Purchased by the U. S. Department of Agriculture
For Official Use

Reprinted from AGRONOMY JOURNAL
Vol. 68, Nov.-Dec. 1976, p. 979-982

Higher Fatty Acid Composition of Immature Forages as Affected by N Fertilization[1]

H. F. Mayland, L. F. Molloy, and T. W. Collie[2]

ABSTRACT

High levels of both N and total higher fatty acids (HFA) in forage have been associated with increasing the grass tetany hazard to grazing cattle. The objective of this study was to determine the relationship between forage N, total HFA, and HFA species distribution in several forages.

Forage N, HFA, HFA species concentration, and total chlorophyll were determined in immature vegetative growth of *Agropyron desertorum* (Fisch.) Schult., *Cynodon dactylon* L., *Lolium perenne* L., *Trifolium repens* L., and *Triticum aestivum* L. established with soil fertility levels up to 500 ppm N in the growth chamber. Forage HFA concentrations were positively and linearly related to forage N levels, but regression coefficients were not the same for all species. The HFA concentrations were as high as 16 mmol COOH/100g DM at 6% total N in first cutting *Lolium perenne* L. The relative HFA species distribution was the same within a given forage, even though total N concentrations ranged from 2 to 6%. The mean HFA specie concentrations (determined by gas-liquid-chromotography relative to mean total HFA concentrations determined by titration) when expressed as percent for the grasses were: C14:0 — 2%, C16:0 — 13%, C16:1 — 1%, C18:0 plus C18:1 — 1%, C18:2 — 11%, and C18:3 — 67%. The total HFA concentrations were positively correlated with chlorophyll a + b concentrations which was expected, since the HFA of green plants is largely associated with chloroplast membrane.

Additional index words: **Hypomagnesemia, *Agropyron desertorum* (Fisch.) Schult., *Cynodon dactylon* L., *Lolium perenne* L., *Trifolium repens* L., *Triticum aestivum* L.**

GRASS tetany, a Mg deficiency in ruminants, may result when animals graze rapidly growing forage low in Mg or containing factors which reduce forage Mg availability to the animal. Forage with high N concentration has been associated with reduced Mg availability and an increased incidence of grass tetany in grazing cattle (7, 8, 10). However, present evidence indicates that the reduction in apparent Mg availability to the animal may not be a direct function of forage N levels per se, but rather of other factors, including the higher fatty acid (HFA, aliphatic carboxylic acids having more than eight carbon atoms) levels found in high-N forage.

Kemp et al. (9) suggested that forage HFA's reduce the availability of forage Mg to animals by forming insoluble soaps. Animal studies have supported this hypothesis. Clarke and Roberts (2) observed that an appreciable amount of the HFA found in lamb feces was in the form of soaps. They noted that hydrogenation of unsaturated HFA by rumen microorganisms enhanced the formation of soaps. Kemp et al. (9) reported that the addition of animal fat to the diets of dairy cows reduced Mg availability and increased the amount of Mg excreted in the feces and recovered as earth-alkali soap. Wilson et al. (15) found that plasma Mg concentrations of peanut oil supplemented cows were significantly lowered when compared to controls. This reduction was greater in older than in younger cows and paralleled observations that older cows were more likely to develop grass tetany than younger cows.

A high correlation has been shown between N and HFA concentrations in forages with a wide range of

[1] Contribution from the Western Region, ARS, USDA, in cooperation with the Soil Bureau, Dep. of Scientific and Industrial Research, New Zealand. Received 2 Sept. 1975.

[2] Soil scientist, Snake River Conservation Research Center, Kimberly, ID 83341; soil scientist; and chemist (deceased), Soil Bureau, Lower Hutt, New Zealand, respectively.

Table 1. Effect of N fertilization on maximum NO_3-N concentrations in forages and on relative dry matter yield per pot for five N fertility levels.

