RELATIONSHIPS BETWEEN ALFALFA LEAF CHLOROPLASTS AND BLOAT

by

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INTRODUCTION

Bloat is a digestive disturbance of ruminants characterized by distension of the rumen and reticulum. The distension noted in bloat is attributed, in most cases, to the accumulation of large amounts of gases entrapped in a stable foam. The formation of a stable foam within the rumen is generally accepted as the major cause of legume bloat (Quinn, 1943; Weiss, 1953; Johns, 1954; Ferguson and Terry, 1955; Hungate et al., 1955; Reid and Johns, 1957). The foam functions by preventing the normal eructation of gases (Jacobson et al., 1957; Johns, 1958; Cole and Boda, 1960). The effectiveness of many anti-foaming agents in bloat prophylaxis gives additional support to the foam theory (Reid and Johns, 1957; Reid, 1958, 1959; Johnson, 1959; Reid et al., 1961; Kassir, 1962).

To gain a better understanding of the bloat syndrome, researchers have attempted to identify the plant and animal factors which contribute to the formation of a stable foam. Most workers recognize that bloat is a complex syndrome resulting from a balance between foam-stabilizing and foam-inhibiting factors. Proteins, lipids and minerals are three important plant factors contributing to foam stability and these are discussed in considerable detail in the review of literature.

The objective of this research was to elucidate the possible roles of these three plant constitutents in the bloat syndrome. Particular attention was devoted to the structure and chemical composition of alfalfa leaf chloroplasts because the chloroplast is one of the primary sites of protein, lipid and mineral metabolism in plants.

The specific objectives of the research were:

1. To determine the relationships between leaf and chloroplastic calcium, magnesium, lipids and protein and bloat severity in cattle and sheep.
2. To determine the relationship between calcium and magnesium binding to Fraction I chloroplastic protein and bloat in cattle and sheep.
3. To determine the magnitude of diurnal changes in the chemical composition and ultrastructure of alfalfa leaf chloroplasts and its relationship to bloating patterns.
4. To correlate chemical changes in alfalfa leaf chloroplasts with changes in the chloroplastic ultrastructure as shown by electron microscopy.
5. To determine the extent of chloroplastic degradation in bolus juices, rumen fluid and rumen foam.

REVIEW OF LITERATURE

Relationship of Plant Constituents to the Etiology of Bloat

Several reviewers have suggested that bloat is a complex syndrome resulting from a combination of both plant and animal factors. One can think of the bloat syndrome as the result of a delicate ruminal balance between foam-stabilizing and foam-inhibiting factors. When the balance is shifted in favor of the foam-stabilizing factors bloat ensues. The relationship of the chemical composition of plants has been reviewed by Cole et al. (1956) and Warner (1960). In the past few years considerable attention has been devoted to further elucidation of the relationship of chemical plant constituents to foam stability and bloat. The scope of this literature review will be limited primarily to the relationship of three important plant components, protein, lipids and minerals, which contribute greatly to the production of a stable foam.

Protein

In recent years considerable research has been conducted to isolate factors contributing to the production of a stable foam which characterizes legume bloat. Bartley and Bassette (1961) analysed rumen foam from nine fistulated cows on alfalfa pasture. Following isolation and precipitation with absolute alcohol, the foam composition was found to contain

the following: 63.3% crude protein, 18.2% ash, 17.0% carbohydrates and 1.5% ether extract. Fifteen amino acids were identified in the precipitate. These workers concluded that protein was probably the major foam constituent.

Johns (1954, 1956) postulated that protein and its degradative products may contribute to bloat, but questioned whether the protein would be released rapidly enough from the ingested material to be a significant factor. Ferguson and Terry (1955) also questioned the importance of the contribution of plant protein due to the slow release of protein from plant cells.

Recently Reid *et al*. (1962) devised a method for measuring the rate and extent of release of plant components through mastication of ingested forage. These workers collected boli of chewed forage at the cardia of the rumen from fistulated cows and analyzed the boli for various plant constituents. Ranges of 33 to 68% of the cellular soluble protein and 22 to 38% of the chloroplastic material were liberated. The soluble protein was released almost twice as rapidly as the foam-inhibiting chloroplastic material. The authors concluded that, "The prevailing ideas about causes of bloat suggest that a rapid release of soluble protein from plant cells should increase the likelihood of foam production, with the onset of a bloating condition".

Mangan (1959) found significant correlations between

cytoplasmic protein in red clover and foam strength and pH values normally found in the rumen. Maximum foam stability was observed between pH 5 and 6. Based upon these findings, it was suggested that cytoplasmic proteins are the primary foaming agents.

By drenching with fresh egg white (a cytoplasmic protein, Boda et al. (1957) produced bloat in cattle fed dehydrated alfalfa. A stable foam was produced and persisted for 90 minutes following the administration of egg white. The authors suggested that water-soluble plant proteins may play a prominent role in legume bloat. The research of Shinozaki and Sugawara (1957) and Boda (1958) gave further substantiation to the importance of water-soluble plant proteins in the etiology of legume bloat.

Lyttleton (1956) extracted the soluble cytoplasmic proteins from leaf cells of white clover and ryegrass. Through electrophoresis and ultracentrifugation, he isolated and characterized from clover a single soluble protein fraction known as Fraction I or 18S protein with a molecular weight of approximately 600,000. Dorner et al. (1958) demonstrated by precipitation and ultracentrifugal analyses that Fraction I protein is ubiquitous in higher plants, often comprising a majority of the total soluble leaf protein.

Following extraction of alfalfa leaf protein with a phosphate buffer, McArthur and Miltimore (1964) chromatographed

the protein on agar gel columns and obtained a protein fraction with strong foaming properties. Sedimentation patterns identified the foaming agent as the homogenous Fraction I or 18S protein. The other fraction separated from the gel columns did not contain a surface denatured agent. These workers proposed the following theory regarding the activity of the Fraction I protein: "In the rumen liquor, the protein is in solution as an almost spherical particle. Molecules which diffuse to the surface, without being subjected to proteolytic activity, immediately uncoil and become insoluble. This surface-denatured protein will stabilize the liquid films in a foam." It is suggested that quantitative differences in the 18S protein content of plant species or diurnal changes in this fraction might account for differences in the bloat potential of forages. Previous workers have shown that the 18S protein content does vary with plant maturity (Dorner et al., 1958) and the amount of light (Kupke, 1962).

In an attempt to identify the site of Fraction I protein in green spinach leaves, Lyttleton and Ts'o (1958) compared the yields of Fraction I protein obtained in extracts from the whole cell and from its constituent chloroplasts. By means of direct ultracentrifugal analyses and comparisons of chlorophyll concentrations, these workers found that at least 65 to 70% of the Fraction I protein in the plant cell is

associated with the chloroplasts. Contamination with soluble protein liberated from the leaf cells accounted for less than 0.5% of the concentrations obtained. However, no speculation was made concerning the actual location of the Fraction I protein within the chloroplasts.

By means of differential centrifugation and electron microscopy, Park and Pon (1961) identified the stroma as the primary site of Fraction I protein in spinach chloroplasts. Dried preparations of the isolated 18S protein appeared as oblate spheres 100Å in height and 200Å in diameter. Lichtenthalter and Park (1963) identified 18S protein in both the lamellar grana and stroma of chloroplasts.

The recognition of the chloroplast as the major site of Fraction I protein within leaf cells is paralleled by the recent discoveries that the chloroplast is the major site of protein synthesis in plants. Utilizing aqueous isolation procedures, Granick (1938) found that tobacco chloroplasts contained a maximum of 35 % of the total plant protein. Employing non-aqueous isolation techniques and electron microscopy, Smillie (1963) observed that more than 50% of the soluble protein was leached from the chloroplasts during isolation in an aqueous medium.

Studies of soluble chloroplastic enzymes (Avron and Jagendorf, 1957; Stocking, 1959) and isolation of spinach chloroplasts with large quantities of extractable protein

(Whatley et al., 1956; Sissakian, 1958) have shown that a majority of both soluble and insoluble leaf proteins are found in the chloroplasts. Measuring protein to chlorophyll ratios of chloroplasts and leaf homogenates from evening primrose leaves, Zucker and Stinson (1962) demonstrated that chloroplasts contain 75% of the leaf protein. A borate buffer was found to be the most efficient extractant of chloroplastic and leaf proteins. These authors state conclusively that chloroplasts are the major sites of protein synthesis in the primrose leaf.

Recent metabolic studies by Bove and Raacke (1959) identified amino acid activating enzymes in isolated chloroplasts from spinach leaves. Rhodes and Yemm (1963) observed that chloroplasts are extremely active centers for protein synthesis which are closely associated with high levels of ribonucleic acids in young leaves of barley seedlings. Heber (1962) found that labeled amino acids were incorporated into chloroplastic protein as soon as photosynthesis was initiated at a rate of 1 μg amino acid/mg protein/hour. The incorporation of amino acids into chloroplastic protein preceded incorporation into soluble cytoplasmic protein.

Lipids

In recent years researchers have studied the possible relationships between plant lipids and bloat. Interest in

the lipid components of plants was stimulated by the known anti-foaming properties of various oils and surface-active lipids. Blake *et al*. (1957), Brown *et al*. (1957) and Reid and Johns (1957) showed that various vegetable oils and detergents effectively reduced bloat through a reduction of surface tension and a reduction in foam stability.

Fraser (1961) found that non-bloat-provoking ryegrass contained a higher lipid content than either red or white clover. However, no significant correlation was observed between the lipid content of the clovers and the incidence of bloat. This worker observed that phospholipid levels were 80% higher in non-bloat-versus bloat-provoking clover. The phospholipid levels ranged from 4.2 to 8.3% on a dry matter basis. No marked changes were seen in lipid levels in the rumen liquor of bloating versus non-bloating cows.

