-formic acid solution (formic acid was triple distilled). The injection hole was immediately covered with another layer of parafilm, each tube was agitated vigorously on a vortex mixer, and then the samples were allowed to stand for 15 minutes before chromatography.

Extraction procedure no. 6. Individual mucus samples were weighed and adjusted to pH 9-10 with 0.05 N NaOH before mixing with 40 ml isopropyl alcohol. Solutions were centrifuged at 2900 rpm for 30 minutes, then the supernatants were decanted into beakers where they were evaporated to several ml. The last few ml of solution were

transferred to small test tubes and evaporated to dryness. Parafilm was used to seal each tube and 9:1 diethyl ether-formic acid was injected exactly as in procedure no. 5.

Extraction procedure no. 7. Individual samples were extracted three times with 5 ml diethyl ether. To the ether was added 40 ml isopropyl alcohol and 3 ml 0.01 N NaOH. The mixture was thoroughly agitated, then centrifuged, evaporated, and taken up in solvent exactly as in procedure no. 5. This procedure was subsequently used in quantitation of fatty acids recovered from mucus samples.

Gas chromatography of sample extracts was performed initially on an F and M Scientific Corporation Model 810 Research Chromatograph which used the recorder from a Dohrman Instruments Company Model C-200A Microcoulometer. Samples were injected into a 6 foot by 2 mm (inside diameter) stainless steel column packed with Supelco 10 percent SP 1200/1 percent H_2PO_4 on 80/100 Chromosorb W AW. The column was plugged with glass wool and conditioned overnight at 125°C before use.

Chromatographic analysis was transferred, when the instrument became available, to a new Bendix Model 2600 gas chromatograph with a Model 1300 recorder. The Bendix column was packed with the same packing as was used on the F and M instrument in a 6 foot by 2 mm glass column which was plugged with glass wool and conditioned overnight before use. Operating conditions for both chromatographs were optimized at 40 ml/minute nitrogen flow, 40 ml/minute hydrogen flow, and 320 ml/minute air flow. Both machines kept an injection port temperature of 180°C and a detector temperature of 185°C. The F and M oven was kept

at 115°C. The Bendix oven was programmed through a temperature range of 68-125°C at a rate of 32°/minute (temperature increase was not begun until formic acid was detected).

For quantitation of fatty acid recovery in sample extracts, a standard solution was prepared by dissolving in 100 ml of 9:1 diethyl ether-formic acid, the following amounts of acid:

Acetic	0.2992 gm
Propionic	0.1168 gm
Isobutyric	0.0619 gm
Butyric	0.0990 gm
Isovaleric	0.0497 gm
Valeric	0.0591 gm

Four successive injections of 0.5 μ l aliquots of standard solution were made under operating conditions exactly as used for sample injections.

Peak area was averaged for each acid using the formula:

$$(1) \text{ Peak area (cm}^2\text{)} = \text{Peak height (cm)} \times \text{Peak width (cm) at } 1/2 \text{ height}$$

Electrometer attenuation was kept at 200 for calculation of standard solution concentrations. A factor was then calculated to correlate the peak area of each acid in the standard solution with the concentration (mg/100 ml) of each acid in the standard:

$$(2) \text{ Factor} = \frac{\text{Concentration (mg/100 ml) of acid in standard solution}}{\text{Average area (cm}^2\text{) of acid peak in standard solution}}$$

Use of formula (2) allowed calculation of the following factors for each fatty acid in the standard solution:

<u>Fatty Acid</u>	<u>Concentration (mg/100 ml)</u>	<u>Peak Area (cm²)</u>	<u>Conversion Factor</u>
Acetic	299.2	291.11 ±	1.03
Propionic	116.9	197.55 ±	0.59
Isobutyric	61.9	110.54 ±	0.56
Butyric	99.0	186.63 ±	0.53
Isovaleric	49.7	134.38 ±	0.37
Valeric	59.1	126.58 ±	0.47

When a 0.5 µl injection of sample was made, the peak area of each acid was calculated using formula (1). Peak area for each acid was converted to acid concentration by the following formula:

$$(3) \text{ Concentration (mg/100 ml) acid} = (\text{Peak area}) \times (\text{Factor for that Acid})$$

If electrometer attenuation (sensitivity) was increased or decreased during the sample run, the peak area at attenuation = X was converted to an attenuation = 200 basis as follows:

$$(4) \text{ True peak area (cm}^2\text{)} = \left(\frac{X}{200}\right) \times (\text{Peak area at attenuation} = X)$$

Then, concentration of the acid was calculated using formula (3). Since each single extract was taken up in 200 µl of solvent, the concentration of acids in each sample was converted to µg acid/200 µl solvent basis as follows:

$$(5) \text{ µg acid/200 µl solvent} = (\text{mg acid/100 ml solvent}) \times 2$$

With use of the standard solution, 0.5 µl injections of sample extracts, and formulas as given, an approximation of the acid content of each sample could be made.

