NITRATE TOXICITY IN DAIRY HEIFERS. I. EFFECTS ON REPRODUCTION, GROWTH, LACTATION, AND VITAMIN A NUTRITION

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SUMMARY

Forty-five dairy heifers were fed nitrate at levels of 0, 440, and 660 mg/kg body weight daily beginning three estrous cycles before breeding, or at 40, 150, or 240 days of pregnancy, continuing until they were killed at 30 days after parturition. Conception rate was lower in heifers fed 660 mg nitrate. One abortion occurred in those fed the lower level of nitrate and two abortions and two deaths occurred in those fed the higher level. Growth, length of estrous cycles, length of gestation, birth weight and performance of calves, vitamin A and carotene nutrition, and milk production were similar in all groups. Nitrate feeding increased the nitrate content of milk and meat, but the contamination was so small that it did not appear to constitute a hazard to human health.

The various conditions which may cause nitrate to accumulate in relatively high concentrations in various crops and weeds were reviewed by Wright and Davison (40), who stressed that level of soil nitrogen, drought, intensity of light, and species of plant were major factors influencing nitrate accumulation under practical conditions. Nitrate is found in highest concentration in the plant stem, and in very small quantities, only, in the seed.

The quantity of nitrate required to produce acute toxicity in cattle is still controversial. Estimates of the LD_{50} range from 330 mg/kg body weight daily when given as a drench (3) to 990 mg/kg when fed on hay (7). Bradley et al. (3) arbitrarily set a limit of 0.92% nitrate,[1] or 1.5% KNO_3, as the maximum that could be safely fed in hay, and apparently intended this level to apply when hay was the major, if not the only, dietary constituent. The work of Kretschmer (22) showed that cattle could survive on a diet consisting entirely of forage containing 4.0% nitrate, if they were not overly hungry before given access to the high nitrate forage.

Consumption by cattle of sublethal levels of nitrate, usually meaning 0.5 to 1.0% of the diet, has reportedly caused abortion, lowered milk production, loss in body weight or slower growth, and vitamin A deficiency (4, 11, 15, 23, 30, 31). Many of these reports were based on the detection of nitrate in forages being consumed rather than on controlled experimentation. In preliminary investigations at this station, the feeding of forages containing up to 2.3% nitrate did not cause abortion, lower milk production, or slower growth (7). Investigations were, therefore, intensified. The effects of various levels of nitrate fed for various portions of the reproductive cycle on conception, survival of the embryo and fetus, vitamin A nutrition, growth, and lactation in dairy heifers were studied.

EXPERIMENTAL PROCEDURE

The 20 grade Holstein heifers comprising Replicate 1 (1961-62) were estimated to be 16-20 months old and weighed 600 to 850 lb at the beginning of the experiment. Their reproductive tracts appeared free of abnormalities upon rectal palpation.

During a preliminary 6-wk period the animals were accustomed to the experimental ration. It consisted of first-cutting alfalfa-grass hay, mostly alfalfa, to appetite and 4 lb of concentrate containing 80% ground shelled corn, 18% soybean meal (44% protein), 1% trace mineralized salt,[2] 1% dicalcium phosphate and 2,000 IU vitamin A palmitate per pound. All estrous periods were recorded. Antibody titers were determined in serum for brucellosis and leptospirosis, and in vaginal mucus for vibrio-

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[1] All quantitative expressions have been converted to nitrate ion, and are on a dry matter basis for feeds.

[2] Guaranteed to contain 96-99% NaCl, 0.25% Mn, 0.1% Fe, 0.05% $SO_4^{=}$, 0.33% Cu, 0.015% Co, 0.008% Zn, and 0.007% I by the processor.

sis; all heifers were free of these diseases.

The 20 heifers comprising Replicate 2 (1962-63) were more uniform in weight and age than those of Replicate 1, being about 16 months old and weighing 550 to 660 lb. They were managed as previously described, except that vitamin A was not added to the concentrate.