Rumen microorganisms were observed to hydrolyze lipids such as olive oil, linseed oil, monostearin and tributyrin at varying rates (Wright, 1961). The addition of either penicillin or terramycin considerably depressed the lipolytic activity of whole rumen fluid. Due to known bacteriostatic effects of antibiotics, Wright suggested that bacteria are responsible for most of the lipolysis occurring in the rumen. His results suggest that one mode of action of penicillin in controlling bloat might be due to a decrease in bacterial degradation of plant lipids which act as anti-foaming agents *in vivo*.

Mangan (1958) showed that plant chloroplastic lipids have definite anti-foaming properties, presumably due to a particularly high content of surface-active lipid components. Using column chromatography on DEAE-cellulose followed by silicic acid column chromatography, Allen et al. (1966) isolated and identified the lipids of spinach chloroplastic lamellae. The molar distribution of chloroplastic lipids was as follows: 45 monogalactosyl diglycerides, 32 digalactosyl diglycerides, 17 phosphatidyl glycerol, 14 sulfolipid, 6 lecithin, 3 phosphatidyl inositol and 74 non-polar lipids. The polar, surface-active lipids accounted for more than 60% of the total chloroplastic lipids.

The phospholipid, sulpholipid and glycolipid fractions have all received considerable attention by bloat researchers in the past 10 years. Nichols et al. (1957) found that a granular plant lecithin consistently gave a great reduction in surface tension of paunch fluid and good control of mechanical foaming. Lecithin was found to decrease the surface tension and viscosity up to 7% with no depression in gas production or cellulose digestion.

Benson and Maruo (1958) found that chloroplastic phospholipids accounted for almost 50% of the total lipid phosphorus in sweet clover and barley. The distribution of each phospholipid class varied with the plant species. The greatest difference between the phospholipid classes of

sweet clover and barley occurred in the lecithin fraction, barley containing 22% more lecithin then clover. Eberhardt and Kates (1960) noted that the phosphatide content of primary runner-bean leaves increased linearly with plant maturity. The increase in phosphatides occurred at a rate proportional to the growth of leaves.

Kates (1953) demonstrated that the choline-liberating activity of juices from spinach, sugar-beet and cabbage leaves was associated with the chloroplastic fractions. The cytoplasm possessed no lecithinase activity. Hydrolysis of lecithin and lysolecithin by rumen microorganisms of sheep has also been observed (Dawson, 1959). Following incubation with washed suspensions of rumen microorganisms, lecithin-P^{32} was degraded and the P^{32} appeared in a water-soluble fraction mainly as inorganic phosphorus. The enzyme activity appeared to depend mainly on the diet of the sheep, although micro-organisms from sheep on a synthetic diet showed some enzymic activity. Dawson suggested that the surface activity of lecithin may play a prominent physio-chemical role in ruminant digestion, particularly with regard to its vigorous anti-foaming properties.

A sulpholipid, 1-O-oleoyl-3-(beta-galactopyranosyl-6-sulphate)-1-glycerol, was isolated and identified in barley, clover and spinach chloroplasts by Benson _et al_. (1959). These workers found that this sulpholipid fraction was

rapidly labeled with S^{35} and C^{14} during photosynthesis. The origin of the sulpholipid was speculated to occur through the peroxidation of a disulfide linked glycolipid. Russell and Bailey (1966) found that the concentration of lipid-bound sulphoquinovose and galactose in red clover leaves fluctuated from 14 to 40%, respectively, during one growing season. Both the sulpholipid and galactolipids were released rapidly into the rumen within 2 hours following ingestion of clover. Eight hours after ingestion the concentration returned to the pre-feeding levels. The sulpholipid showed strong surface activity with a direct linear relationship to concentration. The anti-foaming properties of the sulpholipid resembled those of several surface active compounds which have been successfully used in bloat control by breaking high viscosity protein-type foams (Reid et al., 1961). The authors concluded that the sulpholipids may be an important factor influencing the degree of foam production in the rumen due to the rapid release from plant material, the slow rate of rumen hydrolysis and the ability to depress surface-tension in aqueous solutions.

Bailey (1964) observed that clover leaves possessed significantly higher levels of lipid-bound sugars than the stems plus petioles. The levels of lipid-bound sugars showed a significant variation with the season, being lowest

in mid-to-late summer, but the differences appeared independent of the stage of maturity of the plant. No significant correlation was noted between the leaf levels of lipid-bound sugars and bloat observed on pasture. The galactolipids were found to be rapidly released and degraded within the rumen.

Minerals

Only recently has the relationship of minerals to the etiology of legume bloat received much attention. Because the research reported in this thesis is limited to interrelationships between calcium and magnesium and legume bloat, the review of literature will also be confined mainly to these two alkaline earth metals. The interest in these two elements is of particular importance due to the extreme quantitative differences in the levels of calcium and magnesium in legumes and grasses. Legumes normally contain 3 to 4 times as much calcium and magnesium as do grasses (Thomas et al., 1952; Thompson, 1953).

The availability of minerals from legumes is of primary importance in assessing their role in the etiology of bloat. Garton (1951) observed soluble calcium and magnesium levels of 15 and 11 mg/100 ml rumen fluid, respectively, in the rumen of sheep fed chopped meadow hay. Utilizing an in vitro experiment with artificial saliva, Garton showed that 20 mg of calcium and 14 mg of magnesium per 100 ml rumen fluid were released from 10 g of hay in 5 hours at 39 C.

The levels of soluble ruminal calcium and magnesium were found to decrease with time.

Garner (1949) reported that 85% of the magnesium in grass powder was liberated during a 72-hour *in vitro* digestion in rumen fluid. It was concluded that rumen microorganisms do play a prominent role in liberating magnesium from plant cells.

Phelps *et al.* (1958) observed a threefold increase in the level of magnesium ammonium phosphate precipitate from fistulated animals in a frothy versus non-frothy state. The level of magnesium ammonium phosphate was dependent on both the ruminal hydrogen ion concentration and the diet fed.

Johnson (1959) observed a significant positive correlation between bloat severity and total ash content of alfalfa. During two grazing seasons, calcium was found to be both negatively and positively correlated with bloat severity. Jackson *et al.* (1962) found no significant correlation between cattle bloat on Ladino clover pasture and the phosphorus, potassium or calcium levels in the forage. Smith (1963) reported no significant correlation between the total amount of calcium and magnesium in whole alfalfa plants and the severity of bloat in lambs grazing alfalfa pasture.

Smith (1963) observed that calcium and magnesium salts significantly increased bloat whether administered by foliar

application to the growing alfalfa or by drenching the animal prior to grazing. Spraying alfalfa with calcium carbonate prior to grazing was shown by Warner *et al.* (1962) to increase bloat severity in lambs. Slaked lime additions to drinking water also resulted in a marked increase in bloat (Reid *et al.*, 1961). These findings suggest that the action of calcium and magnesium is mediated through the rumen. This view is partially substantiated by the findings of Smith (1963) that the ruminal magnesium level increased as bloat increased. However an inverse relationship was observed with ruminal calcium levels.

Sodium and calcium ions were found to be essential for development of strong stable foams *in vitro* (Perri and Hazel, 1947; Mangan, 1959). Mangan found that the ruminal salt concentration markedly influenced the foaming properties of legume forages. Following exhaustive dialysis, cytoplasmic protein solutions produced only small increases in foam expansion with zero foam strength. The addition of as little as 0.063M sodium chloride increased the foam capacity tenfold and resulted in maximum foam stability. A linear increase in foam strength was observed with salt additions from 0 to 0.33M. On a molar basis, calcium was found to be twice as efficient as sodium in enhancing foam stability. Mangan stated that divalent cations do not become significant foam stabilizers until reaching a minimum concentration of

0.1M. The normal rumen cation concentration ranges from 0.1 to 0.2M with calcium and magnesium being the predominant cations present.

Perri and Hazel (1947) observed that calcium and sodium additions up to 0.5M cause a marked increase in the foaming capacity of alpha-soybean protein. Calcium was more effective at increasing foam stability than sodium, particularly at low concentrations. These workers attributed the physical phenomenon to a "salting-in" effect of the electrolytes upon the protein molecule.

The importance of calcium in maintaining foam stability is also seen in its relationship to two other plant foaming constituents, saponins and pectin methylesterase. Mangan (1959) observed that calcium was essential for the formation of rigid saponin foams. Precipitation of calcium from alfalfa saponins reduced the foam strength to virtually zero. A similar relationship has also been observed with pectins and pectin methylesterase. Pressey (1962) demonstrated that calcium is an essential cofactor for the activity of bacterial pectinase enzymes. Gupta et al. (1962) reported that calcium is required for maximum foam production and stability in vitro with pectins and pectin methylesterase.

A clue to the possible significance of minerals in foam formation is seen in a recent paper by Harris and Sebba

(1965). Polarographic examination of foam fractions at pH 4.5 revealed traces of nickel and zinc concentrated in the foam. During the first 10 minutes of foaming, the concentration of nickel in young, immature alfalfa was five times greater than that in mature alfalfa. Zinc concentrations were similar in the foam regardless of the maturity of the plant source. The authors concluded that in young, immature alfalfa nickel appeared to be available for protein attachment, while in the mature plant, it was no longer in an available form. The cause of the variation in metal-protein binding might also be due to steric or chemical alterations in the protein structure. It is readily conceivable that calcium and magnesium are also concentrated in the foam in a manner similar to zinc and nickel. The concept of competitive binding sites for different metals is also a possibility. However, no such observations have yet been made.

