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CHEMICAL AND ULTRASTRUCTURAL RELATIONSHIPS BETWEEN ALFALFA LEAF CHLOROPLASTS AND BLOAT*

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Abstract—Total and soluble leaf chloroplast protein levels were directly related to severity of bloat in cattle and sheep. The highest correlation was found between Fraction I chloroplast protein and bloat. Chlorophyll to protein ratios demonstrated a preferential synthesis of protein in chloroplasts during high-bloat stages. Progressing from non- to high-bloat-provoking forage, the percentage of soluble protein localized in chloroplasts increased from 45 to 77 per cent. Chloroplast lipids were inversely related to bloat. The percentages of total lipids in the chloroplasts suggest that preferential synthesis of chloroplast lipids occurs in non-bloat forage. Progressing from non- to high-bloat forage, the percentage of total leaf lipids localized in chloroplasts decreased from 71 to 27 per cent. Electron micrographs showed numerous large osmiophilic lipid granules in non-bloat chloroplasts. The number and size of the granules were highly correlated with changes in chloroplast lipids and bloat. Chloroplast fragments containing osmiophilic granules, starch grains and grana lamellae were observed in bolus juice and rumen fluid. Lamellae and osmiophilic granules were the only identifiable plant components found in rumen foam, while the chloroplast stroma was dispersed. These results strongly suggest that a stable foam is produced by an imbalance of protein and lipid chloroplast components which are specifically located within the stroma.

INTRODUCTION

BLOAT is a digestive disorder of ruminants characterized by extreme distension of the reticulorumen due to the entrapment of fermentation gases in a stable foam. The foam is the result of a delicate balance between foam-stabilizing and foam-inhibiting plant factors. A chloroplast protein, Fraction I or 18S, is the major foaming agent in alfalfa,[1,2] while the highly polar chloroplast lipids are the major anti-foaming agents.[3] Chloroplasts are also the major site of calcium and magnesium accumulation;[4] both cations are essential for the formation of a stable foam.[5,6] This background information prompted us to further examine several relationships between alfalfa leaf chloroplasts and bloat in cattle and sheep. Our specific objectives were: (a) to determine the relationships between leaf and chloroplast proteins, lipids, calcium and magnesium and bloat severity in cattle and sheep; (b) to determine the magnitude of diurnal changes in the chemical composition and ultrastructure of alfalfa leaf chloroplasts and the relationship to bloat; (c) to correlate chemical changes in alfalfa leaf chloroplasts with changes in the chloroplast ultrastructure as shown by electron microscopy; and (d) to determine the extent of chloroplast degradation following ingestion by the animal.

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RESULTS

Relationships between Alfalfa Leaf Constituents and Bloat

Leaf and chloroplast protein. As shown in Table 1, leaf and chloroplast protein values were directly related to bloat severity in cattle and sheep. The chloroplast protein components were more highly correlated with bloat than total leaf values. The percentages of all chloroplast protein components increased with increasing bloat. However, the most marked change occurred in the Fraction I component of the chloroplast protein which increased sevenfold from 3 to 21 per cent during increasing bloat. Protein to chlorophyll ratios of both total leaf and leaf homogenates demonstrated a preferential synthesis of protein in chloroplasts during

TABLE 1. COMPOSITION AND CORRELATION VALUES OF ALFALFA LEAF CONSTITUENTS WITH BLOAT

Chemical components[a]	Non-bloat	Low-bloat	Medium-bloat	High-bloat	Correlation coefficient[b]
			%		
Protein:					
Total leaf	21·7	34·0	33·9	37·2	0·77†
Chloroplast	0·56	0·84	1·23	1·70	0·85†
Soluble chloroplast	0·22	0·37	0·55	0·75	0·84†
Fraction I chloroplast	0·02	0·05	0·21	0·42	0·98†
Lipid:					
Total leaf	4·93	5·55	4·96	3·85	−0·62†
Chloroplast	3·50	3·27	3·11	1·03	−0·47*
Soluble chloroplast	2·58	2·00	1·72	0·73	−0·43
Total leaf phospholipids	0·90	0·86	0·52	0·27	−0·48*
Chloroplast phospholipids	0·49	0·39	0·13	0·05	−0·33
Calcium and magnesium:					
Leaf calcium	1·42	1·22	1·01	0·83	−0.61†
Leaf magnesium	0·17	0·27	0·37	0·68	0·60†
Chloroplast calcium	0·34	0·28	0·15	0·12	−0·65†
Chloroplast magnesium	0·03	0·04	0·18	0·43	0·96†

[a] Expressed on total leaf dry matter basis.
[b] Correlation coefficient for both cattle and sheep.
† $P < 0.01$.
* $P < 0.05$.

TABLE 2. RELATIONSHIP BETWEEN THE PERCENTAGE OF ALFALFA LEAF PROTEIN LOCALIZED IN THE CHLOROPLAST AND BLOAT

Protein component ratios[a]	Non-bloat	Low-bloat	High-bloat
		%[b]	
Chloroplast protein/leaf protein	46·7	59·9	75·5
Soluble chloroplast protein/soluble leaf protein	44·9	60·2	76·8

[a] Ratios based upon the average of two percentage values expressed on total leaf dry matter basis.
[b] Based upon protein to chlorophyll ratios.

high-bloat stages (Table 2). Progressing from non- to high-bloat forage, the percentage of soluble protein localized in chloroplasts increased from 45 to 77 per cent.

Leaf and chloroplast lipids. The levels of leaf and chloroplast lipids were inversely related to bloat (Table 1). Total leaf lipids showed a higher correlation with bloat than did chloroplast lipid values. The most marked change, however, occurred in the phospholipid fraction of the chloroplast lipids, which decreased tenfold from 0·5 to 0·05 per cent during increasing bloat. The percentages of total lipids in the chloroplast suggest, therefore, that a preferential synthesis of chloroplast lipids occurs in non-bloat forage. The percentage of leaf lipids (total and soluble chloroplast lipids) also decreased with increasing bloat. Total and soluble chloroplast lipids composed as much as 71 and 54 per cent of the total leaf lipids, respectively, in non-bloat forage. These percentages decreased to 19 per cent in high-bloat samples. Soluble chloroplast lipid levels ranged from 41 to 86 per cent of the total chloroplast lipids.