After the preliminary period, the animals were randomly assigned to the following experimental groups of ten animals each: (1) control, (2) fed nitrate beginning three estrous cycles before breeding, (3) fed nitrate beginning the 40th day of pregnancy, and (4) fed nitrate beginning the 150th day of pregnancy. Groups 2, 3, and 4 were subdivided so that three animals in each group received 440 mg nitrate per kilogram body weight per day and two animals in each group received 660 mg during Replicate 1, and this order was reversed in Replicate 2.

Since heifers in Replicates 1 and 2 appeared to adapt to nitrate feeding by increased erythropoiesis, five Holstein heifers of similar age and weight to those of Replicate 2 were purchased when seven months pregnant and fed nitrate at the 660 mg level beginning at the 240th day of pregnancy. These heifers were sold after 30 days of lactation.

The amounts of nitrate fed were adjusted every 28 days for changes in body weight. Fertilizer grade synthetic sodium nitrate was dissolved in hot water and sprinkled on chopped hay just before each of two equal daily feedings. All animals were permitted to exercise in a small paddock for about 2 hr each morning and evening, at which time they had access to water, trace mineralized salt, and dicalcium phosphate, and were watched for estrus. The heifers were bred artificially, beginning at the fourth observed estrus. Development of the fetus was followed by rectal palpations at 28-day intervals.

Shortly after parturition, all placentae were examined and one calf from each nitrate level within each group was necropsied. The remaining calves, and calves purchased to replace those necropsied, were fed milk from their respective dams at a rate of 4 lb twice daily to 14 days of age, and 5 lb to 30 days when both cow and calf were necropsied. Immediately following parturition, the concentrate was increased at a rate of 1 lb per feeding until the heifers were consuming 18 lb per day.

Daily milk production records were kept. Milk samples were taken at weekly intervals and analyzed for nitrate by the xylenol method of Greweling et al. (14), fat by the Babcock method, total solids with the Watson lactometer and protein by the Orange G dye method previously described by Castillo et al. (5). Per cent solids-not-fat was determined by difference.

Plasma from jugular blood samples taken at 4-wk intervals was analyzed for carotene and vitamin A by the method of Kimble (21). Samples of liver tissue were obtained at the start of the experiment and near the 40th and 150th days of pregnancy by the technique of Udall et al. (34) and immediately analyzed for vitamin A by the method of Davies (8).

Samples of meat were collected from the round (semimembranosus and semitendinosus muscles and fascia) and rib (longissimus dorsi muscle and fascia) areas at slaughter, freeze dried, and analyzed for nitrate by the xylenol method (14).

Samples of the basic feeds were collected biweekly and pooled for a single monthly sample. Nitrate was determined by the phenoldisulfonic acid method of Harper (16), carotene by the AOAC method (1), and crude protein by the Kjeldahl method. Calcium and phosphorus were determined after ashing by flame photometric (13) and colorimetric (2) methods, respectively. Total soluble carbohydrate was determined by refluxing 0.5 g samples for 1 hr in 2% sulfuric acid and analyzing the filtrate by the method of Heinze and Murneek (17).

RESULTS

Results of the feed analyses are given in Table 1. Hay fed in Replicate 1 contained much less carotene than that fed in Replicate 2. The hay of Replicate 1 was also lower in soluble carbohydrate and calcium, and higher in crude protein and nitrate. At no time did nitrate in any sample exceed 0.1%.

The productive and reproductive performance of the heifers in Replicates 1 and 2 are summarized in Table 2. Average hay consumption was similar among groups. The amount of hay offered was carefully controlled throughout to attain maximum consumption with minimum refusal, since the hay was the nitrate carrier. Most refusals were less than 5%. One heifer offered 660 mg nitrate/kg, Group 4, consistently left hay when the nitrate was added, despite severe restriction of the quantity offered, and averaged 15% refusals to the end of the trial. Other heifers refused the nitrate-treated hay at first; therefore, the quantity of hay offered was reduced. They adjusted rapidly to it, and in most cases were consuming hay at their original levels within 2 wk.