CHAPTER IV

RESULTS AND DISCUSSION

A. RESULTS

All attempts to identify fatty acids in extracts of mucus from dairy cows using the F and M instrument were negative. This failure was presumably due to a lack of sensitivity of the instrument. Injection of water solutions on high concentrations of the acids did yield peaks which indicated presence of the acids. However, the quantities of acids required to obtain detectable peaks were of the order of 10 or more times greater than those subsequently found to be needed for detection in the Bendix instrument.

When duplicate samples prepared by the same extraction procedures (Procedures 1-4) and from the same samples tested on the F and M instrument were applied to the rumps of nonestrous cows, they failed to elicit any signs of interest on the part of the bull. Thus, there remains the question of whether these extracts did, in fact, contain any pheromonal activity. In view of the later demonstration, when using the Bendix instrument, of presence of fatty acids in mucus samples, it seems likely that failure to detect fatty acids at this stage was due to the methods of extraction used.

Initial analysis on the Bendix chromatograph was performed on two different pools of dairy mucus. In Table I, sample 1 contained mucus from four different cows and was extracted via procedure 5. Sample 2 contained mucus from three cows and was extracted by procedure

Table I
Concentrations (μg Acid/gm Sample) of Fatty Acids
Recovered from Two Pooled Batches of Cervical
Mucus from Estrous Dairy Cows

Source of Mucus ^a	Fatty Acids					
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric
Sample 1	28.3	2.02	0.54	1.05	0.24	0.34
Sample 2	18.7	3.21	0.57	0.85	0.43	0.52

^aSample 1 was pooled mucus samples from four dairy cows, extracted via Procedure 5, while Sample 2 was pooled mucus samples from three dairy cows, extracted via Procedure 6.

6. The acid peaks from sample injections were quantitated as described earlier. Considering that the results shown in Table I represented mucus from different sets of animals (possibly from different stages of estrus) and considering also that the two samples were extracted differently, the values obtained appear reasonably similar. Perhaps the most striking feature of Table I is the high concentrations of acetic and, to a lesser extent, propionic acids. Butyric acid appears to rank third in order of concentration.

Table II shows the fatty acid recovery from both ovariectomized beef heifers following estrogenic Treatment 2. Each heifer received a tampon 12 hours following the first estrogen injection, with removal and replacement of the tampon at 24 and 32 hours following the first dose of estrogen. However, the Hereford expelled the second and third tampons inserted. Therefore, while samples 1A-3A represent, chronologically, the three tampon collections from the Angus during this treatment period, sample 1H represents only the first tampon collection from the Hereford. Table II shows, as does Table I, a strikingly higher concentration of acetic acid than the other acids, with propionic acid again present in next highest concentrations. The cause of the marked variation of propionic acid within the estrus period of the Angus is unknown although it may be associated with the cyclic change during the estrous period, this seems unlikely. Following Treatment 2, concentrations of other than acetic and propionic acids remained below 1 $\mu\text{g/gm}$ of sample except in a single instance for butyric acid.

In the third and final treatment period, a tampon was inserted

Table II
Concentrations (μ g Acid/gm Sample) of Fatty Acids
in Cervical Mucus Recovered from Ovariectomized
Beef Heifers Subsequent to Hormone Treatment
(Treatment 2)^a

Source of Mucus ^b	Fatty Acids					
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric
Sample 1A	77.02	11.57	0.02	0.02	0.02	0.37
Sample 2A	29.52	0.97	0.04	0.30	0.28	0.08
Sample 3A	79.79	47.11	0.20	2.37	0.23	0.07
Sample 1H	29.26	3.68	0.25	0.13	0.36	0.03

^aThe hormone treatment consisted of five consecutive daily subcutaneous injections of 10 mg of progesterone followed seven days later by four subcutaneous injections of 500 μ g estradiol benzoate at six hour intervals.