Electron micrographs of alfalfa leaf chloroplasts confirmed the inverse relationship between lipids and bloat (Figs. 1–4). Both the number and size of the osmiophilic granules increased with decreasing bloat. Numerous large osmiophilic granules were present in

TABLE 3. RELATIONSHIP BETWEEN ALFALFA LEAF PROTEIN TO LIPID RATIOS AND BLOAT

Protein to lipid ratio	Non-bloat	Low-bloat	Medium-bloat	High-bloat	Correlation coefficient[a]
Leaf protein/leaf lipids	4·60	6·23	8·08	9·96	0·81†
Chloroplast protein/chloroplast lipids	0·18	0·26	0·41	1·82	0·89†
Soluble chloroplast protein/soluble chloroplast lipids	0·09	0·25	0·42	1·07	0·90†
Chloroplast protein/leaf phospholipid	0·61	0·97	2·72	8·94	0·95†
Chloroplast protein/chloroplast phospholipid	1·12	2·19	10·00	40·80	0·98†
Soluble chloroplast protein/chloroplast phospholipid	0·46	0·95	5·50	19·67	0·98†

[a] Correlation coefficient for both cattle and sheep.
† $P < 0·01$.

non-bloat chloroplasts (Figs. 1–2). Conversely, chloroplasts in high-bloat alfalfa contained osmiophilic granules that were much smaller and less numerous (Figs. 3–4).

Because of the importance of both chloroplast protein and lipids to foam stability, we thought that the ratios of these components might give extremely high correlations with bloat. This was found true (Table 3). The chloroplast protein/soluble chloroplast lipid ratio increased markedly with increasing bloat. The ratios of total and soluble chloroplast protein to chloroplast phospholipids showed a 40-fold increase from non- to high-bloat alfalfa.

Calcium and magnesium. Total leaf and chloroplast calcium were inversely related to bloat severity (Table 1). As bloat severity increased, the percentage of total leaf calcium in the chloroplasts decreased from 24 to 12 per cent. Total leaf and chloroplast magnesium were directly related to bloat severity (Table 1). As bloat severity increased, the percentage of total leaf magnesium in the chloroplasts rose from 20 to 60 per cent.

Relationships between Diurnal Changes in Alfalfa Leaf Constituents and Bloat

Marked diurnal chemical changes (expressed on dry matter basis) occurred in the protein, lipid and mineral content of alfalfa leaf chloroplasts. Trends among the diurnal samples of

the four bloat stages were quite uniform. The three major groups of chemical constituents generally were high at 4 a.m. to 7 a.m., declined at 10 a.m. to 1 p.m. and then increased progressively until 7 p.m. (Figs. 5–9). Both chloroplast calcium and magnesium levels were highly correlated with chloroplast phospholipid and chloroplast protein changes, respectively.

FIGS. 5–8. RELATIONSHIPS OF DIURNAL CHANGES IN THE CHEMICAL COMPOSITION OF ALFALFA LEAF CHLOROPLASTS TO BLOAT SEVERITY.

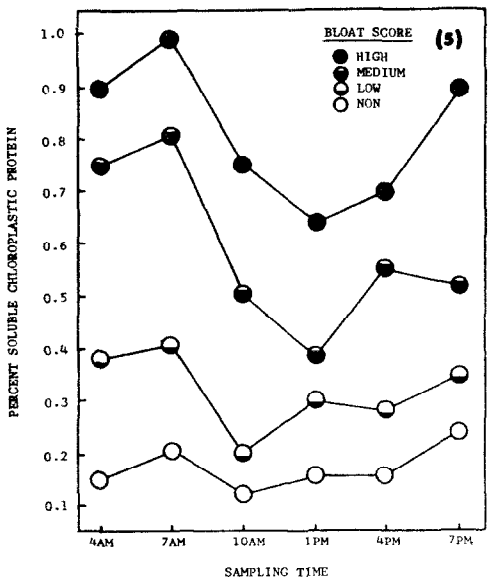


FIG. 5. PER CENT SOLUBLE CHLOROPLAST PROTEIN.

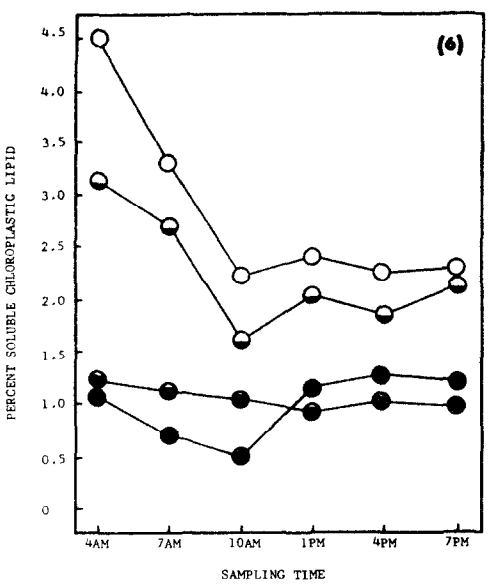
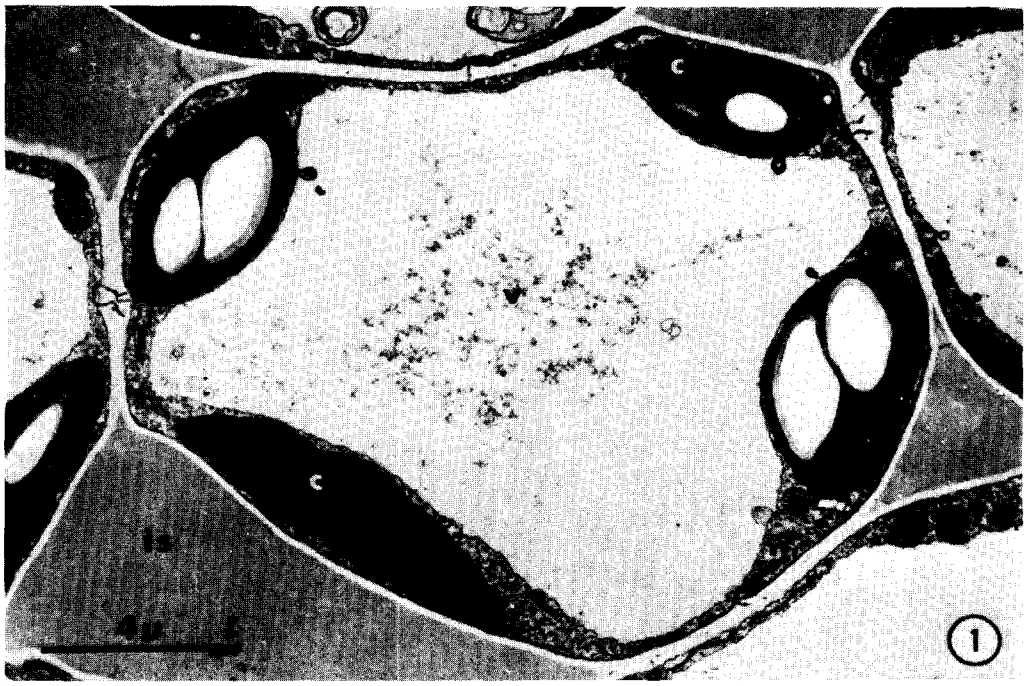