Data from Groups 3 and 4 were averaged

TABLE 1
Nutrient content of the basic feeds

	Hay		Concentrate	
	1961-1962 Replicate 1	1962-1963 Replicate 2	1961-1962 Replicate 1	1962-1963 Replicate 2
Carotene *(μg/g)*[a]	5.4	18.2	1.9	1.2
Crude protein *(%)*	13.5	11.6	16.9	16.2
Nitrate *(%)*	0.07	<0.01	0.02	<0.01
Calcium *(%)*	0.80	1.13	0.24	0.24
Phosphorus *(%)*	0.21	0.19	0.45	0.51
Ash *(%)*	6.6	6.8	4.1	4.3
Soluble carbohydrate *(%)*	12.6	15.2		

[a] Carotene analyzed on an as-fed basis, other nutrients determined after drying overnight at 70 C, with a moisture loss of 8 to 10%.

TABLE 2
Productive and reproductive performance of dairy heifers fed various levels of nitrate beginning at various stages of reproduction

	Level of nitrate fed in Replicates 1 and 2, mg/kg			Nitrate beginning 240th day gestation
	0	440	660	
Avg hay consumption *(lb/day)*	18.1	18.0	17.8	13.5
Avg hay refusal *(% of offered)*[a]	4.5	4.2	5.2	23.7
Avg estrous cycle length *(days)*[b]	21.0	20.9	21.1	....
No. of conceptions to first service [b]	25	3	1	....
Avg no. of services per conception [b]	1.3	1.4	2.6	...
No. of abortions	0	1	2	0
No. of deaths	0	0	2	0
Avg daily gain to calving *(lb)*	1.44	1.51	1.41	1.48
Avg gestation length *(days)*[c]	278	275	274	278
Avg birth weight of calves *(lb)*	86	80	77	86
Avg daily gain of calves *(lb)*	0.67	0.91	0.85	...
Avg milk production *(lb/day)*	43.6	42.6	40.8	38.9
Avg milk composition				
Nitrate *(μg/ml)*[d]	5	9	15	9
Total solids *(%)*	12.5	13.0	12.8	13.2
Fat *(%)*	3.8	4.0	3.7	4.3
Protein *(%)*	3.4	3.4	3.6	3.6
Avg nitrate in meat *(μg/g)*[d]	5	16	21	

[a] Not corrected for moisture gain. Analyses of periodic samples indicated that refusals contained 30 to 40% water.
[b] Values at this stage represent averages of 30, 5, and 5 heifers, respectively. Heifers fed 660 mg nitrate required more services than controls or those fed 440 mg ($P < 0.05$).
[c] Heifers that aborted not included.
[d] Values significantly different from each other, $P < 0.005$; Column 4 not included in comparisons.

with those of the control group for comparing lengths of estrous cycles and conception rates. Nitrate feeding did not alter the length of the estrous cycle. The conception rate of heifers fed 660 mg nitrate was lower than either the controls or those fed 440 mg nitrate, but this difference was probably not due entirely to the nitrate (see discussion).

One heifer fed 440 mg nitrate, Group 3, aborted at 257 days of gestation. Two heifers fed 660 mg nitrate aborted; one at 237 days of gestation in Group 2, and one at 179 days in Group 3. *Pseudomonas aeruginosa* was cultured from the stomach of the 237-day fetus. Abortions were confined to the second replication and, judging from autolysis of the fetal organs, occurred a few days after fetal death. The exact methemoglobin levels in the dams when fetal death occurred are not known. However, the maximum values recorded were 39% seven days before abortion in the heifer fed 440 mg nitrate, and 84 and 77%, respectively, several months before abortion in those fed 660 mg. These latter heifers had consistently high methemoglobin values.

A heifer fed 440 mg nitrate, Group 3, had a stillborn calf weighing 67 lb at 266 days of gestation. The calf was born during the night with no attendants present. Its lungs showed fetal atalectasis, indicating that respiration had not occurred. Upon slaughter of the cow 30 days later, a double uterine cervix was discovered. The wall separating the two cervices had been partially torn during calving. It is possible that this condition slowed the birth process, causing death of the calf. The dam was the smallest cow in the experiment, weighing only 800 lb at slaughter. The calf also had a double cervix.