The importance of changes in the mineral content of legumes upon plant metabolism must be considered. Magnesium and calcium are both known to be essential in primary metabolic pathways, magnesium for protein synthesis and calcium for certain phospholipid syntheses. Because the chloroplast has been identified as the primary site for both protein and phospholipid synthesis, it is significant to note that non-aqueously isolated bean and tobacco chloroplasts

contained from 40 to 75% of the total leaf potassium, magnesium and calcium (Stocking and Ongun, 1962). The high levels of mineral elements found in chloroplasts isolated non-aqueously from leaves replaces the old concept of the vacuole as the major site of ion accumulation (Hoagland, 1944).

EXPERIMENTAL PROCEDURES

Assay Animals and Forage Sampling

Assay animals

Cattle and sheep were used as assay animals in the bloat studies. In both years the two species received alfalfa soilage from the same plots. Both groups of 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.

Twenty crossbred wether lambs averaging 57 lb in 1965 and 67 lb in 1966 were used for the assays. The lambs were group-fed 6 to 8 lb of alfalfa soilage twice daily at 7 AM and 2 PM in bunk-type feeders. The soilage was composed of 6 to 18-inch alfalfa tops. At the conclusion of the 1-hour feeding periods, the remaining soilage was removed, weighed and the lambs scored for bloat severity according to the scale shown in Table 1 (Johnson *et al.*, 1958). Lambs with bloat scores of three or more were drenched with 50 ml of emulsified soybean oil by means of a stomach tube. Correlation studies were made between bloat scores and various chemical components as described later in Laboratory Methods. The bloat scores were based upon average afternoon bloat per lamb in 1965 and average morning bloat per lamb in 1966.

Twenty steers of dairy breeding averaging 950 lb in

1965 and 875 lb in 1966 were used as assay animals. The steers were group-fed from 600 to 1000 lb of alfalfa soilage twice daily in fence-row feed bunks at 7 AM and 2 PM. The steers were scored at hourly intervals following feeding of the soilage.

Table 1. Description of scale used in assigning bloat scores to lambs during feeding trials

Score		Description
0	No bloat	No distension on left side
1	Slight	"Puffy" distension on left side
2	Mild	Marked distension on left side, slight distension on right side
3	Moderate	Extreme distension on both sides; drum-like, minor distress symptoms
4	Severe	Extreme distension on both sides; discomfort, difficult breathing, excessive urination, etc.
5	Terminal	Extreme abdominal distension; severe distress, down, death unless treated

Steers showing any evidence of bloat were placed in a restraining pen. Those animals scoring two or more were drenched with 500 ml of emulsified soybean oil using a stomach tube. The average maximum morning bloat scores for 1965 and 1966 were used for correlation studies with chemical data.

Forage sampling

One-hundred alfalfa plants were collected at 20 different locations from the same plot being cut for alfalfa soilage. The plants were cut by hand at the same time and the same height as the flail chopper for each respective feeding. The samples were placed immediately inside a plastic bag in an insulated chest containing 5 lb of dry ice. The sample was taken directly to a cold room at 4 C where the leaves and flowers were cut from the stems and weighed. The leaves were stored in a deep-freeze until the chemical analyses could be run. Prior to weighing the sample for chemical studies, the frozen samples were thawed for 16 hours.

From June to September of 1965, forage samples were collected periodically at the 1 PM cutting. From May to August of 1966, samples were collected periodically at the 7 AM cutting. Chemical analyses conducted on these samples were correlated with the bloat severity of the lambs and steers fed alfalfa soilage cut at the same time and from the same plot. Samples were collected at 4 AM, 7 AM, 10 AM, 1 PM, 4 PM and 7 PM on five different days to show diurnal changes in plant composition. Simple correlation coefficients were calculated between individual chemical values and bloat severity (Snedecor, 1956).

Laboratory Methods and Miscellaneous Procedures

Chloroplast isolation

All steps described in the following procedures were conducted in a cold room at 4 C. One g of thawed leaves was ground with 20 ml of ice-cold carbon tetrachloride/hexane (D = 1.32) in a Waring Blendor for 3 minutes. The grinding was done in 30-second intervals followed by a 30-second rest to prevent overheating and possible denaturation of the protein. Following mixing, the homogenate was filtered through three layers of cheesecloth and centrifuged in a refrigerated centrifuge for 15 minutes at 12,000 x g. The green suspension on the upper surface was collected and added to a tube containing 10 ml of ice-cold hexane. The density of the resulting solution was 1.10. The mixture was centrifuged for 30 minutes at 1,000 x g. The sedimented pellet represents the nonaqueously isolated chloroplasts.

In the aqueous isolation technique, 1 g of thawed leaves was homogenized for 2 minutes with 20 ml of a 0.15M sucrose buffer in a Waring Blendor. One ml of Antifoam A Silicone Spray (Dow Chemical) was added to prevent foaming in the blendor. Following homogenization the solution was filtered through three layers of cheesecloth and spun at 500 x g for 5 minutes to remove the heavier cellular particles. The supernatant was spun for 30 minutes at 15,000 x g. The resulting pellet represented the aqueously isolated chloroplasts.

Percent leaves, stems and flowers

Following cutting and freezing with dry ice, the 100 alfalfa plants were taken to the cold room where the leaves and flowers were cut by hand from the stems. Following the physical separation, the leaves, stems and flowers were weighed immediately on a fresh-weight basis. Dry matter measurements were made on triplicate 1 g samples of thawed alfalfa leaves by heating for 24 hours in an oven at 85 to 90 C.

Leaf and chloroplastic protein

In all protein analyses, protein was determined by the micro-Kjeldahl technique. The samples were digested for 2 hours with 5 ml concentrated sulfuric acid and catalyzed by copper and potassium sulphate (2:1 w/w). The contents of the micro-Kjeldahl flask were transferred into a distillation apparatus as described by Markham (1942) with three successive 5 ml aliquots of ion-free water. Following the addition of 20 ml of 10N NaOH, the digestion mixture was distilled into 20 ml of 4% boric acid containing one drop of methyl red methylene blue indicator. The flask was then titrated with 5.61×10^{-4}N HCl and the readings corrected with a blank.

For total leaf protein analyses, 100 to 300 mg of thawed leaves were homogenized in a Waring Blendor for 2 minutes with 20 ml of ion-free water. Following precipitation with 20% trichloroacetic acid (TCA) and centrifugation, the

precipitate was transferred to a micro-Kjeldahl flask with three successive 5 ml aliquots of ion-free water, digested, distilled and titrated as described earlier.

For total chloroplastic protein analyses, the non-aqueously isolated chloroplastic pellet was dissolved in 3 ml of 0.1M sodium borate buffer, pH 8.3. The protein was precipitated with 2 ml of 20% TCA and spun at 15,000 x g in a refrigerated centrifuge for 15 minutes. The precipitated protein was transferred to the micro-Kjeldahl flask with three successive 5 ml aliquots of ion-free water and analyzed as described earlier.

In the total soluble chloroplastic protein analysis, the nonaqueously isolated chloroplastic pellet was extracted for 20 hours with 3 ml of a 0.1M sodium borate buffer, pH 8.3. The solution then was filtered through three layers of cheesecloth with 10 ml of ion-free water. Beginning with the precipitation by TCA, the remaining steps were identical to those used for total chloroplastic protein.

Leaf and chloroplastic lipids

A standard procedure was used for the final extraction and weighing procedure for all lipid analyses. The sample was placed in a paper extraction thimble and extracted for 6 hours with 50 ml of anhydrous ether. Following the extraction, the thimble was replaced with a pyrex vial and the ether was refluxed into the vial. After evaporation of

the ether, the vials were allowed to cool to room temperature in a dessicator and weighed.

A 1 g sample of thawed leaves was used for determining the total leaf lipids. In the total chloroplastic lipid analysis, the aqueously isolated chloroplastic pellet from 1 g of leaves was extracted overnight with 1 ml of 0.1M sodium borate buffer, pH 8.3. The mixture was further dissolved in 1 ml of the buffer and transferred to the extraction thimble. Following the air drying of the thimble, the lipids were extracted as described in the preceding paragraph.

In the determination of total soluble chloroplastic lipids, the aqueously isolated chloroplastic pellet was extracted for 20 hours with 3 ml of 0.1M sodium borate buffer, pH 8.3, filtered through three layers of cheesecloth into the extraction thimble and allowed to air dry. All remaining steps were identical to those used for total chloroplastic lipids.

Leaf and chloroplastic phospholipids

Total phospholipids were determined in leaf and chloroplast samples. For the determination of total leaf phospholipids, a 1 g sample of thawed leaves was homogenized in 20 ml of a 0.15M sucrose buffer for 3 minutes in a Waring Blendor. For total chloroplast phospholipids, the aqueously isolated chloroplastic pellet was dissolved in 20 ml of 0.15M

sucrose. Following these preliminary homogenization and isolation steps, the extracts were transferred to 100 ml separatory funnels.

The solvent fractionation technique used was a modification of the procedure described by Biezenski and Spaet (1961). Twenty ml of a methanol-ethanol mixture (4:1, v/v) were added to the separatory funnel and mixed thoroughly with the homogenate. An equal volume of chloroform was added to make the extraction mixture of chloroform-methanol-ethanol (5:4:1, v/v). Lipids in the homogenate were extracted three times for 30 minutes, 2 hours and overnight at room temperature with continuous shaking. Following filtration through Whatman No. 2 filter paper, filtrates were pooled, evaporated to dryness in vacuo and the lipids taken up in 5 ml of chloroform and filtered. A 1 ml aliquot of the resulting filtrate was used for the lipid phosphorus determination.

The lipid phosphorus determination was a modification of the technique of Fiske and Subbarow (1925). The lipid filtrate was digested for 45 minutes with 1 ml of $HClO_4$: HNO_3:H_2SO_4 (70:20:10) on a micro-Kjeldahl digestion rack. Following cooling, the acid was evaporated off by means of aspiration. The resulting salts were neutralized with 0.5 ml of 8% NaOH. Four ml of a color reagent containing ammonium molybdate and ascorbic acid were added and the tubes were

incubated in a 38 C water bath for 1.5 hours. Aliquots of the solution were read in a Beckman 505 Spectrophotometer. Lipid phosphorus values were calculated from the standard curve obtained using a standard potassium dihydrogen phosphate solution.