^bSamples 1A, 2A, and 3A were consecutive tampon collections from the Angus heifer; sample 1H was from the Hereford heifer. Tampons were first inserted 12 hours after the first estrogen injection and were replaced at 12 and 8 hour intervals. However, the Hereford female expelled the last two tampons resulting in only one collection.

into each heifer at 6, 12, 24, and 30 hours following the first injection of estrogen. In addition, each animal was bled at 24 hours post injection, and blood samples were analyzed along with tampons. No tampons were lost from either animal during this treatment period. Table III shows the result of extractions of mucus and blood samples following Treatment 3. Fatty acid recovery was generally lower than was obtained from Treatment 2, although the same extraction procedure (Procedure 7) was used in analysis of both sets of samples. The discrepancy may be due to the shorter, more intense progesterone treatment during Treatment 3. Nevertheless, the data show some interesting figures. Acetic acid again appears to be present in highest concentration in both blood and mucus, with propionic generally next in order of concentration. However, although all acids tend to show lower concentrations than following Treatment 2, propionic acid appears disproportionately so. Blood samples showed higher fatty acid concentration than did mucus samples. The second mucus collection for each cow showed the highest total fatty acid content; one could postulate that the time of the second collection was the period of most intense estrus.

The data in Table IV were obtained by averaging the concentrations of each fatty acid for each heifer over Treatment periods 2 and 3. Thus sample A is the mean of six values obtained from the Angus (sample 3A of treatment 2 was deleted) over two treatment periods. Sample H is the mean of five values obtained from the Hereford. The Angus appears (Table IV) to have had a strikingly higher concentration of acetic and propionic acids than the Hereford. However, most of this

Table III

Concentrations of Fatty Acids in Cervical Mucus and Blood Plasma Recovered from Ovariectomized Heifers Subsequent to Hormone Treatment (Treatment 3)^a

Source of Mucus ^b	Fatty Acids					
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric	Valeric
Sample 1A	2.93	0.19	0.07	0.07	0.07	0.05
Sample 2A	19.38	0.48	- ^c	-	-	0.35
Sample 3A	0.85	0.16	0.04	0.03	0.08	-
Sample 4A	3.89	0.31	0.25	0.16	-	-
Sample 5A	53.56	2.19	0.88	0.33	2.06	-
Sample 1H	1.85	0.24	-	-	0.19	-
Sample 2H	18.22	1.08	3.29	0.09	0.10	0.61
Sample 3H	3.67	0.38	0.16	0.07	0.18	0.09
Sample 4H	2.10	0.15	0.04	-	0.05	-
Sample 5H	63.09	1.69	0.80	0.32	2.37	0.19

^aThe hormone treatment consisted of three consecutive daily injections of 25 mg progesterone with one injection on the next day of 10 mg progesterone, followed three days later with 2.0 mg (in three injections) estradiol benzoate.

^bSamples 1A-4A (Angus heifer) and 1H-4H (Hereford heifer) were consecutive tampon collections at 12, 24, 30, and 36 hours, respectively, following the first injection of estrogen. Samples 5A (Angus) and 5H (Hereford) were extracts of blood plasma drawn at 24 hours after the first estrogen injection.

^cDash marks indicate either that no acid peaks could be detected or that peak areas were too small to permit quantitation.

Table IV
Mean Concentration (μ g Acid/gm Sample) of Fatty Acids in Cervical Mucus
Recovered from Each Ovarietomized Heifer for
Both Treatment Periods

Source ^a of Mucus	Fatty Acids				
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric
Sample A	35.42 $\pm$ 8.27	10.10 $\pm$ 3.54	0.12 $\pm$ 0.03	0.58 $\pm$ 0.23	0.15 $\pm$ 0.05
Sample H	11.02 $\pm$ 3.29	1.11 $\pm$ 0.37	0.94 $\pm$ 0.46	0.10 $\pm$ 0.03	0.18 $\pm$ 0.04
					0.24 $\pm$ 0.13

^aSample A represents the mean (with standard error) of six values obtained from the Angus heifer over two treatment periods. Sample H represents the mean of five values obtained from the Hereford heifer during the same treatment periods.

discrepancy was probably due to the fact that the Angus yielded three mucus collections during the high acid production period of Treatment 2, while the Hereford yielded but a single sample during the same period (note that the one sample obtained from the Hereford during Treatment 2 contained high concentrations of acetic and propionic acids). Otherwise, fatty acid concentrations in the two heifers for a comparable time period were within the same order of magnitude.