Forage	Relative dry matter yield** N level N_1	N_2	N_3	N_4	N_5	Max. dry matter yield/pot	Max. NO_3-N concentration
	%					g	%
Wheatgrass	1 e†	35 d	71 c	93 b	100 a	11.0	0.04
Ryegrass, 1st 1st harvest	44 d	65 c	94 b	100 a	76 c	2.6	1.33
Ryegrass, 2nd harvest	58 d	62 c	76 b	76 b	100 a	8.1	1.12
Wheat	43 d	84 c	100 a	93 b	82 c	4.4	1.20
Bermudagrass, 2nd harvest‡	20 e	37 d	58 c	100 a	96 b	11.3	0.36
Clover§	56 b	100 a	98 a	50 b	0 c	7.5	0.81

** Data were processed by the analysis of variance and Duncan's multiple range test. Row means followed by no letters in common are significantly different at the 1% level. † Insufficient sample for chemical analysis.
‡ 1st harvest bermudagrass not analyzed. § Yield reductions for N_4 and N_5 treatments resulted from dampening-off disease (*Pythium* spp.).

morphological maturities (1, 9, 12). However, both N and HFA concentrations decrease during plant maturation, and published data on HFA vs. N have been generally confounded by differences in morphological maturities of samples.

The objectives of this study were to determine: first, the effect of added fertilizer N on the concentrations of N, and total HFA, and their interrelationships in forage samples of similar morphological maturity; and second, the effect of added N on the relative distribution of HFA species.

METHODS AND PROCEDURES

'Nordan' crested wheatgrass [*Agropyron desertorum* (Fisch.) Schult.], 'common' perennial ryegrass (*Lolium perenne* L.), 'Wanser' hard red, winter wheat (*Triticum aestivum* L.), 'common bermudagrass (*Cynodon dactylon* L.), and 'ladino' clover (*Trifolium repens* L.) were germinated in pots containing 3 kg of a calcareous loam soil (Durixerollic Camborthid, pH = 8.0, CEC = 17 meq/100 g, 1% O.M.) similar to the Orovada series. Plants were grown in chambers with day/night temperatures of 18/6, 25/20, 25/20, 30/25, and 25/20 C for the above species, respectively, and a 15/9-hour light/dark cycle. The light intensity was 580 μeinsteins m^{-2} sec^{-1} (400 to 700 nm range) at the top of forage canopy. Soil moisture was adjusted daily to −0.3 bar potential. Time from seeding to first harvest was 100, 20, 40, 20, and 60 days for the five species listed above, respectively. Second harvests were sampled from ryegrass and bermudagrass after 18 and 22 additional days, respectively. This harvest schedule produced forage having similar morphological maturity, except for clover. All grasses were harvested at the immature vegetative stage of growth, while the clover was in the bud to early flowering stage.

Before seeding, 50 ppm K (as K_2SO_4), 50 ppm Mg (as $MgSO_4 \cdot 7H_2O$), 50 ppm P (as H_3PO_4), and selected levels of N (as NH_4NO_3) were mixed with the soils. The N levels were: wheatgrass — 0, 50, 100, 150, 200 ppm N; ryegrass — 50, 100, 150, 200, 500 ppm N; and for bermudegrass, wheat, and clover — 0, 50, 100, 300, 500 ppm N. Ryegrass was fertilized with an additional 50 ppm N and 50 ppm K after the first harvest. The N levels were adjusted in an attempt to have maximum dry matter yields at intermediate N fertilizer levels.

Forages were clipped at a 1-cm stubble height and immediately frozen in a forced-draft freezer at −30 C. Six replications each of wheatgrass, first and second harvest ryegrass, and four replications of wheat where freeze-dried at regular intervals and immediately analyzed by the USDA-ARS laboratory for concentrations of total HFA, total N, and chlorophyll a + b. Four replications each of wheatgrass, second-harvest ryegrass, and second-harvest bermudagrass, and two replications each of first-harvest ryegrass, wheat, and clover were periodically dried to constant weight in a forced-draft oven at 108 C for about 1 hour, sealed in polyethylene bags, and air mailed to the Soil Bureau, D.S.I.R., New Zealand laboratory where, upon arrival, the samples were ground and analyzed for total and individual HFA and total N. Total HFA concentrations were determined within 2 days of forage drying at the USDA-ARS laboratory and provided a check on possible HFA loss by autooxidation (11) encountered during the 24-day average time lapse between oven-drying in the U.S. and analysis at the Soil Bureau laboratory in New Zealand. Oven-drying of whole plant samples before shipment was selected as the best drying procedure to minimize autooxidation under conditions of this study (11).