Leaf and chloroplastic calcium and magnesium

The calcium and magnesium contents in leaf and chloroplastic samples were determined. Following wet oxidation with nitric, sulfuric and perchloric acids as described by Reitz et al. (1960), the salts were dissolved in 3 ml of concentrated hydrochloric acid and 10 ml of ion-free water. The solution was transferred to a 50 ml volumetric flask with three successive 10 ml washings of ion-free water. Calcium and magnesium were determined by EDTA titration by a modification of the technique of Hildebrand and Rilley (1957). Care was taken in the calcium determination to adjust the pH above 12. In the magnesium determination, the pH was maintained between 10.1 to 10.4. Calcein and calmagite were used as the calcium and magnesium indicators, respectively.

For total leaf calcium and magnesium, 1 g samples of thawed leaves were used. Calculations were adjusted to a dry matter basis. For total chloroplastic calcium and magnesium, the nonaqueously isolated chloroplastic pellet was transferred with two 5-ml aliquots of ion-free water to a 250 ml Erlenmeyer flask. All remaining steps were the same as those described earlier.

Protein to chlorophyll ratios

The protein to chlorophyll ratios were determined for the total chloroplast extracts and for the total soluble chloroplast extracts isolated nonaqueously. Chlorophyll was measured by the method of Arnon (1949) using acetone as a solvent. Protein was determined by micro-Kjeldahl digestion of 20% TCA precipitates as described earlier.

Isolation and properties of Fraction I chloroplastic protein

Separation of Fraction I protein The nonaqueously isolated chloroplastic pellet from a 1 g sample of thawed leaves was dissolved for 20 hours in 3 ml of a 0.15M sodium borate buffer, pH 8.3. Following filtration through three layers of cheesecloth, the soluble protein was precipitated with 2 ml of 20% TCA, spun at 20,000 x g for 15 minutes and then redissolved in 2 ml of the borate buffer. The soluble chloroplastic extract was transferred to a 1 cm (I.D.) x 22.5 cm glass column packed with Sephadex G-50 Medium and with ion-free water. One ml aliquots were collected with a fraction collector, diluted to 3 ml and analyzed for optical density at 280 and 260 $m\mu$ with a Beckman 505 Spectrophotometer. The three aliquots comprising the Fraction I peak were used for binding studies to be described later.

Spectral and ultracentrifugal analyses A spectral analysis from 240 to 340 $m\mu$ using the Beckman 505 Spectrophotometer was conducted on the three aliquots comprising

the Fraction I chloroplastic peak. The relative purity of the Fraction I protein was checked by observing the sedimentation patterns at a maximum speed of 47,700 rpm using the Spinco model E Analytical Ultracentrifuge with schlieren optics and a standard 12-mm cell in an AND rotor.

Foaming properties of fraction I protein The effect of foaming was determined on the optical density of the effluent following gel-filtration of soluble chloroplastic protein extracts on a Sephadex G-50 column. Two ml of each aliquot eluted through the Sephadex column were added to 2 ml of 1.0M sodium acetate-acetic acid buffer, pH 5.3, as described by McArthur and Miltimore (1964). The mixture was drawn into a 10-ml hypodermic syringe, foamed by sucking in air and then ejected. The procedure was repeated for 5 minutes or until the solution would not form a stable foam. After the precipitate was filtered off, the optical density was determined at 280 m_{μ} for each aliquot using a Beckman 505 Spectrophotometer.

Amino acid analyses The amino acid content was determined for total soluble chloroplastic protein and Fraction I chloroplastic protein on two bloat and two non-bloat samples. One ml samples containing approximately 0.1% protein, calculated from previous data, were refluxed with 50 ml of concentrated sulfuric acid for 72 hours. The refluxed mixture was concentrated to approximately 1 ml in a

rotary flask evaporator. The amino acids were separated on a Dowex-1 Column of a Beckman Auto-Analyser and the optical densities of the peak heights were recorded at 280 m_{μ} using ninhydrin. Amino acid concentrations were determined by comparing sample peak heights and areas with those of a 1-μ concentration of a known standard.

Calcium and magnesium binding to fraction I chloroplastic protein Ten-inch lengths of Visking cellulose casing, 0.5 inch in diameter, were used as the dialysis tubing. The casing was initially heated for three successive 1-hour intervals on a steam bath. Following each heating, the casing was transferred to fresh ion-free water. The pre-conditioned casings were kept in ion-free water in the cold room until used. Prior to dialysis the tubing was removed from the water and the excess liquid stripped from the casing. Ten ml of 0.1M calcium chloride or 0.1M magnesium chloride, pH 5.5, were added to 50 ml pyrex test tubes. Added to the casing were 10 ml of the Fraction I protein and 1 ml of Antifoam A Silicone Spray (Dow Chemical). The casing was closed with two overhead knots and suspended into the salt solution. For the control sample, 10 ml of the ion-free water were used.

The solutions were dialyzed for 24 hours. Five-ml aliquots from the internal solutions of the sample were used to determine total protein by means of the micro-Kjeldahl

technique following precipitation with 2 ml of 20% TCA. Four-ml aliquots were collected from the internal and external solution of the control and of the samples for the determination of calcium and magnesium. The extent of binding was calculated on the basis of 1×10^{-5} moles of calcium or magnesium per g of Fraction I chloroplastic protein. Apparent stability constants were also calculated assuming a molecular weight for the protein of 5×10^{5}.

Effect of salt concentration upon binding The effect of four different magnesium concentrations upon metal-Fraction I chloroplastic protein binding was studied in one non-bloat and one high-bloat sample. Prepared with magnesium chloride and ion-free water, magnesium concentrations of 0.02M, 0.04M, 0.08M and 0.10M were used. The techniques used for dialysis were identical to those described in the previous heading.

Effect of lecithin and fatty acid additions The effects of lecithin, palmitic acid and linolenic acid upon calcium-Fraction I chloroplastic protein binding were studied. The lecithin used was α,β-dipalmitoyl phosphatidyl choline. All three lipids were synthetic products purchased commercially from Nutritional Biochemicals (Cleveland, Ohio). The lecithin and fatty acids were prepared in ion-free water at five concentrations corresponding to lipid/protein mole ratios of 4, 8, 16, 32 and 64. The mole ratios were based

upon the Fraction I chloroplastic protein levels determined in previous analyses. In each case the lipid solutions were stirred for a minimum of 24 hours at 25 C. For each of the three lipids, one sample in each of the four bloat ranges was studied. The lipid suspensions were prepared so that 1 ml would give the desired mole ratios in the internal dialysis solution. Dialysis techniques were the same as above.

Chloroplast fragility measurements

To determine chloroplast fragility, chlorophyll analyses were conducted on both alfalfa leaves and alfalfa leaf homogenates following ?-minutes of homogenization in a Waring Blendor. For the total leaf analysis, 1 g of thawed leaves was successively extracted three times for 2 hours each with 100 ml of acetone and ethanol (4:1, v/v) and filtered through a Büchner funnel between each extraction. Following the three extractions, the remaining leaves were devoid of green color. For the homogenate analysis, 1 g of thawed leaves was homogenized in 20 ml of 0.15M sucrose buffer for 2 minutes. The homogenate was filtered through four layers of cheesecloth into a 250-ml Erlenmeyer flask. The filtrate was extracted for 2 hours with 100 ml of acetone and ethanol (4:1, v/v) and centrifuged at 1,200 rpm for 20 minutes to remove any particulate matter. The supernatant was poured off and used for the spectrophotometric analysis. For both the total leaf and homogenate extracts, the chlorophyll concentration was determined by measuring optical

density at 645, 652 and 663 $m\mu$ in a Beckman 505 spectrophotometer according to the method of Arnon (1949).

EXPERIMENTAL METHODS

Electron Microscopy

Fixation, embedding and sectioning techniques

Fixation of the alfalfa leaf discs was accomplished by 6.5% glutaraldehyde in a phosphate buffer, pH 7.2, for 1 hour as described by Sabatini et al. (1963). Following the glutaraldehyde fixation, the discs were rinsed three times for 10 minutes each with the phosphate buffer. Post-fixation was completed by immersing the discs in OsO_4 in a phosphate buffer, pH 7.2, for 1 hour according to the method of Millonig (1961). A 2% stock solution of OsO_4 diluted 1:1 with the phosphate buffer was used. All of the fixation steps described above were performed at 4 C using ice. After dehydration with alcohols, the discs were embedded in Epon 812 using 3 parts of DDSA-Epon mixture to 2 parts of NMA-Epon mixture (Luft, 1961). Discs were sectioned with a diamond knife using an LKB Ultra-microtome. Examination was with an RCA EMU3F electron microscope.

Sampling procedures

Whole, flailed and bolus leaves Electron micrographs were made on sections taken from whole, flailed and bolus leaves of alfalfa plants at different bloating stages. Whole alfalfa plants were collected from the field and brought into the laboratory wrapped in wet newspaper. Discs 0.8 mm in diameter were punched out of whole leaves taken from the

upper 6 inches of plant growth with a circular punch fashioned from a hypodermic needle. Discs from flailed leaves were taken at random from alfalfa soilage in the feedbunks following flail chopping in the field. Following emptying of the rumen contents, a bolus was collected from a fistulated Holstein steer by inserting an arm into the rumen and catching the bolus at the cardia as it entered the rumen. Discs were made from the leaf fragments present in the bolus.