The mean concentration of each fatty acid, based on all but one of the determinations on samples collected from both beef and dairy females is shown in Table V. The mean for the heifers is based on 11 individual determinations, while the mean for the cows are based on only two values (obtained from pooled samples of four and three dairy cows, respectively). Thus, the high standard error apparent on the acetic acid mean of the dairy pool might have been much smaller had individual dairy mucus extractions been performed. Because of the variability of (1) time of collection of samples relative to time of initiation of estrus in dairy cows or first dose of estrogen in beef heifers, (2) pooling of samples from different dairy cows, (3) methods of extraction of mucus, and (4) methods of collection of mucus, statistical analyses of the data do not appear appropriate.

B. DISCUSSION

A better understanding of the reproductive mechanisms of cattle could lead to higher reproductive efficiency, which would be economically beneficial to both the producer and the consumer. Reproductive

Table V

Comparison of Mean Concentration ($\mu\text{g Acid/gm Sample}$) of
Fatty Acid Recovery from Ovariectomized Beef
Heifers and Dairy Cows

Source ^a of Mucus	Fatty Acids				
	Acetic	Propionic	Isobutyric	Butyric	Isovaleric Valeric
Sample B	24.33+3.42	6.01+1.39	0.48+0.13	0.40+0.11	0.16+0.02 0.21+0.04
Sample D	23.5+12.23	3.21+1.37	0.55+0.28	0.95+0.48	0.34+0.18 0.43+0.22

^aSample B represents the mean (with standard error) of 11 individual determinations from both the Angus and Hereford heifers. Sample D represents the mean from two determinations (obtained from pooled samples of four and three dairy cows, respectively) from dairy animals.

efficiency remains well below optimum in American dairy and beef cows (in the vicinity of 80 percent according to Hafez, 1962, or 60-80 percent according to Nalbandov, 1964). Numerous problems plague the cattle producer in his efforts to effect maximum reproductive performance in his herd; not the least significant problem faced is that of identifying the estrous cow for breeding.

The process by which the bull identifies an estrous cow is poorly understood. In many mammalian species, an interplay of mechanisms such as sight, odor and female receptivity appear to contribute to estrous discrimination. The olfaction plays a role in determination of sexual status has been shown in studies of mice and rats (Whitten et al., 1968; Bronson, 1971), swine (Signoret and du Mesnil du Buisson, 1961; Signoret and Mauleon, 1962; Reed et al., 1974), sheep (Kelly, 1937; Schinckel, 1954; Lindsay, 1965), cattle (Hart et al., 1946), and monkeys (Michael and Keverne, 1968, 1969). In only the monkey has the chemical identity of a female-produced mammalian sex attractant been established (Michael et al., 1971), although substances with sex attractant properties have been identified in great detail in many insect species (Jacobson, 1965).

The isolation and identification of bovine sexual pheromones could have several commercial applications. An extract or a synthetic mixture containing the attractant(s) in volatile form might lessen the problems encountered in semen collection from an inexperienced bull. To arouse the semen donor, the extract could be sprayed in the working area as an aerosol. Another possible use of the bovine sex pheromone

could be in the identification of cows experiencing "silent estrus" (Nalbandov, 1964; Hafez, 1962). Cows exhibiting silent estrus ovulate but fail to express any overt signs of estrus; therefore, the bull does not have an opportunity to mate with the cow, nor is there an opportunity to artificially inseminate her. Similarly to the manner in which Reed et al. (1974) sprayed boar odor to elicit lordosis in estrous sows, the bovine pheromone possibly could be used to enhance the overt expression of estrus in the cow.

Collection and analysis of cervical mucus from an estrous cow would seem to be the logical first step in the identification of a bovine sex attractant. Urine from estrous cows has been claimed to possess sex attractant properties, but no confirming data have been presented. Whether the sex attractant response of urine could be due to contamination by mucus or attractant response of urine could be due to contamination by mucus or by a urine-mucus interrelation awaits further investigation. In fact, the possibility must exist that the attraction of cervical mucus for bulls (Hart et al., 1946) could be due to contamination of the mucus by urine. Resolution of this question awaits more sophisticated collection procedures which eliminate the possibility of sample contamination.