Two heifers in Replicate 2 died, and one heifer in Replicate 1 was near death; all three were receiving the higher level of nitrate. Both deaths occurred during the night; one after 270 days of nitrate feeding, and the other after 249 days of nitrate feeding and ten days postpartum. Both heifers died suddenly, with no previous illnesses. Another heifer collapsed after 162, and again after 181 days of nitrate feeding. She had 93% methemoglobin on the first occasion; no measurement was made on the second. Other signs were a grayish-brown discoloration of the visible mucous membranes, a staggering gait before collapse and during recovery, urination (clear) without attempting to lift the tail, and a weak sway-backed stance when standing. She was fed hay without nitrate both evenings and recovered without treatment. Nitrate feeding was resumed the following morning. She delivered a normal 73 lb calf at 273 days.

Nitrate feeding did not alter the length of gestation in heifers that did not abort. The birth weight of calves from heifers fed nitrate did not differ significantly from controls. Birth weight varied from 65 to 100 lb, with both extremes from heifers fed nitrate. In general, the heifers of Group 4 had lighter calves, probably because all but one of these heifers conceived to the first service and they therefore calved at younger ages and lighter weights. The heifers of Group 2, Replicate 1, had the heaviest calves, averaging 90 lb. The growth rate of calves from nitrate-fed heifers was not significantly greater than control calves.

Milk production was not significantly reduced in the nitrate-fed cows. Average daily milk production ranged from 30 to 53 lb in control heifers, 26 to 57 lb in heifers fed 440 mg nitrate, and 30 to 48 lb in heifers fed 660 mg nitrate. The only change in milk composition associated with treatment was a significant increase in nitrate content. The 15 μg/ml present in milk from those fed 660 mg nitrate does not appear to constitute a health hazard to humans, in view of tolerances of 200 μg nitrite/g in processed meats (35) and 45 μg nitrate/ml in water (36). Apparently, there are no federal regulations specifying safe limits for nitrate in milk.

Nitrate feeding increased the nitrate content of the carcass. The values were the same in samples from the round and rib areas. These increases were so small, however, that they present no health hazard.

The growth curves, shown in Figure 1, were nearly identical for all treatment groups. The

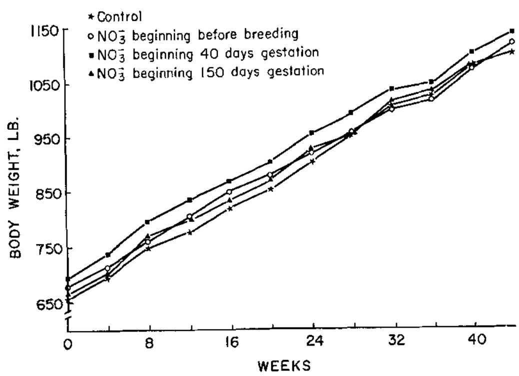

FIG. 1. Growth curves of the heifers of Replicates 1 and 2 as related to grouping.

daily rates of gain, given in Table 2, were similar among nitrate levels and indicate that a near-lethal level of nitrate does not by itself alter growth.

Because of differences between replicates in the carotene and vitamin A content of the feeds, the plasma vitamin A and carotene values of each replicate are presented separately. Plasma vitamin A was moderately low to normal in the heifers of Replicate 1 at the beginning of the experiment (Figure 2) and it decreased slowly to parturition, regardless of

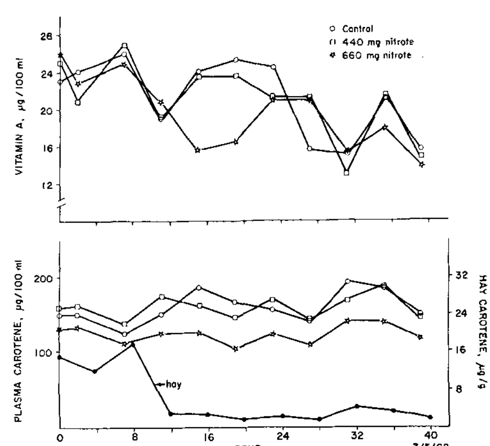

FIG. 2. Vitamin A and carotene in the plasma of the heifers of Replicate 1 as related to level of nitrate and carotene fed.