FIG. 6. PER CENT SOLUBLE CHLOROPLAST LIPID.



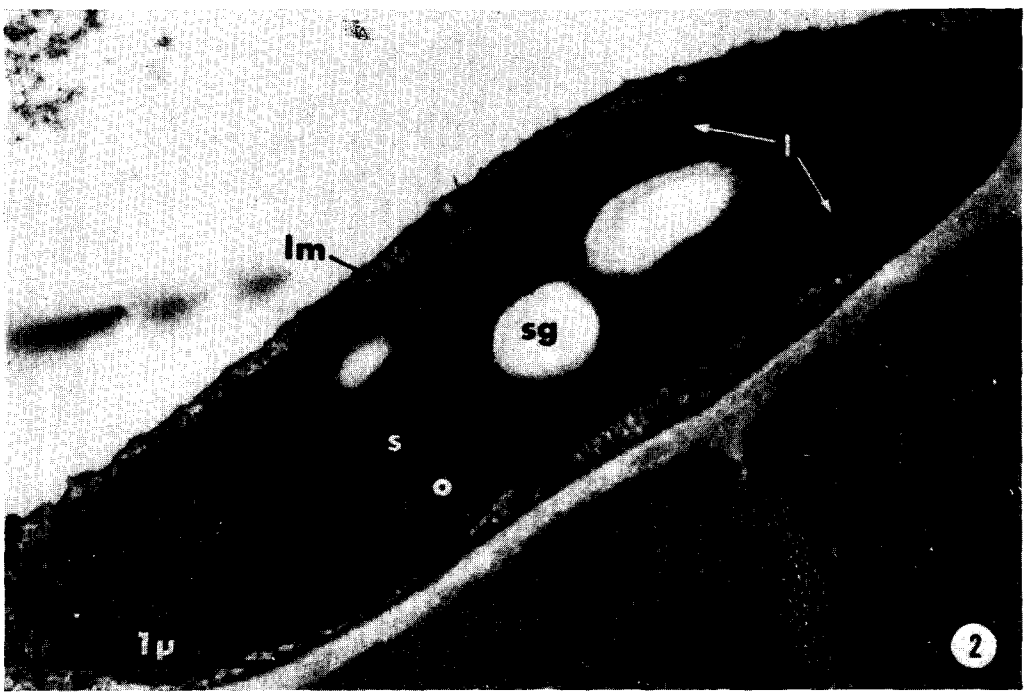


FIG. 1. MESOPHYLL CELLS FROM NON-BLOAT PROVOKING ALFALFA LEAVES. EACH CELL CONTAINS A LARGE VACUOLE (v), CHLOROPLASTS (c), MITOCHONDRIA (M) AND OTHER CELLULAR INCLUSIONS. INTERCELLULAR SPACES (is) ARE SHOWN BETWEEN CELLS.

FIG. 2. A SINGLE CHLOROPLAST FROM A MESOPHYLL CELL OF NON-BLOAT PROVOKING ALFALFA CONTAINING LARGE AND NUMEROUS OSMIOPHILIC GRANULES (o) OR LIPID BODIES, STARCH GRAINS (sg), GRANA AND STROMA LAMELLAE (l), STROMA (s) AND OUTER LIMITING MEMBRANES (lm).

