Relationship between Calcium and Magnesium Binding to Fraction I Chloroplast Protein and Bloat

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Both the extent and strength of Ca^{+2} and Mg^{+2} binding to fraction I chloroplast protein isolated from alfalfa were determined. These parameters were correlated with the bloat potential of the alfalfa plant when fed to cattle and sheep. The maximum number of binding sites in nonbloat and high-bloat fraction I protein were 56 and 446, respectively. The eightfold difference in binding sites corresponds to differences noted in extent of Mg^{+2} binding with the two proteins. The effect of lecithin, palmitic acid, and linolenic acid on the extent of Ca^{+2} and Mg^{+2} binding was also determined. Low molar ratios of lecithin-fraction I chloroplast protein decreased Ca^{+2} binding to fraction I chloroplast protein in nonbloat samples. The inhibitory effect of lecithin upon metal to protein binding diminished as bloat increased. Palmitic acid inhibited Ca^{+2} to fraction I chloroplast protein binding less than did lecithin at all bloat stages, but consistently more than linolenic acid.

Bloat is a digestive disorder of ruminants characterized by extreme distension of the reticulo-rumen, which, if untreated, may result in the death of animals grazing legume pasture. The distension noted in bloat is mainly attributed to the accumulation of fermentation gases entrapped in a stable foam. The formation of a stable ruminal foam is generally accepted as the major cause of legume bloat (Ferguson and Terry, 1955; Hungate *et al.*, 1955; Reid and Johns, 1957). The foam prevents the normal eructation of ruminal gases (Cole and Boda, 1960; Johns, 1958).

Many researchers believe that bloat occurs because of a delicate imbalance between foam-stabilizing and foam-inhibiting factors of plant origin. McArthur *et al.* (1964) identified fraction I or 18S protein as the primary agent in legumes responsible for ruminal foam formation. They observed that only surface-denatured 18S protein stabilized foam. Lyttleton and Ts'o (1958) found 70% of the plant fraction I protein in chloroplasts. Conversely, Mangan (1958) showed that the surface-active chloroplast lipids are vigorous antifoaming agents. The chloroplast lipids are rich in galactolipids, phospholipids, and sulfolipids (Allen *et al.*, 1966).

Many salts are foam-stabilizing agents. Calcium and Na^{+1} additions up to 0.5*M* increased the foaming capacity of alpha-soybean protein (Perri and Hazel, 1947). Additions of as little as 0.063*M* of NaCl increased the foaming capacity of cytoplasmic proteins and resulted in maximum foam stability (Mangan, 1959). On a molar basis, Ca^{+2} was twice as efficient as Na^{+1} in enhancing foam stability.

The purpose of these studies was to determine the relationship between Ca^{+2} and Mg^{+2} binding to fraction I chloroplast protein isolated from alfalfa and bloat in cattle and sheep.

METHODS

Forage Sampling and Assay Animals. For two consecutive years, alfalfa plants (5 from each of 20 randomly selected sites) were handcut at the same time and height as similar material being machine-harvested to feed assay animals. The plants were placed immediately inside a plastic bag in an insulated chest with dry ice. Samples were taken directly to a 4° C. cold room, where leaves were separated from stems and stored frozen for later analysis.

In both years, 20 wether lambs and 20 steers were used as assay animals. The animals were fed freshly chopped alfalfa twice daily at 7 A.M. and 2 P.M. At hourly intervals after feeding, the animals were scored for bloat severity according to a five-point scale developed by Johnson *et al.* (1958).