treatment. Plasma carotene remained static and animals with higher carotene at the beginning remained higher to the end of the experiment. The hay was very low in carotene from the third month to the end of the replicate. Some of this hay was fed in the beginning of Replicate 2. Plasma vitamin A levels were higher initially in the heifers of Replicate 2 (Figure 3), but they decreased until

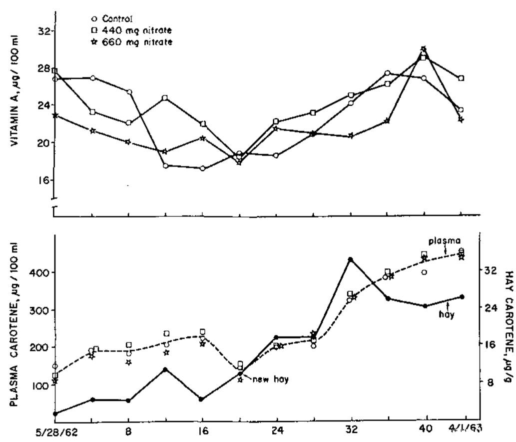

FIG. 3. Vitamin A and carotene in the plasma of the heifers of Replicate 2 as related to level of nitrate and carotene fed.

the new, higher carotene hay was introduced at the 20th week. Plasma carotene and vitamin A then increased to 40 wk, when vitamin A fell as parturition approached, which is a normal occurrence (32).

Liver vitamin A data given in Figure 4 are on a fresh weight basis. Liver storage was somewhat low at the start of both replications and remained low throughout, but this is probably to be expected, because the heifers were

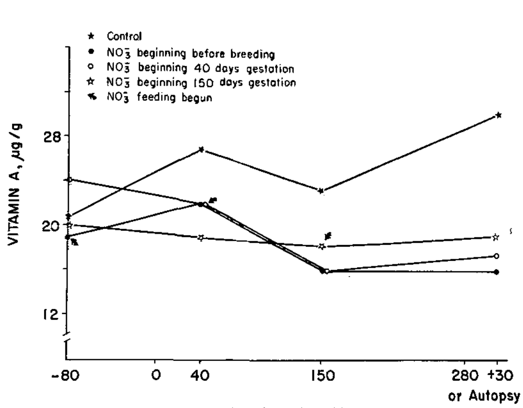

FIG. 4. Vitamin A in the livers of the heifers of Replicates 1 and 2 as related to grouping.

purchased in early spring and kept in drylot thereafter. Although liver vitamin A in the control animals appears to have increased, whereas that of all other animals decreased slightly, the differences were not statistically significant. Liver vitamin A in the animals of Groups 3 and 4 was declining before nitrate feeding was begun at 40 and 150 days of pregnancy, respectively, when that of Group 4 plateaued. One abscessed liver was observed at necropsy, and liver weight did not differ significantly among treatments.

Liver vitamin A had apparently increased over initial levels in all heifers of Replicate 1 when samples were taken at 40 days of pregnancy. This increase was probably an artifact caused by losses of vitamin A in the earlier samples, since they were stored for a short period while the project group was moving into a new laboratory. Vitamin A storage had decreased by 150 days and apparently remained unchanged thereafter.

By the time a few liver samples had been taken at 150 days of pregnancy from the heifers of Replicate 2, it was apparent that liver vitamin A was decreasing in all groups. All heifers were, therefore, biopsied again on December 1. Data are shown in Figure 5. While the data

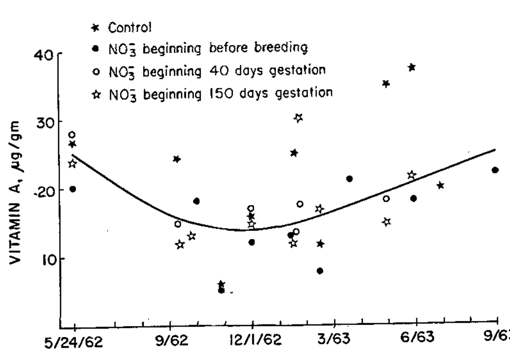

FIG. 5. Variation observed in the vitamin A content of the livers of heifers of Replicate 2, and the changes associated with the level of dietary carotene.