Essentially two methods of mucus collection were attempted in the present study. In the dairy herd, cows in estrus were massaged per rectum to expel cervical mucus. This method had the advantage of being a simple technique in which the presence of mucus could be rapidly determined and quantitated. In the present study, however, several

problems were encountered with use of this collection procedure. The cow has a short (13-17 hours) estrous period, and maximal mucus production occurs early in the period (Herman and Horton, 1948). The optimum time of mucus collection is as early as possible after the cow has entered estrus, before she has an opportunity to expel accumulated mucus from her tract and during the period when (presumably) pheromone production is high. Indeed, since a herd bull may be observed to follow a cow around several days before she enters estrus, pheromone production may begin some time before estrus is exhibited (Murphree, 1975, personal observation). In the current study, the samples collected from dairy cows were often from late estrus or perhaps from postestrus cows, with little, if any, obtainable mucus present. No reliable estimate of the stage of estrus at time of collection could be made under these conditions. A technique was needed which would allow repeated sampling from animals throughout the estrus period in order to investigate any possible cyclic variation of pheromone production.

The use of ovariectomized heifers resulted in more control over events associated with estrus. The heifers were maintained in a stall at a convenient location, and could be observed as often as necessary. Ovariectomy permitted replacement therapy which would bring the heifers into a state of estrus as desired. Successive hormone treatments were given in slightly different regimens in an attempt to elicit intense estrus response. Estrus onset was stimulated by estrogen injection following progesterone priming, except immediately following ovariectomy, when the presence of corpora lutea in an ovary of each heifer indicated that both were in the luteal phase of the estrous cycle.

Perhaps the greatest advantage of tampon collection over massage collection was the opportunity to separate mucus produced in different stages of estrus. Additionally, tampons may have decreased the frequency of spontaneous mucus expulsion from the vagina. Even using tampon bundles, however, occasional tampon expulsion was experienced. Perhaps the loss of tampons was due to improper insertion. The effect, if any, of the tampons on uterine, cervical, and vaginal environments needs further investigation, but spotting of tampons with blood was a persistent problem. The bleeding may have been caused by estrogen-induced hyperemia compounded by tissue irritation caused during tampon insertion. The Angus appeared more susceptible to this problem than the Hereford. The contribution of blood to the total fatty acid content of the sample extracts is difficult to quantitate, but the small amounts of blood present were probably not a significant factor.

Since in the monkey (the only mammal in which a pheromonal component of cervical mucus has been identified) short chain fatty acids constitute the sex attractant, attention was directed toward extraction procedures which would confirm and quantitate the presence of any fatty acids present in the bovine cervical mucus tested. Michael et al. (1971) extracted monkey mucus samples with diethyl ether after NaH_2PO_4 buffering, and used the extracts for behavioral testing. They were able to remove basic and neutral components of mucus while retaining behavioral activity by extracting the acids in salt form from the ether extract. In subsequent analyses, Michael et al. (1974) extracted tampons with methanol. Peterson (1973) extracted urine from estrus cows with diethyl ether washes, mixed the extract with mineral oil,

then evaporated the ether from the mixture. Gardner and Thompson (1974) extracted fatty acids in their salt form from blood plasma with isopropyl alcohol. Ideas from these different scientists were utilized in the current study.

Pooled dairy mucus samples were extracted using extraction procedures 1 through 4 as outlined earlier. When extracts were mixed with mineral oil and applied to recipient non-estrus cows in a small Angus herd, no behavioral response could be discerned either by herd-mates or the herd bull. Analysis of sample duplicates on the F and M chromatograph failed to indicate the presence of any fatty acids. Subsequent extracts of solutions containing known amounts of fatty acids, with chromatograph tests at different stages of the extraction process, showed one particular point of acid loss. Extraction of ether extracts by NaOH solution was found to be of low efficiency. Whether varying the molarity of the NaOH solutions or whether altering other experimental conditions was needed is unknown to the author. A poor understanding of the extraction principles involved is probably responsible for the failure of these extraction procedures. Subsequent to these early failures, efforts were concentrated on extraction procedures 6 and 7. After finding fatty acid recovery (in proportions which delighted the author) on chromatographic analysis of samples which were extracted via procedure 7, this method was utilized throughout the remainder of the study.