Total N (including NO_3) was determined by semimicro Kjeldahl, NO_3-N by nitrate electrode, chlorophyll a + b by spectrophotometry (6), and total HFA by titration (11). Individual HFA's were quantified by gas-liquid-chromotography (GLC) after methylation of HFA (11). Results are expressed on a dry-matter basis. Total N and total HFA concentrations were not different ($P < 0.05$) between the two laboratories, therefore data were composited for statistical analyses. Significance of difference between treatment means was determined using Duncan's multiple range test, while the significance of differences between regression coefficients was measured by the t-test (14). The variation in HFA and chlorophyll a + b concentrations within an N-treatment was attributed to experimental error, therefore regression analysis of HFA concentrations on chlorophyll a + b concentrations were performed on means of the N-treatments.

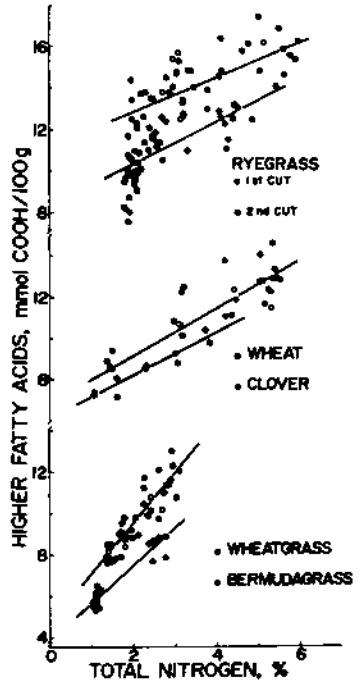

Fig. 1. Regression of total HFA concentration on total N concentration in five forage species. The forage, number of observations (n), regression equation and correlation coefficient are as follows:

Ryegrass (1st cut) n = 39, Y = 11.2 + $_c$0.83X, r = 0.66;
Ryegrass (2nd cut) n = 50, Y = 8.3 + $_c$1.02X, r = 0.63;
Wheat n = 29, Y = 6.8 + $_{bc}$1.17X, 4 = 0.84;
Clover n = 8, Y = 6.1 + $_{bc}$1.06X, r = 0.96;
Wheatgrass n = 41, Y = 4.6 + $_a$2.45X, r = 0.90;
Bermudagrass n = 19, Y = 3.8 + $_b$1.83X, r = 0.94.
Regression coefficients preceded by no subscript letters in common are significantly different ($P \leq 0.01$).

RESULTS AND DISCUSSION

Forage yield of grasses was increased by N fertilization (Table 1), with maximum yields obtained at the highest N rate for wheatgrass and second-harvest rye-

Table 2. Distribution of higher fatty acids (HFA) determined by GLC expressed as percent of total HFA determined by titration for five forage species.**

Forage	N†	C14:0	C16:0	C16:1	C18:0 C18:1	C18:2	C18:3
		%					
Wheatgrass	16	1.9 a	12.9 b	0.8 a	0.4 b	12.3 b	63.6 bc
Ryegrass, 1st harvest	10	2.3 a	13.5 ab	0.9 a	1.5 a	10.1 c	74.9 a
Ryegrass, 2nd harvest	20	2.1 a	14.1 ab	0.4 b	1.6 a	11.9 b	65.0 b
Wheat	10	2.3 a	12.5 b	0.7 a	1.1 a	8.5 d	68.1 b
Bermudagrass, 2nd harvest	10	1.3 b	14.0 a	0.4 b	1.2 a	14.6 a	58.7 c
Clover‡	8	2.8	26.0	0.7	7.9	16.5	35.8

** Data were processed by the analysis of variance and Duncan's multiple range test. Column means followed by no letters in common are significantly different at the 1% level. † Number of observations.
‡ Clover data not included in Duncan's test.

grass. Maximum yields of other species, including the first-harvest ryegrass, were obtained at intermediate N levels. The array of fertilizer N levels produced forage with total N concentrations ranging from 1 to 6% (Fig. 1). Fertilization rates may not have been high enough, however, to produce maximum N levels in the forage, especially in wheatgrass and bermudagrass which contained less than 3% total N.

Higher fatty acid concentrations increased linearly as total N concentrations increased (Fig. 1). The correlations (r) of forage HFA concentrations with N concentrations ranged from 0.63 for ryegrass to 0.96 for clover. The correlation coefficients were not significantly improved by using nonlinear regression analyses even for second-harvest ryegrass.