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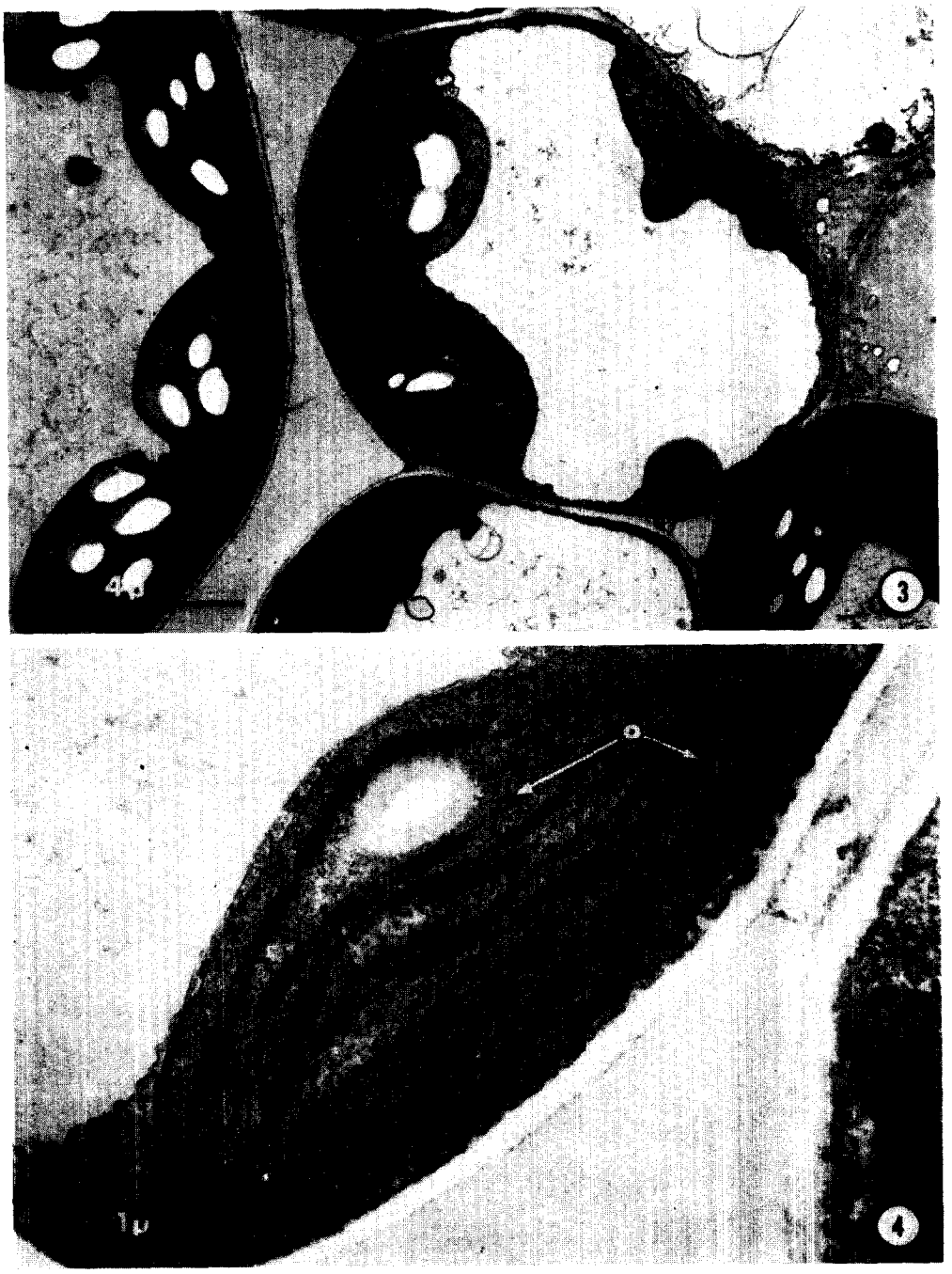

FIG. 3. MESOPHYLL CELLS FROM HIGH-BLOAT PROVOKING ALFALFA LEAVES.

FIG. 4. A SINGLE CHLOROPLAST FROM A MESOPHYLL CELL OF HIGH-BLOAT PROVOKING ALFALFA. OSMIOPHILIC GRANULES (O) ARE SMALLER AND LESS NUMEROUS THAN IN NON-BLOAT PROVOKING CHLOROPLASTS.

The greatest diurnal variation in the levels of the different proteins occurred in the high-bloat samples (Fig. 5). Changes were quite small in the non-bloat samples for soluble

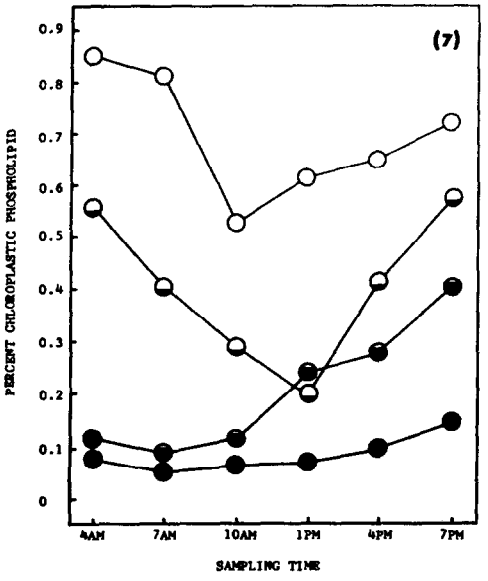

FIG. 7. PER CENT CHLOROPLAST PHOSPHOLIPID.

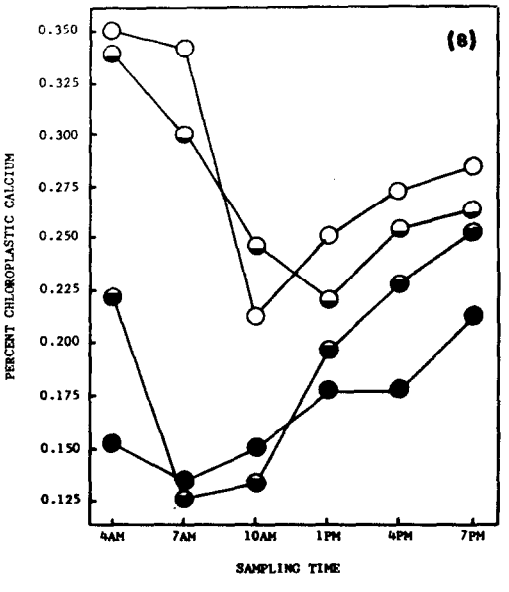

FIG. 8. PER CENT CHLOROPLAST CALCIUM.

chloroplast protein and all of the protein/lipid ratios (Figs. 5, 10, 11). The greatest variation in the non-bloat samples occurred in the various chloroplast lipid and calcium levels. No observable diurnal changes in starch grains, osmiophilic granules, grana and stroma lamellae and stroma were apparent in the chloroplast ultrastructure during each of the four bloat

stages (non-, low-, medium- and high-bloat). However, the number and size of the osmiophilic lipid granules were consistently large in all diurnal stages of the non-bloat samples, but they became progressively smaller in the low- to high-bloat samples. The granules were uniformly smallest and least abundant in all high-bloat samples.

FIGS. 9–11. RELATIONSHIPS OF DIURNAL CHANGES IN THE CHEMICAL COMPOSITION OF ALFALFA LEAF CHLOROPLASTS TO BLOAT SEVERITY.

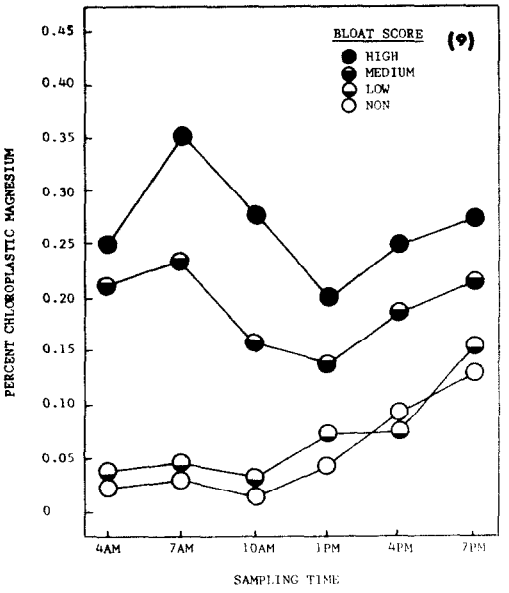

FIG. 9. PER CENT CHLOROPLAST MAGNESIUM.