Early analysis of mucus extracts was performed on the F and M chromatograph. Operator inexperience and unfamiliarity with the

idiosyncrasies of this machine were probably partially responsible for the low acid recovery from samples injected into the equipment. When the new Bendix chromatograph became available, operations were immediately switched to the latter instrument. In several respects, the Bendix apparatus was superior to the F and M chromatograph. First, the old machine contained tube electronics which took hours to warm up and seemed perpetually susceptible to outside "noise." The new machine contained solid state electronics, warmed up quickly, and yielded consistent results. Second, the F and M instrument utilized a stainless steel column, which had a tendency to absorb acids, causing peak "tailing." Tailing decreased acid separation efficiency and made quantitation of acid recovery difficult. The Bendix was fitted with a glass column which allowed unimpeded acid flow through the system. Finally, sensitivity on the F and M machine could be increased only to a point where baseline determination became impossible and peaks became unreliable. The new machine presented no such problem within the desired sensitivity range. Injections with dilutions of fatty acid standard solutions showed that the Bendix chromatograph could detect the presence of fatty acids in concentrations of nanograms per 0.2 ml.

The relationship (if any) of volatile fatty acids in cervical mucus to estrus in cows awaits clarification. The current study does indicate that fatty acids are present in cervical mucus during estrus, but no role for these acids has been demonstrated. Among the deficiencies of the present work, several should be listed:

1. Other than in early attempts, no behavioral tests were made

using extracts of mucus. Clearly, no function can be attributed to the presence of fatty acids in mucus without use of this very essential tool. The fact that fatty acids in monkeys constitute a sex attractant is interesting, and one might be tempted to draw a parallel between species, but such associations are clearly dangerous. One is reminded, in two species as closely related as the rat and the mouse, of exactly opposite responses to the same stimulus. (Hypothyroidism increases the ability of rats, and decreases the ability of mice, to respond to gonadotrophin; Nalbandov, 1964.)

2. The efficiency of extraction was not determined for any extraction technique. Therefore, values given for concentrations of fatty acids must be considered as minimum values. The actual concentrations of fatty acids may be considerably higher than reported.

3. Considerable reliance was placed upon ovariectomized heifers as a source of mucus. Although efforts were made to simulate natural body conditions, one may not assume that the heifers were in "normal" estrus with "normal" mucus production having "normal" pheromone content. Perhaps the best indication that the heifers were stimulated into a state closely resembling estrus was the "riding" response which was observed on several occasions following estrogen stimulation.

4. A method needs to be perfected which will allow collection of mucus without contamination by blood. Blood is known to contain fatty acids in cows, but its contribution to total fatty acid content of samples in the current study cannot be estimated. That blood is not the sole contributor of fatty acids is certain, however, as several uncontaminated samples (both dairy and beef) yielded fatty acids.

5. Tampon collection of mucus may be undesirable in several respects. Mucus is difficult to extract from absorbant cotton. In the present study, three washes with diethyl ether may have missed considerable mucus. Tampons may upset the natural environment of the uterus, cervix, or vagina. Microbial disruption may occur, or tissue irritation could take place. Heifers appeared uncomfortable after most tampon insertions; whether their normal processes were interrupted is unknown to the author. Tampons may not be a realiable means of quantitating mucus production. On several occasions in the present study, rectal massage immediately following tampon removal expelled additional mucus.

6. The scarcity of data collected in the current study allows little speculation even of the relative amounts of fatty acids present in mucus, much less speculation of any periodicity of fatty acid production throughout estrus. Many more samples must be analyzed with reliance upon statistical methods before any significance may be achieved.

7. A more thorough understanding of the principles involved in the various extraction techniques used is needed. Many deficiencies of the extraction processes were undoubtedly caused by improper application of materials and methods. Use of tests to determine extraction efficiencies would have forced some clarification of principles involved.

CHAPTER V

SUMMARY AND CONCLUSIONS

Cervical mucus was collected, either by massaging the mucus from the reproductive tract of estrus dairy cows, or by tampon absorption of mucus from ovariectomized beef heifers brought into physiological estrus by hormone treatment.

Mucus was extracted with isopropyl alcohol and the extract subjected to gas-liquid chromatographic analysis. Acetic, propionic, isobutyric, butyric, isovaleric, and valeric acids were identified in pooled samples of mucus from seven estrous dairy cows and in individual samples collected at 6-32 hours subsequent to hormone treatment from two ovariectomized beef heifers.

Cervical mucus from an estrus cow was found to contain mixtures of short chain fatty acids. Acetic acid was found in highest concentration, and propionic acid was next in order of abundance. Relative proportions of fatty acids were similar in both dairy cows and beef heifers.

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