The rate of increase in HFA concentrations in relation to forage N concentrations ranged from 0.83 to 2.45 mmol COOH/100 g for each 1% increase in total N. These values were generally lower than those reported for field-grown forage. Molloy et al. (11) reported regression coefficients ranging from 2.3 to 3.2 for a wide variety of species in New Zealand, while Barta (1) reported values ranging from 2.0 to 2.7 in a series of grasses grown in Ohio. Values averaging about 4.6 mmol HFA/100 g have been reported for mixed forages in the Netherlands (9). The smaller regression coefficients of HFA on N for growth chamber versus field-grown forage may have resulted because the forages were not light-saturated under conditions of this study (5). The effect of light intensity on HFA concentrations needs to be investigated further.

The HFA concentrations in the luxuriant-appearing first-harvest ryegrass were higher (about 3 mmol/100 g/unit of N) than in the second harvest. However, the regression coefficients were not different ($P \leqslant 0.01$) from each other. The correlation of HFA vs. total N in ryegrass was lower than for other forages in this study. The regression of HFA on total N minus NO_3-N did not improve the correlation values.

In each of the forages, N fertilization increased total HFA and total N concentrations but did not significantly ($P \leqslant 0.01$) alter the distribution of the various HFA species within any given forage or harvest (data not shown). The HFA species concentration values were then averaged across N treatments for each forage and harvest, and calculated relative to total titratable HFA (Table 2). The sum of individual HFA as recovered by GLC ranged from 84 to 103% of total titratable HFA.

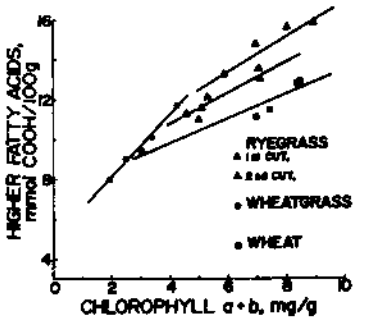

Fig. 2. Regression of total HFA concentration on chlorophyll a + b concentration in three forage species. Data are means within N-fertility treatments. The forage, number of mean observations (n), regression equation, and correlation coefficient are as follows:

Ryegrass (1st cut) $n = 5, Y = 7.94 + {}_{s}0.90$ Chl.a+b, $r = 0.89$

Ryegrass (2nd cut) $n = 5, Y = 7.64 + {}_{s}0.76$ Chl.a+b, $r = 0.93$

Wheatgrass $n = 4, Y = 5.01 + {}_{s}1.55$ Chl.a+b, $r = 1.00$

Wheat $n = 5, Y = 7.35 + {}_{s}0.61$ Chl.a+b, $r = 0.96$

Regression coefficients preceded by no subscript letters in common are significantly different ($P \leqslant 0.01$).

Palmitic (C16:0), linoleic (C18:2), and linolenic (C18:3) acids accounted for most of the total HFA measured. The relative concentrations of each HFA specie were generally similar among the grass species, except for the linolenic (C18:3) acid values. Linolenic acid predominated in all forages and its concentrations were highest in the first-harvest ryegrass (Table 2), which had the most "luxuriant" or "lush" appearance of the forages in this study. The amount of linolenic acid measured in the second-harvest ryegrass was significantly less ($P \leqslant 0.01$) than in first harvest, perhaps resulting from some physiological change in the plants. Hawke's (5) report that linolenic acid concentrations in forage were inversely related to temperature does not account for the difference in C18:3 found between these two ryegrass harvests because they both were grown at the same temperature.

The HFA species distribution for clover was excluded from the analysis of variance (Table 2) because plants were in bud and early flowering stage which resulted in a HFA-specie shift to the more saturated forms (i.e., C16:0, C18:0, C18:1, and C18:2 at the expense of C18:3). This shift accompanies flowering in legumes, as well as in grasses (13).

The HFA found in plants are primarily associated with lipids in the chloroplast membrane. Because of this relationship, there is often a positive correlation between the HFA and chlorophyll concentrations (5). The physiological relationship between HFA concentration and chlorophyll is certainly more

direct than that of HFA and N concentrations, because the HFA in forages is primarily in the chloroplast membrane which envelopes the chlorophyll molecules. In this study, a good correlation between total HFA and chlorophyll concentrations was found (Fig. 2). Perhaps an adequate supply of N provides growing conditions such that the plant has a high density or mass of chloroplasts and their associated lipids and HFA, giving them the "lush" appearance often noted coincident with grass tetany.