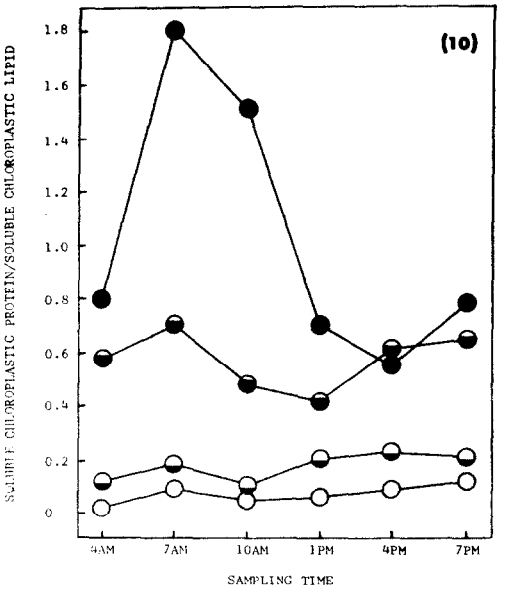

FIG. 10. SOLUBLE CHLOROPLAST PROTEIN/SOLUBLE CHLOROPLAST LIPID.

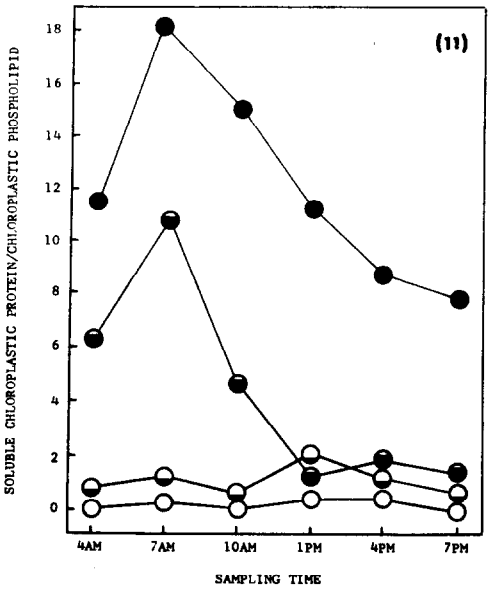

FIG. 11. SOLUBLE CHLOROPLAST PROTEIN/CHLOROPLAST PHOSPHOLIPID.

The observations reported herein show that, chemically, major diurnal changes do occur in chloroplast protein, lipid and mineral values. In summary, the chloroplast components generally showed greater variation than the corresponding leaf values. The chloroplast protein/lipid ratios appear to reflect bloat severity. The magnitude of the diurnal changes in the content of chloroplast protein, lipid and minerals are sufficiently large to help explain differences in time and intensity of bloating. However, additional refinements are needed in this area of research.

Chloroplast Degradation in Bolus Juice, Rumen Fluid and Rumen Foam

Fresh bolus juice was collected at the esophageal-cardial orifice of a rumenfistulated steer during various feedings. Three chloroplast components (starch grains, osmiophilic granules and lamellar membranes) can be identified in the bolus juice (Fig. 12). However, no intact chloroplasts and related stroma were found in the bolus juice suggesting that extensive disruption of the chloroplasts occurs during mastication. Figure 13 depicts a single chloroplast fragment in the bolus juice. The chloroplast lamellar skeleton (minus limiting membranes) is seen enclosing a single starch grain. Several osmiophilic granules appear within the lamellar framework.

Electron micrographs of rumen fluid collected 20 min after feeding alfalfa are shown in Figs. 14 and 15. Chloroplast components in the rumen fluid were similar to those seen in bolus juice, except for the absence of starch grains. The osmiophilic granules appear to coalesce within the chloroplast framework (Fig. 15).

Whole, flailed and bolus leaves from non- and low-bloat alfalfa were incubated in rumen fluid for 1 and 2 hr but leaves from high-bloat alfalfa were incubated for 1 hr only. No differences were observed in the chloroplast ultrastructure of whole, flailed and bolus leaves within each bloat stage prior to incubation. Following incubation in rumen fluid for 1 hr, non- and low-bloat chloroplasts from whole and flailed leaves remained, for the most part, unchanged. Incubation for 1 hr in rumen fluid also failed to radically alter the chloroplasts in non-bloat

bolus leaves (Fig. 16); however, in some instances, the chloroplast-limiting membranes were missing and a portion of the stroma was dispersed.

Figure 18 shows the extreme state of chloroplast disruption in high-bloat bolus leaves following a 1-hr incubation in rumen fluid. The chloroplast-limiting membranes have disappeared, the lamellar system is highly disorganized and the stroma is completely dispersed. Only the starch grains and osmiophilic granules appear unchanged.

Following a 2-hr incubation in rumen fluid, dramatic changes were noted in the non- and low-bloat chloroplasts from whole, flailed and bolus leaves. Figure 17 shows the disrupted state of the non-bloat chloroplast in bolus leaves. The chloroplast-limiting membranes are gone, the lamellar membranes are in a state of disarray and the stroma is dispersed. These changes also typify the conditions noted in the non- and low-bloat flailed and whole leaves following a 2-hr incubation in rumen fluid.

Figures 19 and 20 show rumen foam collected from a fistulated steer 20 min after feeding. An extensive network of lamellar membranes containing osmiophilic granules was found in the foam suggesting that the membranes play a structural role in foam stabilization. The lamellae appear in various states of disorganization. Some lamellae are completely uncoiled as individual strands of membranes, but other lamellae retain the usual grana framework. The stroma was not visible in the foam, and starch grains were found only inside rumen protozoa. The only plant cellular components identified in the foam were chloroplast in nature. No other plant cell structures (mitochondria, ribosomes, nuclei or cell walls) were distinguishable.