Isolation of Chloroplast Fraction I Protein. All steps in the isolation of chloroplast fraction I protein from alfalfa were done at 4° C. One gram of thawed leaves was ground with 20 ml. of ice-cold CCl_4–hexane (p = 1.32) in a Waring Blendor for 3 minutes. Grinding was done at 30-second intervals followed by 30-second rest periods to reduce heating. The homogenate was filtered through three layers of cheesecloth and the filtrate centrifuged for 15 minutes at 12,000 × G. The green suspension on the upper surface was transferred to a tube containing 10 ml. of ice-cold hexane. The mixture was centrifuged for 30 minutes at 1000 × G. The chloroplast pellet was dissolved for 20 hours in 3 ml. of 0.15*M* sodium borate buffer, pH 8.3. Following filtration through three layers of cheesecloth, the soluble protein was precipitated with 2 ml. of 20% trichloroacetic acid, centrifuged at 20,000 × G for 15 minutes, and redissolved in 2 ml. of borate buffer. This solution was added to a 1 × 22.5-cm. glass column packed with Sephadex G-50 Medium and eluted with ion-free water. One-milliliter aliquots were collected, diluted to 3 ml., and the absorbance measured at 280 and 260 mμ with a Beckman 505 spectrophotometer. Three aliquots from the fraction I peak were used for binding studies.

Spectral Analysis and Foaming Properties of Fraction I Protein. The absorption spectrum (240 to 340 mμ, Beckman 505 spectrophotometer) of fraction I protein was measured. The sedimentation pattern was observed at a maximum speed of 47,700 r.p.m. using the Spinco Model E ultracentrifuge with schlieren optics and a standard 12-mm. cell in an An-D rotor.

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Two milliliters of 1*M* sodium acetate–acetic acid buffer (McArthur *et al.*, 1964), pH 5.3, were added to each aliquot from the Sephadex column. The mixture was drawn into a 10-ml. hypodermic syringe, foamed by sucking in air, and ejected. The procedure was repeated until the solution would not foam. Following filtration, absorbance was determined at 280 mμ.

Amino Acid Content of Fraction I Chloroplast Protein. The amino acid content of fraction I protein from two nonbloat and two high-bloat samples was determined. One-milliliter samples containing 0.1% protein were refluxed with 50 ml. of 6*N* HCl for 72 hours and then concentrated to about 1 ml. in a rotary flask evaporator. The amino acids were analyzed by ion exchange chromatography (Technicon AutoAnalyzer) and the concentrations determined by comparison with a standard 1-μmole solution of each amino acid.

Calcium and Mg^{+2} Binding to Fraction I Chloroplast Protein. All steps in the binding studies were done at 4° C. Visking cellulose casings, 25-cm. length × 1.3-cm. diameter, were heated for three successive 1-hour intervals on a steam bath. They were transferred to fresh ion-free water following each heating. The preconditioned casings were kept in ion-free water in the coldroom. Nine milliliters of fraction I protein (three peak aliquots from Sephadex column) and 1 ml. of Antifoam A silicone spray (Dow Chemical Co.) were added to the casing. This was suspended in a 50-ml. borosilicate glass test tube containing 10 ml. of either 0.1*M* $CaCl_2$ or 0.1*M* $MgCl_2$, pH 5.5, and dialyzed for 24 hours. A 10-ml. sample of ion-free water, in a casing, served as a control.

A 5-ml. aliquot from each internal solution was analyzed for total protein by the micro-Kjeldahl technique (Stifel, 1967). Calcium and Mg^{+2} were determined by EDTA titration (Hildebrand and Rilley, 1957) on 4-ml. aliquots collected from internal and external solutions. The extent of binding was expressed as moles of Ca^{+2} or Mg^{+2} per gram of fraction I chloroplast protein. Apparent stability constants (Martell and Calvin, 1952) were calculated assuming a molecular weight of 5×10^5 for fraction I protein.

The effect of Mg^{+2} concentration (0.02, 0.04, 0.08, and 0.10*M*) upon the binding of Mg^{+2} to fraction I protein was studied in nonbloat and high-bloat samples. The dialysis technique was identical to that previously described.

Effect of Lecithin and Fatty Acids on Binding of Ca^{+2} to Protein. Lecithin, palmitic acid, and linolenic acid were prepared at five concentrations (lipid to protein molar ratios of 4, 8, 16, 32, and 64) in ion-free water by stirring for a minimum of 24 hours at 25° C. The lipid suspensions were prepared to give the desired molar ratio in 1 ml. of the internal dialysis solution. Dialysis techniques were the same as previously described. Analyses were made on nonbloat, low-bloat, and high-bloat samples.