for May 24 and December 1 represent averages of all heifers sampled on the same date, the other points represent single observations, or averages of two to three values, and show variation inherent in the technique as well as the trend in vitamin A storage. The increase in vitamin A after the low period between October and December corresponds with the feeding of the higher carotene hay. Much variation was introduced into the final vitamin A values in Figure 4, because of this period of increasing vitamin A storage and the wide variation in times that the heifers were necropsied.

Data from the heifers started on nitrate at 240 days gestation are presented in the last column of Table 2. These heifers consumed only about half as much hay as would be expected of 1,200-lb heifers, but the time between the beginning of nitrate feeding and parturition was too short for them to become hungry enough to eat more. Starvation was attempted briefly but was discontinued. Consequently, an average of 510 mg nitrate/kg body weight was consumed daily. One of these heifers did consume 95% of her hay for a 2-wk period; a 42% methemoglobinemia was the only adverse effect. Their milk production and milk composition compare very favorably with those of the previous heifers.

DISCUSSION

The summer of 1961 was very rainy and humid. Hay purchased then was coarse, and although only a small portion was rained on during curing, it had discolored and molded considerably in storage. The summer of 1962 was droughty. Hay purchased that year had a good green color although it was overmature. Some seed pods had formed in the alfalfa despite the apparent fine stems. These differences explain the higher carotene content of hay fed in Replicate 2.

The ration was formulated to meet the nutrient requirements for normal growth of these heifers, as established by the NRC (24). It is well known that additional energy will increase a ruminant's resistance to nitrate (19, 26) and the grain fed herein undoubtedly offered some protection. The nitrate content of the daily rations, including the grain, averaged slightly over 2% during gestation for heifers fed 440 mg nitrate/kg and slightly over 3% for those fed 660 mg. The nitrate content of the total ration decreased somewhat after parturition because of dilution by the additional grain. Hay consumption decreased slightly during lactation.

There was a small but abrupt drop in hay consumption by all heifers in Replicate 2 when feeding of the overmature hay began, but normal consumption was recovered over a period of several days. Hay consumption also dropped abruptly when nitrate feeding began, but in most cases it returned to the original level within a few days. Heifers fed nitrate beginning at 240 days of pregnancy did not regain normal hay consumption.

Consumption of oat hay containing up to 2.3% nitrate was not lower than that of control hay in previous growth and lactation studies with dairy cattle (7), and nitrate additions at similar levels did not reduce feed consumption in growing fattening studies with lambs (6, 12). Nitrate feeding did not reduce body weight gains in any of the preceding studies. On the other hand, nitrate additions reduced feed consumption and rate of gain of fattening steers when it was fed with grain or mixed in a ration containing cottonseed hulls (15, 37). Therefore, nitrate probably does not slow growth except in cases where it reduces feed consumption.

There was no indication that nitrate lowered milk production, and perhaps the preceding conclusion on growth applies here. High-nitrate oat hay did not lower milk production in the study of Crawford (7). These observations are in contrast with the conclusions of Muhrer et al. (23), Stewart and Merilan (30), and Case (4). In the report of Case, the reduced milk production observed in cattle eating high-nitrate silage may have been caused by an energy deficiency per se, since the cows did not have methemoglobinemia and the feeding of molasses improved milk production. All lactation experiments, including this one, could have been improved by prolonging them, and perhaps additional studies are justified on this basis.