DISCUSSION

Fraction I protein (18S protein) has been identified as the primary foaming agent in alfalfa responsible for production of a stable rumen foam.[1,2,7] The major site of Fraction I protein is the chloroplast.[8] By weight, Fraction I makes up at least 50 per cent of the total soluble chloroplast protein.[8,9] McArthur and Miltimore[2] observed that the 18S content of two bloating forages, alfalfa (*Medicago sativa*) and red clover (*Trifolium pratense*), was four to ten times greater than that of two non-bloating forages, trefoil (*Lotus corniculatus*) and orchard grass (*Dactylis glomerata*). However, no direct correlation was made between the 18S content of the forages and bloat. Our data show a highly significant ($P < 0{\cdot}01$) correlation between the level of chloroplast Fraction I protein in alfalfa and bloat severity in cattle and sheep.

The site of Fraction I protein in the chloroplast is not clearly defined. By differential centrifugation and electron microscopy, Park and Pon[10] identified the stroma as the primary site of Fraction I protein in spinach chloroplasts. Dried preparations of the isolated 18S appeared as oblate spheres 100 Å in height and 200 Å in diameter. In later work, Lichtenthalter and Park[11] found some Fraction I protein in the grana lamellae. Trown[12] found that carboxydismutase (ribulose-1,5-diphosphate carboxylase) activity is superimposable upon the elution profile of Fraction I protein. He concluded that Fraction I protein and carboxydismutase were one and the same. Haselkorn *et al.*[13] observed that Fraction I protein contains

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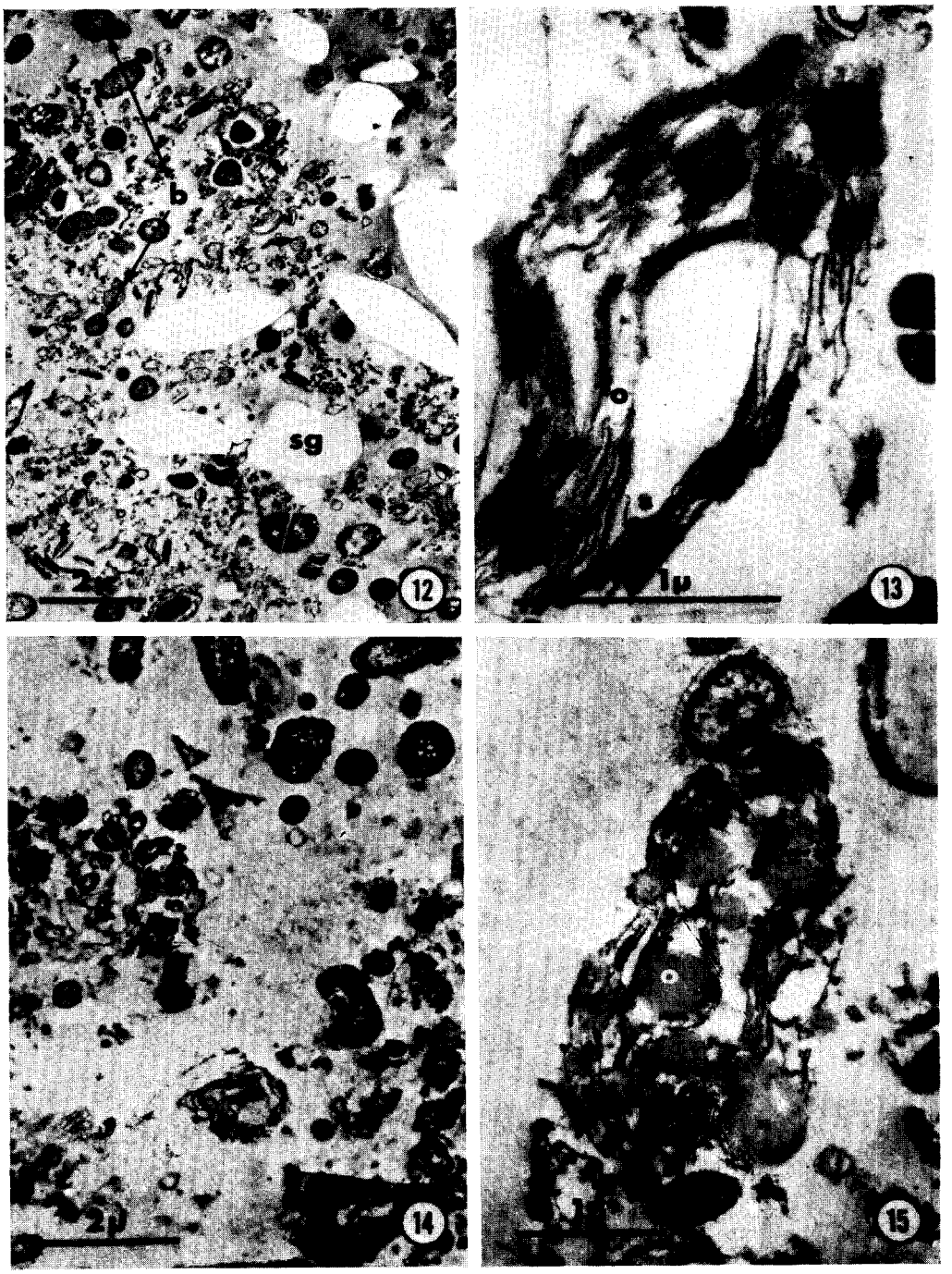

FIG. 12. BOLUS JUICE SHOWS DISRUPTED CHLOROPLASTS BETWEEN BACTERIA (b) AND OTHER DEBRIS, STARCH GRAINS (sg) AND CHLOROPLAST FRAGMENTS (f).

FIG. 13. A SINGLE DISRUPTED CHLOROPLAST FROM BOLUS JUICE LACKING OUTER LIMITING MEMBRANES AND STROMA. OSMIOPHILIC GRANULES (o) ARE PRESENT.

FIG. 14. RUMEN FLUID CONTAINS SIMILAR DEBRIS OF BOLUS JUICE EXCEPT FOR THE ABSENCE OF STARCH GRAINS.