An electron micrographic study was made of diurnal changes in the chloroplastic ultrastructure. Whole leaf samples were collected at 3 hour intervals beginning at 7 AM and ending at 7 PM. Comparisons were made of ultrastructural changes in one non-bloat, one low-bloat and one high-bloat sample.

In vitro incubation in rumen fluid Whole, flailed and bolus leaves were incubated in whole rumen fluid for 1 and 2 hours. The leaves were collected on 3 different days from soilage representing non-, low- and high-bloat. The rumen fluid was collected via aspiration and gravity flow from a fistulated steer. The bolus leaves were collected from the same fistulated steer as the rumen fluid. One g of each type of leaf (fresh weight basis) was incubated with 30 ml of whole rumen fluid. The samples were incubated in a water bath at 38 C with carbon dioxide being constantly

bubbled through the fluid. At the end of 1 and 2 hours, the different types of leaves were blotted dry and taken to the microscopy laboratory where discs were immediately punched. The non- and low-bloat leaf samples were incubated for both 1 and 2 hours whereas the high-bloat leaf samples were incubated for only 1 hour.

Rumen fluid, bolus juices and foam Whole rumen fluid was collected by aspiration from a fistulated Holstein steer prior to feeding alfalfa soilage and 20 minutes after feeding. Bolus juices were collected 20 minutes after feeding at the cardia of the rumen in a plastic bag which was inserted by hand through the open plug of the fistulated steer. Foam was collected by hand from the surface of the rumen following opening of the plug of the fistulated steer. Each of the above samples was squeezed through four layers of cheesecloth into a glass vial containing 6.5% glutaraldehyde. Following the return to the microscopy laboratory, the samples were centrifuged for 5 minutes in a Serval SS3 Ultracentrifuge at 5,000 rpm. The samples were rinsed three times for 10 minutes each with glutaraldehyde, each rinse being followed by centrifugation. The samples were post-fixed for 1 hour with 1% OsO_4 in phosphate buffer, pH 7.2. Following fixation the pellets were centrifuged again for 5 minutes at 5,000 rpm. Chunks of the pellet were placed in agar, trimmed and dehydrated as described earlier. Embedding, sectioning and microscopy were the same as above.

EXPERIMENTAL RESULTS

Relationship Between Alfalfa Leaf Constituents and Bloat

To show the relationship of the chemical composition of different plant constituents to bloat, 20 leaf samples were analyzed for each of two consecutive years. The data from the 20 samples were divided into four groups of five samples each based upon the following ranges of bloat severity as determined with assay animals: 0, 0 to 0.5, 0.5 to 1.0 and greater than 1.0. The adjectives used in this thesis to designate the four bloat ranges were, respectively: non-, low-, medium- and high-bloat. Unless specified otherwise, the correlation coefficients shown in the tables refer to the values calculated using the data from the 20 samples. All data shown are calculated on a dry matter basis with the exception of fresh leaf, stem and flower weights. The individual value for each bloat range shown in the tables represents the mean value for the five samples. Duplicate analyses were run for all samples unless specified otherwise.

Percent leaves, stems and flowers

The relationships between percent leaves, stems and flowers and bloat severity are shown in Table 2. In both 1965 and 1966, the percent fresh weight of alfalfa leaves showed a significant direct correlation ($P < .01$) with bloat severity. The correlation coefficients for cattle and sheep

ranged from 0.68 to 0.84. In 1965, the percent fresh weight of alfalfa leaves in the afternoon ranged progressively from 29.8 to 42.2%. The morning values in 1966 showed less variation, ranging from 38.5 to 44.9%. No consistent differences were noted between the morning and afternoon values at the different bloat ranges.

The percent alfalfa stems on a fresh-weight basis were negatively correlated with bloat in both years. Significant negative correlation coefficient ($P < .01$) for cattle and sheep ranged from -0.73 to -0.88. The percent stems were uniformly higher in the afternoon samples of 1966 than the morning samples in 1965. The percent stems ranged from 49.5 to 52.6% in 1965 and 53.8 to 57.4% in 1966.

The non-significant negative correlation coefficients for percent flowers (fresh-weight) ranged from -.08 to -.38. In 1965 the percent flowers were considerably higher, varying from 17.4 (non-bloat) to 0.5% (high-bloat). The percent ranges for the 1966 samples ranged from 6.0 (non-bloat) to 0.2% (high-bloat).

Leaf dry matter

The relationship between alfalfa leaf dry matter and bloat is shown in Table 2. The percent dry matter of alfalfa leaves was significantly ($P < .01$) correlated with bloat severity in both years, ranging from -.50 to -.71. The correlation coefficients were uniformly higher for sheep

Table 2. Relationship of alfalfa leaves, stems, flowers and dry matter to bloat

Component value	Bloat score range				r	
	0	0 to 0.5	0.5 to 1.0	1.0	Sheep	Cattle
			%			
Alfalfa leaves, fresh wt.						
1965	29.8	45.0	42.3	49.2	0.84**	0.68**
1966	38.5	42.5	44.9	44.3	0.74**	0.83**
Alfalfa stems, fresh wt.						
1965	52.6	52.0	49.5	50.2	- .88**	- .82**
1966	57.2	57.4	53.8	55.4	- .73**	- .82**
Alfalfa flowers, fresh wt.						
1965	17.4	2.7	8.0	0.5	- .38	- .30
1966	6.0	0.2	1.5	0.2	- .08	- .08
Leaf dry matter						
1965	37.4	29.3	27.0	24.9	- .66**	- .50*
1966	27.5	22.2	21.6	20.7	- .71**	- .64**

*$P < .05$.

**$P < .01$.

than cattle. In 1965 percent leaf dry matter varied progressively from 37.4 for non-bloat to 24.9% for high-bloat. A similar, but consistently lower trend was noted for the afternoon samples in 1966, ranging from 27.5 (non-bloat) to 20.7% (high-bloat).

Leaf and chloroplastic protein

The relationships between leaf and chloroplastic proteins and bloat are shown in Table 3. The correlation coefficients for sheep were consistently higher than cattle in 1965. In 1966 the correlation coefficients for both cattle and sheep were similar. All protein components, total leaf, total and soluble chloroplastic and Fraction I chloroplastic, were significantly ($P < .01$) correlated with bloat severity in cattle and sheep. Correlation coefficients ranged from 0.67 to 0.99.

The correlation coefficients for the three different chloroplastic protein values were uniformly higher than those for total leaf protein. The ranges in correlation coefficients for the protein components were as follows: total leaf protein, 0.68 to 0.83; chloroplastic protein, 0.74 to 0.93; soluble chloroplastic protein, 0.67 to 0.94; and Fraction I chloroplastic protein, 0.97 to 0.99.

In 1965 total leaf protein values ranged from 20.6 (non-bloat) to 34.7% (high-bloat). Afternoon sample values in 1966 were uniformly higher than those in 1965, ranging from 22.9 (non-bloat) to 39.8% (high-bloat).

Table 3. Composition and correlation values of alfalfa leaf proteins with bloat

Protein component	Bloat score range				r	
	0	0.0 to 0.5	0.5 to 1.0	> 1.0	Sheep	Cattle
			%[a]			
Total leaf protein						
1965	20.6	33.4	33.4	34.7	0.83**	0.68**
1966	22.9	34.7	34.4	39.8	0.81**	0.79**
Chloroplastic protein						
1965	0.58	0.83	1.16	1.36	0.84**	0.74**
1966	0.55	0.85	1.30	2.04	0.88**	0.93**
Soluble chloroplast protein						
1965	0.22	0.38	0.55	0.58	0.84**	0.67**
1966	0.22	0.35	0.56	0.92	0.90**	0.94**
Fraction I chloroplastic protein						
1966	0.02	0.05	0.21	0.42	0.97**	0.99**

[a]Percentages expressed on leaf dry matter basis.

**$P < .01$.

Chloroplastic protein values ranged from 0.58 (non-bloat) to 1.36% (high-bloat) in 1965 and 0.55 (non-bloat) to 2.04% (high-bloat) in 1966. The mean percentages for the non- and low-bloat samples were virtually identical in both years, whereas the medium- and high-bloat samples were markedly higher in the 1966 samples.

The relationship of bloat to the percentage ratio of different chloroplastic proteins expressed as percentages of leaf and chloroplastic protein is shown in Table 4. In 1965 the protein percentage ratio of the isolated chloroplasts ranged from 2.84 (non-bloat) to 3.95% (high-bloat) of the total leaf protein. The values for the same ratio ranged from 2.41 (non-bloat) to 5.12% (high-bloat) in 1966. The only appreciable difference between the figures occurred in the high-bloat samples which were markedly higher in 1966.

The percentages of soluble chloroplastic protein shown in Table 3 were remarkably similar in both years for the three lower bloat ranges. Only the high-bloat value for 1966 was appreciably higher than that in 1965. The figures varied from 0.22 (non-bloat) to 0.92% (high-bloat) during the two years.

When expressed as a percentage of total chloroplastic protein (Table 4), the soluble chloroplastic protein ranged progressively from 37.9 (non-bloat) to 52.1% (high-bloat) in 1965 and 40.5 (non-bloat) to 45.1% (high-bloat) in 1966. As

Table 4. Relationship between percentages of alfalfa leaf proteins and bloat

Protein component ratios[a]		Bloat scores			
		0	0 to 0.5	0.5 to 1.0	>1.0
		%			
Chloroplastic protein/leaf protein	1965	2.84	2.44	3.48	3.95
	1966	2.41	2.45	3.78	5.12
Soluble chloroplastic protein/chloroplastic protein	1965	37.9	47.2	48.3	52.1
	1966	40.5	41.3	42.7	45.1
Soluble chloroplastic protein/leaf protein	1965	1.08	1.13	1.62	1.82
	1966	0.98	1.01	1.62	2.31
Fraction I chloroplastic protein/chloroplastic protein	1966	2.91	5.41	15.85	20.78
Fraction I chloroplastic protein/soluble chloroplastic protein	1966	7.13	14.65	37.20	46.69

[a]Ratios are based upon two percentage values expressed on total leaf dry matter basis.

a percentage of total leaf protein (Table 4), the soluble chloroplastic protein varied from 1.08 (non-bloat) to 1.82% (high-bloat) in 1965 and 0.98 (non-bloat) to 2.31% (high-bloat) in 1966.