The observations by Muhrer et al. (23) and Case (4), that vitamin A deficiency occurred in forages grown during a drought, and that these forages contained more nitrate than normal, suggested that vitamin A deficiency may have been associated with nitrate consumption. Many studies with rats have since shown that nitrate, and particularly nitrite, interferes with conversion of carotene to vitamin A and storage of vitamin A in the liver [See Wright and Davison (40) for review]. The data reported herein indicate that nitrate consumption has very little, if any, effect on vitamin A nutrition of cattle. Most of the differences observed could be explained by the levels of vitamin A and carotene in the feed, and the levels in the plasma and liver initially. Concurrent investigations at other stations with cattle (37) and sheep (6, 12) have supported these observations. Death would probably occur before ruminants could consume enough nitrate to alter their vitamin A metabolism. This does not preclude the possibility of vitamin A deficiency caused by a shortage of vitamin A or carotene in the diet, which may or may not be related to nitrate. Gaseous reduction products from nitrate have been shown under laboratory conditions to destroy carotene in silage during fermentation (25), but the extent of this destruction should be investigated further under applied conditions. Nitrate apparently does not destroy vitamin A in dry feeds (9).

The extreme variation noted in the vitamin A contents of the liver was surprising, since these heifers were fed and managed in an identical manner for over 18 months. Animals with greater initial reserves tended to maintain their respective position, and correction for this initial level might be used to lessen variation in future work.

The reproductive problems encountered are summarized in Table 3. Failure to deposit semen directly into the uterus probably explains the conception problem with Heifer 287. Many heifers not yet consuming nitrate required two inseminations before conception. The reduced fertility of Heifers 257, 263, and 279, all fed nitrate, could have been caused by the observations listed. Therefore, the role of nitrate in causing these poor conception rates, or the problems associated with conception, cannot be assessed.

The embryonic deaths observed in Heifer 310, and possibly 263, were the only ones detected in the 40 heifers. This small number was well within the 16% reported for normal animals by Kidder (20). Heifer 310 probably should not have been bred following estrus on the 49th day, since this probably did not allow sufficient time for the uterus to return to normal.

Many reports have indicated that consumption of high levels of nitrate may cause abortion in cattle (11, 28, 33, 39). In these reports, the abortions were associated with high levels of methemoglobin in the dam, or death of other animals in the same herd. There are enough experimental data to indicate that nitrate consumed at levels less than 2% of the diet, or 440 mg/kg body weight daily, does not cause abortion (7, 10, 27, 29, 38, 39). Abortions reported in animals eating lower levels of nitrate (11, 31), in the absence of other signs such as methemoglobinemia, collapse, or death, may be due to other factors. In the data collected by

TABLE 3

Summary of all the reproductive problems encountered

Cow no.	Level of nitrate	Problem	Observations
		Control	
287	0	5 inseminations for conception.	Cervix very tortuous; catheter not passed beyond third cervical ring until 5th insemination.
316	0	2 inseminations for conception.	No abnormalities observed.
		Nitrate beginning 3 estrous cycles before breeding	
274	440	2 inseminations for conception.	No abnormalities observed.
310	440	Embryonic death between 40 and 49 days. 2 inseminations for 1st conception; 2 for 2nd.	Palpated pregnant 39th day. Estrus 49th day; did not conceive then, returned to estrus and conceived 20 days later.
257	660	3 inseminations for conception. Aborted at 231 days gestation.	Multiple cysts in left oviduct; fluid could not be forced through. Fetus in right uterine horn, side of prior ovulations unknown. *Pseudomonas aeruginosa* isolated from stomach of aborted fetus.
263	660	3 inseminations for conception.	Only 3 days between 1st and 2nd estrus, 39 days between 2nd and 3rd. Ovulation after 1st unknown, ovulated after second. Prior estrous cycles were normal length.
273	660	4 inseminations for conception.	No abnormalities observed.
279	660	2 inseminations for conception. Died after 270 days on nitrate.	Insemination delayed for 24 hr after estrus detected.
		Nitrate beginning 40th day of pregnancy	
237	660	Aborted at 179 days gestation.	No abnormalities observed.
314	660	2 inseminations for conception. Died 10 days after parturition.	No abnormalities observed.
		Nitrate beginning 150th day of pregnancy	
283	440	2 inseminations for conception.	No abnormalities observed.
307	440	Stillborn calf.	Double cervix, septum partially torn.

Hillman et al. (18), approximately 75% of bovine abortions investigated remained unexplained, illustrating the inadequacy of our present knowledge of the causes of abortion.

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