FIG. 15. A SINGLE DISRUPTED CHLOROPLAST FROM RUMEN FLUID LACKING OUTER LIMITING MEMBRANES, STROMA AND STARCH GRAINS. OSMIOPHILIC GRANULES (o) ARE PRESENT.

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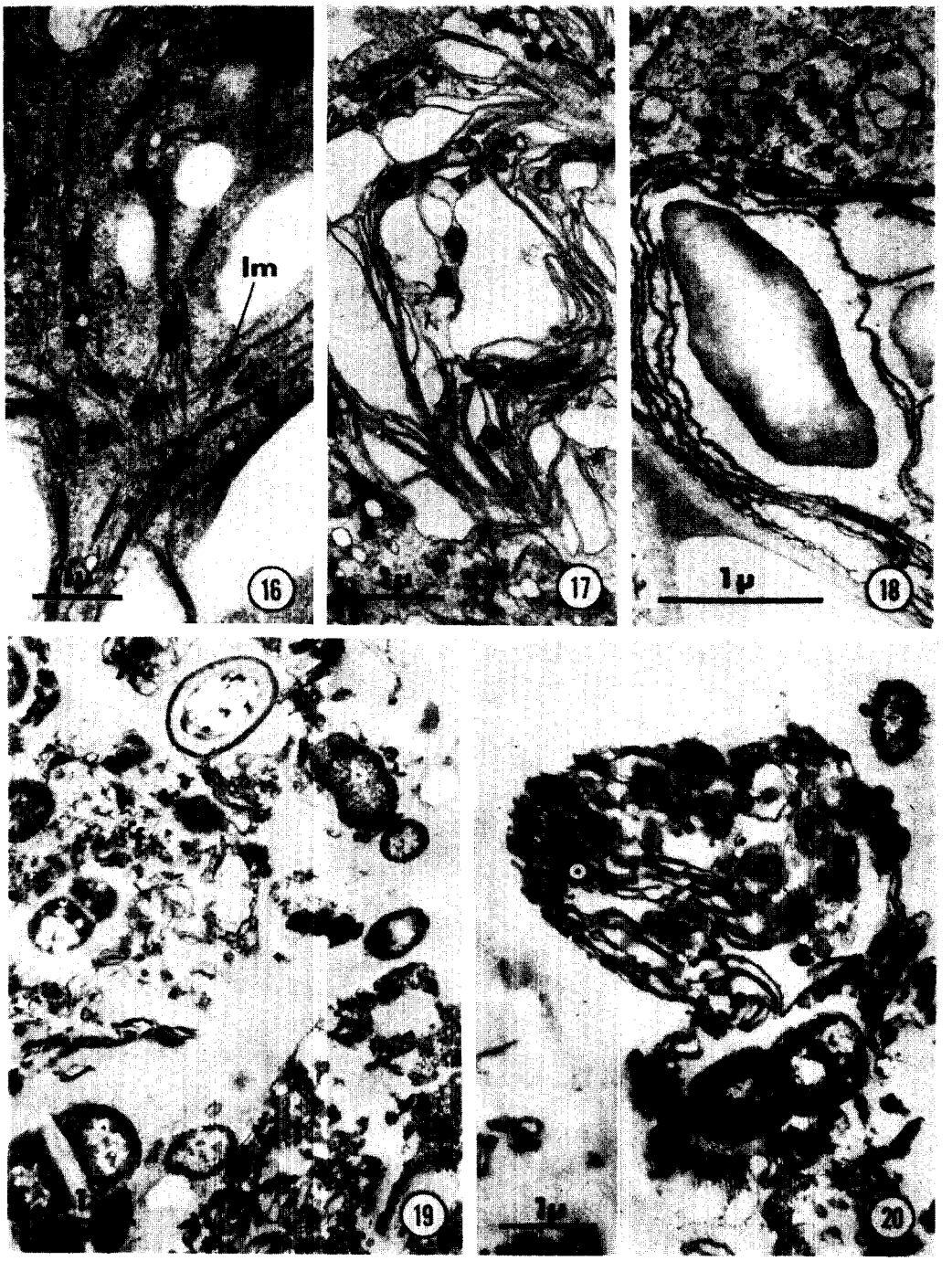

FIG. 16. A PORTION OF A NON-BLOAT PROVOKING BOLUS LEAF INCUBATED 1 hr IN RUMEN FLUID. NOTE PARTIALLY DISRUPTED LIMITING MEMBRANES (lm) AROUND CHLOROPLASTS AND OSMIOPHILIC GRANULES (o).

FIG. 17. A PORTION OF A NON-BLOAT PROVOKING BOLUS LEAF SHOWING A CHLOROPLAST INCUBATED 2 hr IN RUMEN FLUID. LIMITING MEMBRANES ARE DISRUPTED AND STROMA IS GONE. OSMIOPHILIC GRANULES (o) ARE STILL PRESENT.

FIG. 18. A PORTION OF A HIGH-BLOAT PROVOKING BOLUS LEAF SHOWING A HIGHLY DISRUPTED CHLOROPLAST AFTER 1 hr INCUBATION IN RUMEN FLUID. STROMA IS GONE, BUT SOME OSMIOPHILIC GRANULES (o) ARE STILL PRESENT.

FIG. 19. RUMEN FOAM OF A FISTULATED STEER 20 min AFTER FEEDING SHOWS CHLOROPLAST FRAGMENTS (f) AND BACTERIA (b).

FIG. 20. A SINGLE DISRUPTED CHLOROPLAST FROM RUMEN FOAM OF A FISTULATED STEER 20 min AFTER FEEDING LACKING OUTER LIMITING MEMBRANES, STROMA AND STARCH GRAINS. SOME OSMIOPHILIC GRANULES (o) ARE STILL PRESENT.

all the carboxydismutase activity recoverable from glycerol gradients. Carboxydismutase is the vital photosynthetic enzyme that catalyzes the fixation of carbon dioxide to ribulose-1,5-diphosphate in the carbon-reduction cycle. This cycle represents the dark-reaction of photosynthesis that occurs within the stroma of the chloroplast.[10] The identification of carboxydismutase activity with Fraction I protein substantiates the earlier evidence that Fraction I protein is located in the stroma.