As shown in Table 3, Fraction I chloroplastic protein increased from 0.02 (non-bloat) to 0.42% (high-bloat) in 1966.

When expressed as a percentage of total chloroplastic protein (Table 4), the Fraction I content varied from 2.91 (non-bloat) to 20.78% (high-bloat). Table 4 shows that Fraction I comprised from 7.13 (non-bloat) to 46.69% (high-bloat) of the soluble chloroplastic protein.

Protein to chlorophyll ratios

To accurately determine what percentage of the leaf protein was localized in the chloroplasts, protein to chlorophyll ratios were measured on both whole leaf homogenates and isolated chloroplasts. A comparison of the ratios was made on two samples from three bloat ranges for total leaf protein and soluble leaf protein. The results are summarized in Table 5.

The results demonstrate that the percentage of total and soluble leaf protein increases within the chloroplasts as bloat severity increases. This suggests that a preferential synthesis of protein within the chloroplasts favors the incidence of bloat. The percent distribution of both total and soluble leaf protein in the chloroplasts were very similar. From 45.5 (non-bloat) to 76.7% (high-bloat) of the total leaf protein was found in the chloroplasts. The percentage of soluble protein localized in the chloroplasts ranged from 42.7 (non-bloat) to 80.5% (high-bloat).

Table 5. Relationships between the percentages of total and soluble leaf protein localized in the chloroplasts and bloat

Item	PERCENT OF TOTAL LEAF PROTEIN IN CHLOROPLASTS						
	Whole leaf homogenate			Isolated chloroplasts			Total leaf protein in Chloroplasts
	Protein conc.	Chloro-phyll conc.	Ratio: protein/ chloro-phyll	Protein conc.	Chloro-phyll conc.	Ratio: protein/ chloro-phyll	
	mg/g fresh weight			mg/g fresh weight			%
Non-bloat[a]	17.4	1.12	15.5	0.72	0.10	7.0	45.5
Non-bloat[b]	15.5	0.97	16.0	0.95	0.12	7.6	48.0
Low-bloat[c]	18.3	1.03	17.7	1.33	0.12	10.8	61.4
Low-bloat[d]	20.9	1.05	19.9	0.81	0.07	11.7	58.5
High-bloat[e]	19.4	1.17	16.6	2.43	0.19	12.7	76.7
High-bloat[f]	22.3	1.21	18.4	2.67	0.20	13.6	74.3

Item	ORIGIN OF SOLUBLE LEAF PROTEIN IN CHLOROPLASTS						
	Whole leaf homogenate			Isolated chloroplasts			Total sol. protein derived from chloroplasts
	Borate sol. protein conc.	Chloro-phyll conc.	Ratio:sol. protein/ chloro-phyll	Borate sol. protein conc.	Chloro-phyll conc.	Ratio:sol. protein/ chloro-phyll	
	mg/g fresh weight			mg/g fresh weight			%
Non-bloat[a]	5.4	0.63	8.6	0.24	0.07	3.7	42.7
Non-bloat[b]	4.7	0.46	10.2	0.38	0.08	4.8	47.2
Low-bloat[c]	8.4	0.72	11.6	0.52	0.07	6.9	59.7
Low-bloat[d]	6.2	0.50	12.6	0.13	0.02	7.6	60.7
High-bloat[e]	12.5	0.84	15.0	0.80	0.07	12.1	80.5
High-bloat[f]	13.2	0.79	16.6	0.97	0.08	12.2	73.2

[a]Sample taken 7/14/1966 (Cattle and Sheep).
[b]Sample taken 7/18/1966 (Cattle and Sheep).
[c]Sample taken 6/4/1966. Av. daily maximum bloat score = 0.47 (Cattle).
[d]Sample taken 5/30/1965. Av. bloat score = 0.47 (Sheep).
[e]Sample taken 6/7/1965. Av. bloat score = 1.02 (Sheep).
[f]Sample taken 5/31/1966. Av. daily maximum bloat score = 1.80 (Cattle).

Leaf and chloroplastic lipid

The relationships between leaf and chloroplastic lipids and bloat are shown in Table 6. All lipid components were inversely correlated with bloat severity in cattle and sheep. Correlation coefficients ranged from -.32 to -.71.

The correlation coefficients for the chloroplastic components were consistently lower than those for total leaf lipids. The range in correlation coefficients for the various lipid components was as follows: total leaf lipids, -.55 to -.71; chloroplastic lipids, -.37 to -.55 and soluble chloroplastic lipids, -.32 to -.52.

The total leaf lipids were uniformly higher in 1966, ranging from 5.82 (low-bloat) to 3.94% (high-bloat). Morning samples were analyzed in 1966 compared to afternoon samples for 1965. The mean leaf lipid values for the three lowest bloat ranges were virtually identical in 1966. Only the high-bloat mean value was appreciably lower. A similar, but slightly more irregular pattern was observed in the total leaf lipid values in 1965 which ranged from 5.28 (low-bloat) to 3.77% (high-bloat).

The values in 1966 for total and soluble chloroplastic lipids were consistently greater than those for 1965. The percent chloroplastic lipids ranged from 3.07 (medium-bloat) to 0.72% (high-bloat) in 1965, with only a small variation occurring in the lower bloat ranges. A decline in chloroplastic lipids with increasing bloat was observed in 1966,

Table 6. Composition and correlation values of alfalfa leaf lipids and bloat

Lipid component	Bloat score range				Correlation coefficients	
	0	0 to 0.5	0.5 to 1.0	>1.0	Sheep	Cattle
			%[a]			
Total leaf lipids						
1965	4.09	5.28	4.22	3.77	-.70**	-.55*
1966	5.78	5.82	5.71	3.94	-.55*	-.71**
Chloroplastic lipids						
1965	2.89	2.62	3.07	0.72	-.47*	-.37
1966	4.11	3.93	3.18	1.35	-.50*	-.55*
Soluble chloroplastic lipids						
1965	2.03	1.28	1.24	0.71	-.44*	-.32
1966	3.14	2.71	2.20	0.75	-.46*	-.52*
Total leaf phospholipids						
1966	0.90	0.86	0.52	0.27	-.46*	-.51*
Chloroplastic phospholipids						
1966	0.49	0.39	0.13	0.05	-.33	-.34

[a]All percentages expressed on leaf dry matter basis.

*$P < .05$.

**$P < .01$.

ranging from 4.11 (non-bloat) to 1.35% (high-bloat).

When expressed as a percentage of total leaf lipids (Table 7), the chloroplastic lipids decreased from 70.6 (non-bloat) to 19.1% (high-bloat) in 1965. A similar but more gradual depression in chloroplastic lipids was noted in 1966, ranging from 71.1 (non-bloat) to 34.2% (high-bloat).

As shown in Table 6, the soluble chloroplastic lipids declined from 2.03 (non-bloat) to 0.71% (high-bloat) in 1965. A similar decrease was observed in 1966 varying from 3.14 (non-bloat) to 0.75% (high-bloat).

The percentage of total leaf lipids found as soluble chloroplastic lipids followed a similar, but less marked pattern than that for the total chloroplastic lipids (Table 7). The percent soluble chloroplastic lipids decreased from 49.5 (non-bloat) to 18.8% (high-bloat) in 1965 and 54.3 (non-bloat) to 19.0% (high-bloat) in 1966. A majority of the chloroplastic lipids was consistently found in the sodium borate soluble fraction, ranging from 86.4 (high-bloat) to 41.1% (medium-bloat) in 1965. The extremely high percentage of soluble chloroplastic lipids observed in the 1965 high-bloat samples was not found in the 1966 samples. In 1966 the percentages of the total chloroplastic lipids (soluble in borate buffer) decreased from 76.4 (non-bloat) to 55.5% (high-bloat).

Table 7. Relationship between percentages of alfalfa leaf lipids and bloat

Lipid component ratios[a]		Bloat scores			
		0	0 to 0.5	0.5 to 1.0	>1.0
		%			
Chloroplastic lipid/leaf lipid	1965	70.6	49.5	63.5	19.1
	1966	71.1	67.5	55.6	34.2
Soluble Chloroplastic lipid/leaf lipid	1965	49.5	24.3	29.3	18.8
	1966	54.3	46.5	38.5	19.0
Soluble Chloroplastic lipid/ chloroplastic lipid	1965	66.8	47.0	41.1	86.4
	1966	76.4	68.9	69.1	55.5
Leaf phospholipid/leaf lipid	1966	15.5	14.6	9.0	6.7
Chloroplastic phospholipid/leaf lipid	1966	8.4	6.6	2.2	1.2
Chloroplastic phospholipid/ chloroplastic lipid	1966	11.9	9.8	4.0	3.6
Chloroplastic phospholipid/soluble chloroplastic lipid	1966	15.6	14.3	5.9	6.6
Chloroplastic phospholipid/leaf phospholipid	1966	54.4	45.3	25.0	18.5

[a]Ratios are based upon two percentage values expressed on total leaf dry matter basis.

Leaf and chloroplastic phospholipids

The relationship between leaf and chloroplastic phospholipids and bloat are shown in Table 6. Like total lipid values, both phospholipid components were inversely correlated

with bloat severity in cattle and sheep, ranging from -.33 to -.51. The correlation coefficients for the phospholipid values were both lower than those for the corresponding total lipid analyses. The ranges in correlation coefficients were as follows: total leaf phospholipids, -.46 to -.51 and chloroplastic phospholipids, -.33 to -.34.