RESULTS

Separation and Foaming Properties of Fraction I Protein. Fraction I and fraction II proteins from non- and high-bloat samples were separated by gel filtration and identified by absorbance at 280 mμ (Figure 1). Peak heights of both fractions from the high-bloat sample were similar, while the fraction II peak predominated in the nonbloat sample. The following 280 to 260 ratios were found: fraction I, 1.75 to 1.85 and fraction II, 0.85 to 1.02. Following repeated foaming and filtering, the absorbance of each aliquot was recorded at 280 mμ. Foaming and filtering removed the fraction I protein peak, while the fraction II peak was virtually unchanged. This confirms earlier work (McArthur *et al.*, 1964).

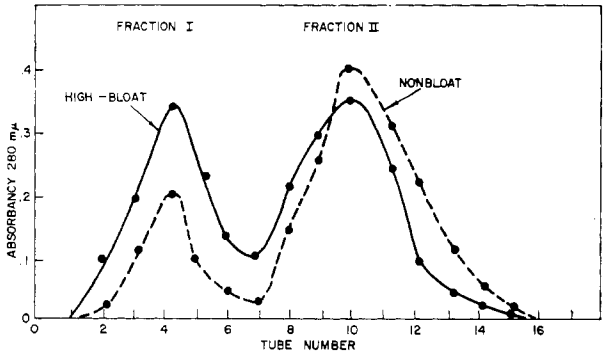

Figure 1. Absorption spectra at 280 mμ of soluble chloroplast protein fractions from nonbloat and high-bloat samples separated by elution through Sephadex G-50

The absorption spectrum (240 to 340 mμ) of fraction I protein revealed one absorption band at 280 mμ. The sedimentation pattern obtained for fraction I protein revealed a single peak (18S protein) after centrifuging for 20 minutes at 44,700 r.p.m. using schlieren optics.

Amino Acid Content of Fraction I Protein. Seventeen amino acids were identified in fraction I protein from nonbloat and high-bloat samples (Table I). Aspartic acid, glutamic acid, glycine, leucine, and alanine constituted about half of the total amino acids. Methionine, cysteine, and histidine were present in only trace amounts. Aspartic acid, serine, and glutamic acid were 22, 22, and 17% higher, respectively, in bloat than in nonbloat fraction I protein. Threonine, proline, and histidine were 29, 52,

Table I. Comparison of Amino Acid Composition of Fraction I Chloroplast Protein in Nonbloat and High-Bloat Samples

Amino Acid	Total Amino Acids, % Nonbloat	High-bloat	Change, %[a]
Aspartic acid	9.7	11.9	+22.1
Threonine	5.5	4.9	−29.0
Serine	5.2	6.3	+21.9
Glutamic acid	10.5	12.8	+17.3
Proline	8.5	4.0	−52.5
Glycine	10.4	11.0	+ 6.0
Alanine	8.9	8.9	...
Valine	7.3	6.4	−12.4
Cysteine	1.7	1.9	+11.8
Methionine	0.4	Trace	...
Isoleucine	5.0	4.6	− 8.1
Leucine	8.2	7.9	− 2.7
Tyrosine	3.4	2.9	−14.0
Phenylalanine	4.0	3.9	− 6.6
Lysine	7.0	7.4	+ 6.5
Histidine	1.9	0.9	−53.7
Arginine	3.9	3.9	...

[a] Change, % = $\frac{\text{(high-bloat − nonbloat)}}{\text{(nonbloat)}} \times 100$.

and 53% lower, respectively, in bloat samples. No other amino acids varied by more than 15%.

Calcium and Mg^{+2} Binding to Fraction I Protein. Calcium and Mg^{+2} binding to fraction I protein were both significantly correlated with bloat severity (Table II). Binding studies were conducted only at pH 5.5 which gives maximum foam stability (Mangan, 1958). Expressed as moles per gram of fraction I protein, Ca^{+2} and Mg^{+2} binding ranged from 0.44 to 3.70 $\times$ 10^{-5}. Magnesium binding was similar to Ca^{+2} except for a larger mean value for Mg^{+2} in high-bloat.