Our electron microscopic studies demonstrate that the chloroplast-limiting membranes are rapidly disrupted during mastication (as shown in the bolus juice) and within the rumen (as shown by *in vitro* incubation of bolus leaf fragments in rumen fluid). In both the bolus juice and rumen fluid, the lamellar membranes remained intact while the stroma was completely dispersed. Because of the rapid occurrence of bloat in cattle and sheep following ingestion of alfalfa, the Fraction I protein must be rapidly liberated into the rumen to exert its foam-stabilizing effect. This gives further indirect evidence that the stroma is the major site of Fraction I protein in the chloroplast. We are presently engaged in attempts to directly identify the exact site of the Fraction I protein using a conjugated antibody technique.

The importance of Fraction I protein as the primary foaming agent responsible for bloat is well substantiated. The protein in a surface-denatured state immobilizes rumen gas by forming foams of high shear strength.[1,2] Recent evidence shows that the extent of calcium and magnesium binding to Fraction I chloroplast protein is directly related to bloat severity in cattle and sheep fed alfalfa.[7] This confirms the importance of the surface-active, surface-denatured Fraction I protein in the etiology of bloat. However, Fraction I protein does not appear to be the only factor contributing to the incidence of bloat. Since the Fraction I protein content does not vary significantly with age (4·4–5·3 per cent of dry weight),[2] some additional plant factor(s) must contribute to the day by day variation in bloating patterns observed when ruminants graze the same alfalfa plot over a single growth cycle.

The chloroplast lipids, known for their anti-foaming properties,[3] are probably such a factor. Both our chemical data and electron microscopic studies confirm the inverse relationship between the level of chloroplast lipids, especially phospholipids, and bloat severity in cattle and sheep. Polar, surface-active lipids (phospholipids, sulpholipids and galactolipids) account for more than 60 per cent of the total chloroplast lipids.[14] Phospholipids, particularly lecithin, reduce the surface tension of rumen fluid and control mechanical foaming.[15] Sulpholipids are rapidly released from plant material, slowly hydrolyzed in the rumen and depress surface-tension in aqueous solutions.[16] To further characterize the relationship between chloroplast lipids and bloat, studies are presently being initiated to determine the chemical composition of the osmiophilic lipid granules and to correlate any changes in the chemical composition with the incidence of bloat.

The ratio of the levels of chloroplast Fraction I protein to chloroplast phospholipids was intimately related to the bloat severity observed in cattle and sheep fed alfalfa. Our data suggest that when chloroplast metabolism favors Fraction I protein synthesis, foam-stabilizing factors predominate and bloat ensues. When chloroplast metabolism shifts to favor lipid synthesis, presumably phospholipids or sulpholipids, foam-inhibiting factors predominate and bloat is prevented. It is the balance of these foam-stabilizing and foam-inhibiting factors that probably determines the ultimate bloat potential of the forage.

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EXPERIMENTAL

Assay animals. Twenty crossbred wether lambs and 20 steers of dairy breeding were used as assay animals. They were fed freshly-chopped alfalfa twice daily at 7 a.m. and 2 p.m. The animals were held throughout the trials in drylot with open-type sheds for shelter. Water and trace-mineralized salt were available free-choice throughout the experiments.

Animals were scored for bloat severity on a basis of 0 to 5 at frequent intervals following the feeding period.[17] The highest score obtained for each animal during a feeding period was used. The sum of these scores was divided by the number of assay animals so that a bloat index was derived; i.e. 0=non-bloat; 0·1–0·5=low-bloat; 0·6–1=medium-bloat; greater than 1=high-bloat.

Forage sampling. Alfalfa plants (5 from each of 20 locations) were randomly handcut from the same plot being mechanically chopped to feed the assay animals. The samples for the chemical and ultrastructural studies were placed immediately inside plastic bags in an insulated chest containing dry ice and taken directly to a cold room at 4° where the leaves were cut from the stems and weighed. Chemical analyses were conducted on 20 different samples collected each year.

Chemical determinations. Chloroplasts were isolated in aqueous 0·5 M sucrose, pH 6·5, for lipid analyses;[4] in hexane/CCl_4 for protein analyses.[18] Analyses for soluble protein and lipids were made following 16 hr extraction with 0·15 M Na-borate buffer, pH 8·3.[19] Lipids were determined by a 6-hr ether extraction (7), protein by micro-Kjeldahl techniques following precipitation with 20 per cent trichloroacetic acid (TCA).[20] Following wet oxidation[21], Ca and Mg contents of leaf and chloroplast samples were determined by ethylenediaminetetraacetic acid (EDTA)-titration.[22] Phospholipids were measured by colorimetric procedures.[23,24] Fraction I protein was determined from 0·15 M Na-borate extract.[19] Following filtration through 3 layers of cheesecloth, the soluble protein, containing Fraction I, was precipitated with 2 ml of 20 per cent TCA, centrifuged at 20,000 × *g* for 15 min and redissolved in 2 ml of the Na-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 ml aliquots were collected, diluted to 3 ml and the absorption measured at 280 and 260 mμ with a Beckman 505 Spectrophotometer to test for Fraction I protein. Protein to chlorophyll ratios were determined on total leaf and chloroplast homogenates.[25] All analyses were adjusted to a leaf dry matter basis. Simple correlation coefficients were calculated between individual chemical values and bloat severity.[26]

Electron microscopy. Alfalfa leaf discs (0·8 mm in dia.) were taken from the top 15 cm of growth. Discs were fixed for 12 hr in 6·5 per cent glutaraldehyde in a 0·1 M phosphate buffer,[27] pH 7·2, at 4°, rinsed in three changes of buffer 15 min each and post-fixed with 1 per cent OsO_4 in the same buffer,[28] pH and temperature for 1 hr. Discs were dehydrated in an alcohol-propylene oxide series and embedded in Epon 812.[29] Sections were stained with uranyl acetate dissolved in methanol.[30] Pictures were taken on an RCA EMU-3F microscope. The bolus leaves, rumen fluid and rumen foam samples were fixed directly in the buffered glutaraldehyde centrifuged at 5000 rev/min for 5 min, rinsed, post-fixed with buffered 1 per cent OsO_4 and placed in agar for dehydration. Embedding, sectioning, staining and microscopy were the same as described previously in this paragraph.

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