Both total leaf and chloroplastic phospholipid values declined uniformly with increasing bloat severity. The total leaf phospholipids decreased from 0.90 (non-bloat) to 0.27% (high-bloat) while the percent chloroplastic phospholipids dropped from 0.49 (non-bloat) to 0.05% (high-bloat).

When expressed as a percentage of the total leaf lipid (Table 7), the percent leaf and chloroplastic phospholipids ranged, respectively, from 15.5 (non-bloat) to 6.7% (high-bloat) and 8.4 (non-bloat) to 1.2% (high-bloat). The chloroplastic phospholipids decreased from 11.9 (non-bloat) to 3.6%(high-bloat) of the total chloroplastic lipids. When expressed as a percentage of the soluble chloroplastic lipids, a similar decline was noted with chloroplastic phospholipids varying from 15.6 (non-bloat) to 6.6% (high-bloat).

The last line in Table 7 shows the relationship between bloat and the percentage of leaf phospholipid localized in the chloroplasts. The data show that a preferential synthesis

of phospholipids in the chloroplasts occurs in the non-bloating forage. The percent distribution declined progressively from 54.4 (non-bloat) to 18.5% (high-bloat).

Protein to lipid and phospholipid ratios

Because of the complex nature of the bloat syndrome, it is difficult to believe that any single plant constituent is the sole cause of bloat. In an attempt to elucidate the relationship of a possible balance of plant components, the ratios of the percentages of foam-stabilizing protein and foam-inhibiting lipids were calculated. These relationships between percent leaf and chloroplastic protein to lipid ratios and bloat are shown in Table 8. In both years the correlation coefficients for the various protein to lipid ratios were highly significant ($P < .01$) for both cattle and sheep, ranging from 0.68 to 0.99. The correlation coefficients were generally similar for both species.

The correlation coefficients were consistently higher for the ratios involving chloroplastic components than for those based on total leaf values. The highest correlation coefficients were found with ratios computed using total and soluble chloroplastic protein, soluble chloroplastic lipids and chloroplastic phospholipids. The ranges in correlation coefficients were as follows: leaf protein/ leaf lipids, 0.68 to 0.89; chloroplastic protein/chloroplastic lipids, 0.76 to 0.99; chloroplastic protein/leaf phospholipid,

Table 8. Relationship between alfalfa leaf protein and lipid ratios and bloat

Component ratios[a]		Bloat score range				r	
		0	0 to 0.5	0.5 to 1.0	>1.0	Sheep	Cattle
Leaf protein/leaf lipids	1965	5.07	6.48	8.00	9.71	0.81**	0.68**
	1966	4.13	5.99	8.16	10.20	0.89**	0.88**
Chloroplastic protein/chloroplastic lipids	1965	0.22	0.32	0.40	2.05	0.76**	0.82**
	1966	0.14	0.21	0.42	1.59	0.99**	0.96**
Soluble chloroplastic protein/soluble chloroplastic lipids	1965	0.11	0.36	0.58	0.82	0.92**	0.76**
	1966	0.07	0.13	0.26	1.32	0.99**	0.96**
Chloroplastic protein/leaf phospholipid	1966	0.61	0.97	2.72	8.94	0.94**	0.96**
Chloroplastic protein/chloroplastic phospholipid	1966	1.12	2.19	10.00	40.80	0.98**	0.97**
Soluble chloroplastic protein/chloroplastic phospholipid	1966	0.46	0.95	5.50	19.67	0.98**	0.97**

[a]Ratios are based upon two percentage values expressed on total leaf dry matter basis.

**$P < .01$.

0.94 to 0.96; chloroplastic protein/chloroplastic phospholipid, 0.97 to 0.98 and soluble chloroplastic protein/chloroplastic phospholipid, 0.97 to 0.98.

In 1965 the ratio of leaf protein/leaf lipid increased approximately twofold from 5.07 (non-bloat) to 9.71 (high-bloat). The ratios for 1966 (morning samples) were lower than those in 1965 except for the high-bloat range. The ratio for 1966 ranged from 4.13 (non-bloat) to 10.20 (high-bloat).

The ratios of chloroplastic protein/chloroplastic lipid and soluble chloroplastic protein/soluble chloroplastic lipid both exhibited greater extremes than the total leaf values. The chloroplastic protein/chloroplastic lipid ratio was characterized by nine- to elevenfold increases from non- to high-bloat samples. In 1965 the ratio varied from 0.22 (non-bloat) to 2.05 (high-bloat). The mean values in 1966 were very similar to those in 1965 ranging from 0.14 (non-bloat) to 1.59 (high-bloat).

The ratio of soluble chloroplastic protein/soluble chloroplastic lipid showed fluctuations to similar total chloroplastic ratios with values characterized eight- to nineteenfold differences. The 1965 and 1966 ratios ranged, respectively, from 0.11 (non-bloat) to 0.82 (high-bloat) and from 0.07 (non-bloat) to 1.32 (high-bloat).

The greatest range in ratios involved the total leaf and

chloroplastic phospholipids, due in large part to the fact that the mean phospholipid values were less than one. A fortyfold difference was found in the ratios involving both total and soluble chloroplastic protein and chloroplastic phospholipids with the ratios ranging, respectively, from 1.12 (non-bloat) to 40.80 (high-bloat) and 0.46 (non-bloat) to 19.67 (high-bloat). A fifteenfold difference marked the chloroplastic protein/leaf phospholipid ratio, ranging from 0.61 (non-bloat) to 8.94 (high-bloat).

Leaf and chloroplastic calcium and magnesium

The relationships between leaf and chloroplastic calcium and magnesium and bloat are shown in Table 9. Both leaf and chloroplastic magnesium were directly correlated with bloat. The 1966 correlation coefficients were significant ($P < .01$). In both years, a significant negative correlation ($P < .01$) was found between leaf and chloroplastic calcium and bloat. The ranges in correlation coefficients for calcium and magnesium with bloat were as follows: leaf calcium, -.46 to -.70; chloroplastic calcium, -.58 to -.73; leaf magnesium, 0.27 to 0.94 and chloroplastic magnesium, 0.96 to 0.97.

In all year, the magnesium levels fluctuated more than the calcium levels. The mean values for leaf calcium and magnesium were remarkably similar for both years, ranging, respectively, from 1.43 (non-bloat) to 0.80% (high-bloat) and 0.16 (non-bloat) to 0.71% (high-bloat).

Table 9. Relationships between alfalfa leaf calcium and magnesium and bloat

Chemical component	Bloat score range				r	
	0	0 to 0.5	0.5 to 1.0	>1.0	Sheep	Cattle
			%[a]			
Leaf calcium						
1965	1.41	1.15	0.80	0.83	- .62**	- .46*
1966	1.43	1.30	1.22	0.83	- .66**	- .70**
Leaf magnesium						
1965	0.16	0.20	0.31	0.66	+0.30	0.27
1966	0.17	0.34	0.42	0.71	0.88**	0.94**
Chloroplastic calcium						
1966	0.34	0.28	0.15	0.12	- .73**	- .58*
Chloroplastic magnesium						
1966	0.03	0.04	0.18	0.43	0.96**	0.97**

[a]Percentages expressed on leaf dry matter basis.

*$P < .05$.

**$P < .01$.

The percentage ratios of total calcium and magnesium found in the chloroplasts are shown in Table 10. The distribution of calcium within the chloroplasts was inversely related to bloat severity, ranging from 23.7 (non-bloat) to 12.3% (medium-bloat). The content of chloroplastic magnesium was directly related to bloat increasing from 11.7 (low-bloat) to 60.6% (high-bloat).

Table 10. Relationship between alfalfa leaf calcium and magnesium percentages and bloat, 1966

Mineral ratio	Bloat score range			
	0	0 to 0.5	0.5 to 1.0	>1.0
		%[a]		
Chloroplastic calcium/leaf calcium	23.7	21.5	12.3	14.3
Chloroplastic magnesium/leaf magnesium	20.0	11.7	42.8	60.6

[a]Percentages expressed on leaf dry matter basis.

Chloroplastic magnesium and protein

Magnesium is an essential component in chloroplastic protein synthesis. Both plant magnesium and protein were shown to be preferentially mobilized into or synthesized within the leaf chloroplasts during bloat. Since both components are directly correlated with bloat, it was of interest to calculate the correlation coefficient between chloroplastic protein and magnesium levels. A significant ($P < .01$)

positive correlation coefficient of 0.90 was found between the two components.

Chloroplastic calcium and phospholipids

Calcium is an essential cofactor in the synthesis of several phospholipids. Both chloroplastic calcium and phospholipids are negatively correlated with bloat. Due to the interrelationships of the two plant components, the correlation coefficient was determined. A significant ($P < .01$) positive correlation of 0.95 was found between chloroplastic calcium and chloroplastic phospholipids.

Isolation and properties of Fraction I chloroplastic protein

Separation and foaming properties of Fraction I The spectral patterns of soluble chloroplastic protein extracts separated by gel filtration are shown in Figures 1 and 2. In both instances, two peaks corresponding to Fraction I and Fraction II protein were separated and identified by optical density at 280 $m\mu$. Figure 1 shows the typical separation of the soluble chloroplastic proteins from a high-bloat sample. Figure 2 shows the same separation from a non-bloat protein sample. In the high-bloat sample, both peak heights were similar. In the non-bloat sample, the Fraction II protein predominated.