Stability constants [(log K_M)(M^{-1})] for Ca^{+2} and Mg^{+2} were significantly correlated with bloat severity (Table II). Both Ca^{+2} and Mg^{+2} mean values were virtually identical for each bloat range. Values for log $K_M(M^{1-})$ of Ca^{+2} and Mg^{+2} increased uniformly with increasing bloat from 3.22 to 4.34 and 3.24 to 4.35, respectively.

Effect of Salt Concentration upon Binding. As Mg^{+2} increased from 0.02 to 0.10M, the extent of Mg^{+2} binding to fraction I protein increased from both the bloat and nonbloat samples (Figure 2). The moles of aspartic acid and glutamic acid necessary for maximum Mg^{+2} binding were calculated making the following assumptions: pure fraction I protein, molecular weight 5 $\times$ 10^5; at pH 5.5, aspartic acid and glutamic acid are the primary binding sites; and fraction I protein from nonbloat and high-bloat samples contains 10 and 15%, respectively, of aspartic acid and glutamic acid with an average molecular weight of 140.

The point where the plotted lines cross the ordinate equals $1/n$, where n = the maximum number of protein binding sites available to the metal (Hughes and Klotz, 1956). The non- and high-bloat fraction I protein possess 56 and 446 maximum binding sites, respectively. The eightfold difference in maximum binding sites agrees with the differences noted in the extent of binding. The estimated number of moles of the two acidic amino acids varied from 714 to 857 moles in non- and high-bloat fraction I protein, respectively. Assuming aspartic acid and glutamic acid are the only Mg^{+2} binding sites, two amino acids are required to bind each Mg^{+2} in the high-bloat sample, while 12 are required in the nonbloat sample.

Effect of Lecithin and Fatty Acids on Ca^{+2} Binding Protein. Lecithin additions to the nonbloat fraction I protein decreased Ca^{+2} binding 14% at the 4 molar ratio, while binding was virtually eliminated at the 64 molar ratio (Figure 3). In the low-bloat samples, lecithin decreased binding 35% at the 64 molar ratio. Lecithin

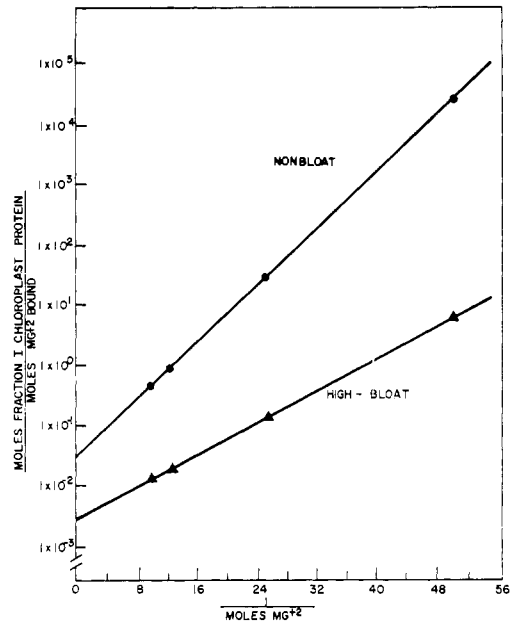

Figure 2. Comparison of effect of four Mg^{+2} concentrations upon the extent of Mg^{+2} binding to fraction I chloroplast protein from nonbloat and high-bloat samples

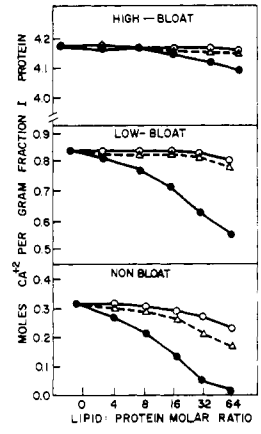

Figure 3. Effect of different molar ratios of lecithin (●), palmitic acid (△), and linolenic acid (○) to protein upon Ca^{+2} binding to fraction I chloroplast protein