What is the relationship of forage HFA to animal metabolism and Mg availability? Hawke (5) noted that the levels of triglycerides encountered in immature, high N forage may exceed the biohydrogenation capacity of rumen microorganisms; however, others do not think this is likely (3). Nonetheless, stearic acid is the major fatty acid found in the rumen with little linolenic acid detected (2, 3). There is very little, if any, degradation of the HFA other than by hydrolysis in the rumen, and there is no evidence that the C16 and longer carbon chain HFA's are absorbed to any appreciable extent from the rumen (4). Thus the HFA may complex with $^{++}$Mg and $^{++}$Ca in the rumen, and then be excreted as water-insoluble soaps, thereby reducing Mg availability to the animal (9, 15). The effect of N fertilization on increasing the tetany hazard may result, in part, from the increased concentrations of forage HFA's which, in turn, reduce Mg availability to grazing ruminants.

ACKNOWLEDGMENT

The authors wish to thank Mr. Rollin Abernathy, Univ. of Arizona, Tucson, for preliminary HFA analysis, and A. R. Florence and H. O. Waggoner for assistance in the experimental phase of the study.

LITERATURE CITED

1. Barta, A. L. 1975. Higher fatty acid content of forages as affected by species and by nitrogen and potassium fertilization. Crop Sci. 15:169-171.
2. Clarke, R. A., and W. K. Roberts. 1967. Ruminal and fecal fatty acids and apparent ration digestibility in lambs as affected by dietary fatty acids. Can. J. Anim. Sci. 47:31-38.
3. Dawson, R. M. C., and N. Hemington. 1974. Digestion of grass lipids and pigments in the sheep rumen. Br. J. Nutr. 32 327-340.
4. Garton, G. A. 1969. Digestion and absorption of lipids in the ruminant. Proc. Nutr. Soc. 28:131-139.
5. Hawke, J. C. 1973. Lipids. p. 213-263. *In* G. W. Butler and R. W. Bailey (Eds.) Chemistry and biochemistry of herbage. Vol. I. 639 p. Academic Press, New York.
6. Horwitz, W. (ed.). 1965. Methods of analysis. Assoc. Off. Agric. Chem., Washington, D.C. 957 p.
7. Kemp, A. 1960. Hypomagnesaemia in milking cows: The response of serum magnesium to alterations in herbage composition resulting from potash and nitrogen dressing on pasture. Neth. J. Agric. Sci. 8:281-304.
8. ———, W. B. Deijs, O. J. Hemkes, and A. J. H. van Es. 1961. Hypomagnesaemia in milking cows: Intake and utlization of magnesium from herbage of lactating cows. Neth. J. Agric. Sci. 9:134-149.
9. ———, ———, and E. Kluvers. 1966. Influence of higher fatty acids on the availability of magnesium in milking cows. Neth. J. Agric. Sci. 14:290-295.
10. Metson, A. J., W. M. H. Saunders, T. W. Collie, and V. W. Graham. 1966. Chemical composition of pastures in relation to grass tetany in beef breeding cows. N. Z. J. Agric. Res. 9:410-436.
11. Molloy, L. F., D. J. Giltrap, T. W. Collie, and A. J. Metson. 1974. Degradation of higher fatty acids in perennial ryegrass and white clover following drying and storage. J. Sci. Food Agric. 25:595-606.
12. ———, A. J. Metson, and T. W. Collie. 1973. Grass tetany. IV. Relationship between higher fatty acids and nitrogen in some New Zealand herbages. N. Z. J. Agric. Res. 16:457-462.
13. Saito, T., S. Takadoma, H. Kasuga, and T. Nakanishi. 1969. Effects on fatty acid composition of lipids in cow's milk by grass and legume fed. II. Differences of fatty acid composition in edible parts of grass and legume. Jpn. J. Dairy Sci. 18:A61-A66.
14. Steel, R. G. P., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co., New York. 481 p.
15. Wilson, G. F., C. S. W. Reid, L. F. Molloy, A. J. Metson, and G. W. Butler. 1969. Grass tetany. I. Influence of starch and peanut oil supplements on plasma magnesium, calcium, and phosphorus levels in grazing cows. N. Z. J. Agric. Res. 12:467-488.