Table II. Relationships between Extent and Strength of Ca^{+2} and Mg^{+2} Binding to Fraction I Chloroplast Protein and Bloat

Chemical Component	Nonbloat	Low-Bloat	Medium-Bloat	High-Bloat	r[a]
Extent of binding[b]					
Ca^{+2}-to-protein	0.44	0.84	1.62	3.12	0.92[c]
Mg^{+2}-to-protein	0.45	0.85	1.68	3.70	0.95[c]
Strength of binding[d]					
Ca^{+2}-to-protein	3.22	3.63	3.96	4.34	0.84[c]
Mg^{+2}-to-protein	3.24	3.64	3.97	4.35	0.85[c]

[a] Correlation coefficient for combined data from cattle and sheep.
[b] Binding expressed as moles of Ca^{+2} ($\times 10^{-5}$) or Mg^{+2}($\times$ 10^{-5}) bound per gram of fraction I protein.
[c] $P < 0.01$.
[d] Binding expressed as stability constants [log $K_M(M^{-1})$].

additions to high-bloat fraction I protein did not appreciably alter binding at any molar ratios.

Palmitic and linolenic acids inhibited Ca^{+2} binding considerably less than did lecithin in all samples (Figure 3). In the nonbloat sample, palmitic acid decreased binding 16 and 45% at the 16 and 64 molar ratios, respectively. In the bloat samples, palmitic acid had little effect upon Ca^{+2} binding. Linolenic acid additions depressed Ca^{+2} binding less than either lecithin or palmitic acid. As with palmitic acid, the major decrease in binding with linolenic occurred at the 64 molar ratio in the nonbloat sample.

DISCUSSION

In the present study, the extent and strength of Ca^{+2} and Mg^{+2} binding to fraction I chloroplast protein were compared with the bloat potential of alfalfa. There was a significant direct relationship between the extent and strength of Ca^{+2} and Mg^{+2} binding to fraction I chloroplast protein, pH 5.5, and bloat. In an attempt to account for binding differences, amino acid contents were analyzed for nonbloat and high-bloat fraction I protein. Aspartic acid and glutamic acid were 17 and 22% higher in high-bloat samples. However, quantitative differences in the two amino acids alone account for only 10% of the binding increase. Using four salt concentrations, a minimum of two acidic amino acids was needed to bind each Mg^{+2} in the high-bloat sample, while 12 acidic amino acids were required in the nonbloat sample. These observations suggest that changes in the steric configuration and conformation of fraction I protein may alter the availability of binding sites and, thereby, account for the observed differences in Ca^{+2} and Mg^{+2} binding.

Akoyunoglou and Calvin (1963) observed Mg^{+2} binding to fraction I protein (carboxydismutase) in spinach extracts. They discovered that the carboxylation of ribulose diphosphate by carboxydismutase involves two separate steps. The first step, the carboxylation of the enzyme, is Mg^{+2} dependent. Other ions (Hg^{+2}, Fe^{+2}, Fe^{+3}, Cu^{+2}, Zn^{+2}, Cr^{+3}, and Co^{+2}) did not activate the enzyme, although Ni^{+2} did to a lesser extent. Calcium was not investigated. These data, coupled with the present data, suggest that changes in the extent of Ca^{+2} and Mg^{+2} binding to fraction I protein may indicate an altered enzymic activity of the chloroplast protein.

The relationship of Ca^{+2} and Mg^{+2} binding to bloat may be thought of in terms of the proposal by McArthur *et al.* (1964) that fraction I protein acts as a ruminal foaming agent only in a denatured state. Thus, any factors promoting protein denaturation would enhance bloat. The metal binding reported herein may act to "salt-out" fraction I protein, thereby stabilizing the rumen foam. Factors (such as polar lipids) actively competing with protein for metal ions would decrease surface denaturation of the protein and, thus, act as antifoaming agents. This agrees with the foam-inhibition theory proposed by Ross and Haak (1958) who observed that antifoamers act by absorbing foaming agents from the surface of interlamellar bulk solutions.

Results reported herein show that lecithin decreases Ca^{+2} binding to fraction I protein in nonbloat samples. The inhibitory effect of lecithin upon metal to protein binding was diminished as bloat increased. The failure of lecithin to reduce binding in high-bloat samples is explained by the log K_M values of fraction I protein. The log K_M for Ca^{+2} to fraction I protein binding in the non- and high-bloat samples were 3.22 and 4.34, respectively. By comparison, the log K_M of Ca^{+2} to phosphatidyl serine was 4.13 (Hendrickson and Fullington, 1965). Assuming a similar stability constant between Ca^{+2} and lecithin, competitive Ca^{+2} binding by lecithin in nonbloat alfalfa would be expected.

Several workers have reported Ca^{+2} binding to phospholipid films. Kimizuka and Koketsu (1962) observed Ca^{+2} binding to lecithin monolayers at physiological pH values suggesting that Ca^{+2} binds with two oxygen sites forming a bridge between radically oriented phospholipids. Rosano *et al.* (1962) found that Ca^{+2} injected under cephalin monolayers breaks the phosphoric acid–amine polar groups intramolecular association, permitting intermolecular associations among neighboring cephalin molecules. The intermolecular bonds allow surface lattice formation.

Both free and bound fatty acids may reduce Ca^{+2} binding to fraction I protein. Palmitic acid, the major saturated chloroplast fatty acid, inhibited Ca^{+2} binding to fraction I protein less than lecithin at all bloat levels. In nonbloat alfalfa, plamitic acid required four times the molar ratio as lecithin to decrease binding to the same extent. In all bloat stages, palmitic acid exerted little influence on Ca^{+2} binding.

Linolenic acid, the major unsaturated chloroplast fatty acid, decreased Ca^{+2} binding to fraction I protein less than either lecithin or palmitic acid. The only apparent decrease in binding occurred in nonbloat alfalfa, in which twice the molar ratio of palmitic acid was required. These results suggest that the extent and rate of hydrolysis of fatty acid ester linkages in phospholipids, sulfolipids, and other complex lipids contribute to the antifoaming capacity of plant lipids.

Deamer and Cornwell (1966) found that Ca^{+2} ion binding to dipalmityl phosphatidyl choline was greater than to lecithins that contained unsaturated fatty acid residues. Klopfenstein and Shigley (1967) reported that phospholipids in mature alfalfa were highest in saturated fatty acids, but immature alfalfa was highest in unsaturated fatty acids. These findings suggest that when saturated fatty acids increase in the phospholipid fraction, Ca^{+2} binding to phospholipid increases in competition with fraction I protein and bloat potential is prevented. With high levels of unsaturated fatty acids, Ca^{+2} binding to phospholipid decreases, surface viscosity increases, and bloat ensues. Other factors competitively binding Ca^{+2} and Mg^{+2} would also function as antifoaming agents. The possible relation of the physical state of the test lipids to their metal ion binding should be recognized also. It is likely these lipids would exist in different states in ion-free water.

Other trace minerals may also be involved in binding reactions in the rumen. Harris and Sebba (1965) observed that the Ni^{+2} concentrated in foam of immature alfalfa was five times greater than that of mature alfalfa. This observation agrees with the present findings with Ca^{+2} and Mg^{+2}. Harris and Sebba (1965) identified protein as the foaming agent which binds Ni^{+2} on its negatively charged sites. They concluded that Ni^{+2} was

available for protein attachment in immature alfalfa, but unavailable in mature plants. Other possibilities involve changes in protein configuration and conformation.

The data reported herein show a direct relationship between Ca^{+2} and Mg^{+2} binding to fraction I protein and bloat in cattle and sheep. However, these relationships have not been adequately studied in vivo. Nevertheless, the authors' observations do substantiate the present theory (McArthur *et al.*, 1964) concerning the etiology of bloat.

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Received for review October 30, 1967. Accepted March 13, 1968. Financial support is acknowledged from Iowa State University Agricultural and Home Economics Experiment Station Project 1267. Journal Paper No. 5723.