The three aliquots from each protein peak were checked for optical density at 260 $m\mu$ and 280 $m\mu$. The following 280/260 $m\mu$ ratios were found: Fraction I, 1.75 to 1.85,

Figure 1. The spectral pattern at 280 $m\mu$ and effect of foaming of the soluble chloroplastic protein fractions from a high-bloat sample separated by elution through Sephadex G-50

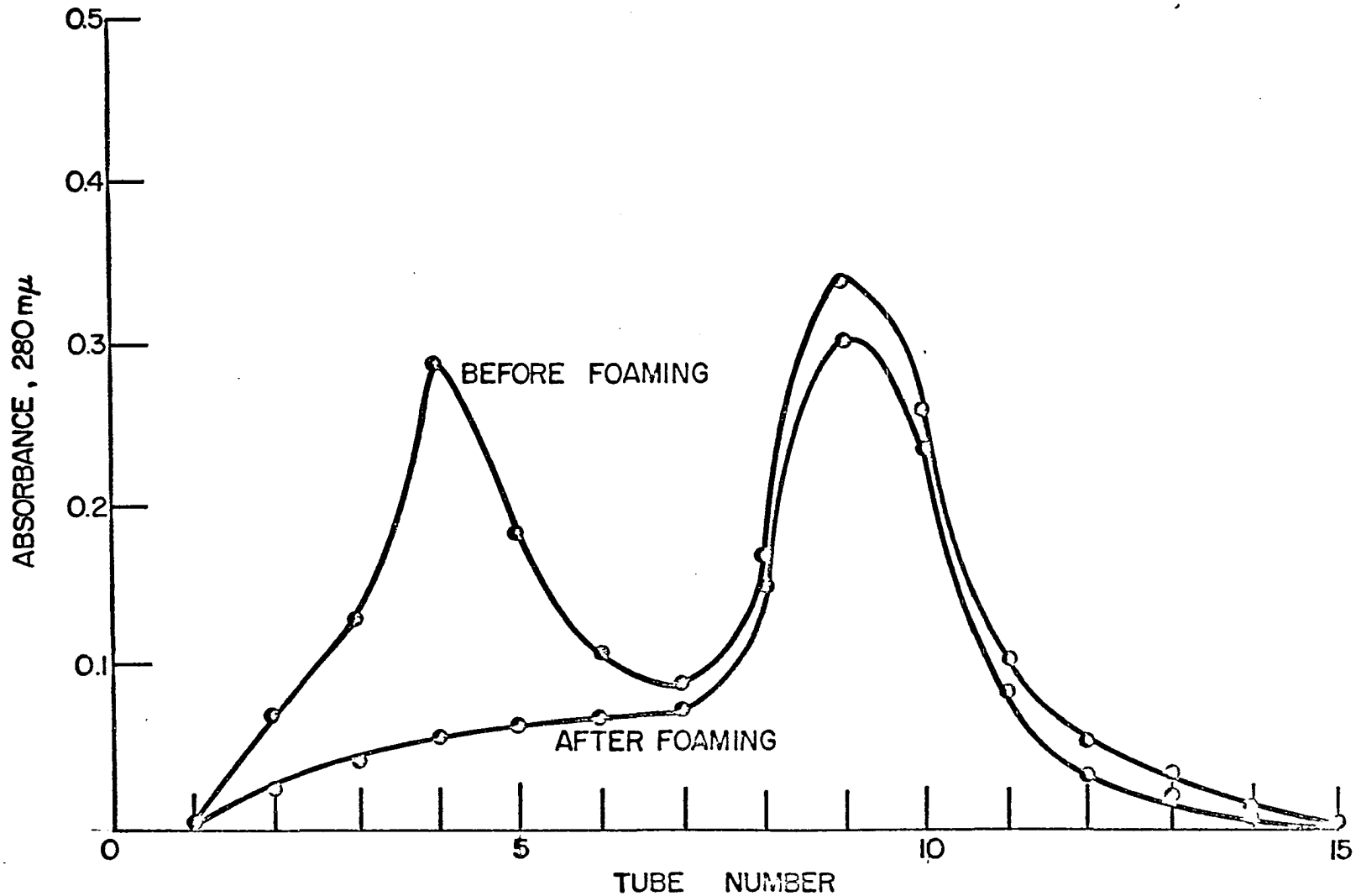
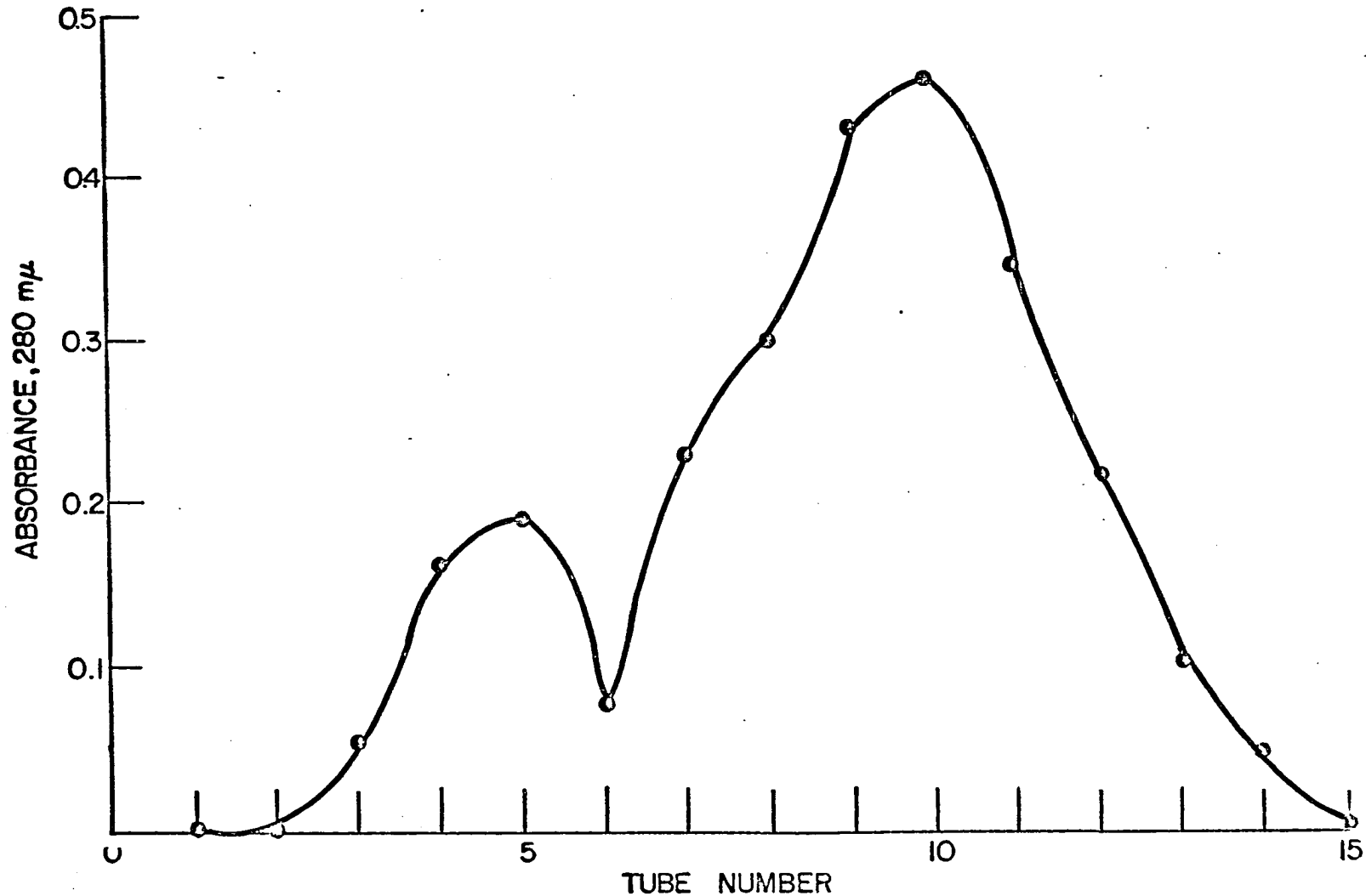

0.5
0.4
0.3
0.2
0.1
ABSORBANCE, 280 mμ
BEFORE FOAMING
AFTER FOAMING
0
5
10
15
TUBE NUMBER

Figure 2. The spectral pattern at 280 m_{μ} of the soluble chloroplastic protein fractions from a non-bloat sample separated by elution through Sephadex G-50


0.5
0.4
0.3
0.2
0.1
ABSORBANCE, 280 mμ
0
5
10
15
TUBE NUMBER

and Fraction II, 0.85 to 1.02. The effect of exhaustive foaming on the two protein fractions is shown in Figure 1. Following repeated foaming and filtering, the optical density of each aliquot was recorded at 280 m_{μ}. Foaming and filtering almost eliminated the Fraction I protein peak, while the Fraction II peak was virtually unchanged.

Spectral and ultracentrifugal analyses Spectral analysis of the aliquots comprising both Fraction I protein peaks is shown in Figure 3. Scanning from 240 m_{μ} to 340 m_{μ} showed one maximum absorption band at 280 m_{μ}.

The purity of the soluble chloroplastic protein extract and fraction I protein separated by gel filtration were checked using the Spinco Model E Analytical Ultracentrifuge. The sedimentation patterns obtained for the two protein solutions in a sodium borate buffer, pH 8.3, are shown in Figures 4 and 5.

The patterns shown in Figures 4 and 5 are drawn from those observed after 20 minutes centrifugation at 44,700 rpm using schlieren optics. The soluble chloroplastic protein revealed two peaks corresponding to the Fraction I and Fraction II peaks isolated by elution through Sephadex G-50 (Figure 4). A single peak, resembling the 18S component, sedimented from the aliquots comprising the Fraction I protein peak.

Figure 3. The absorption spectrum from aliquots of the Fraction I protein peak scanned from 240 $m\mu$ to 340 $m\mu$ with a Beckman Spectronic 505 Spectrophotometer

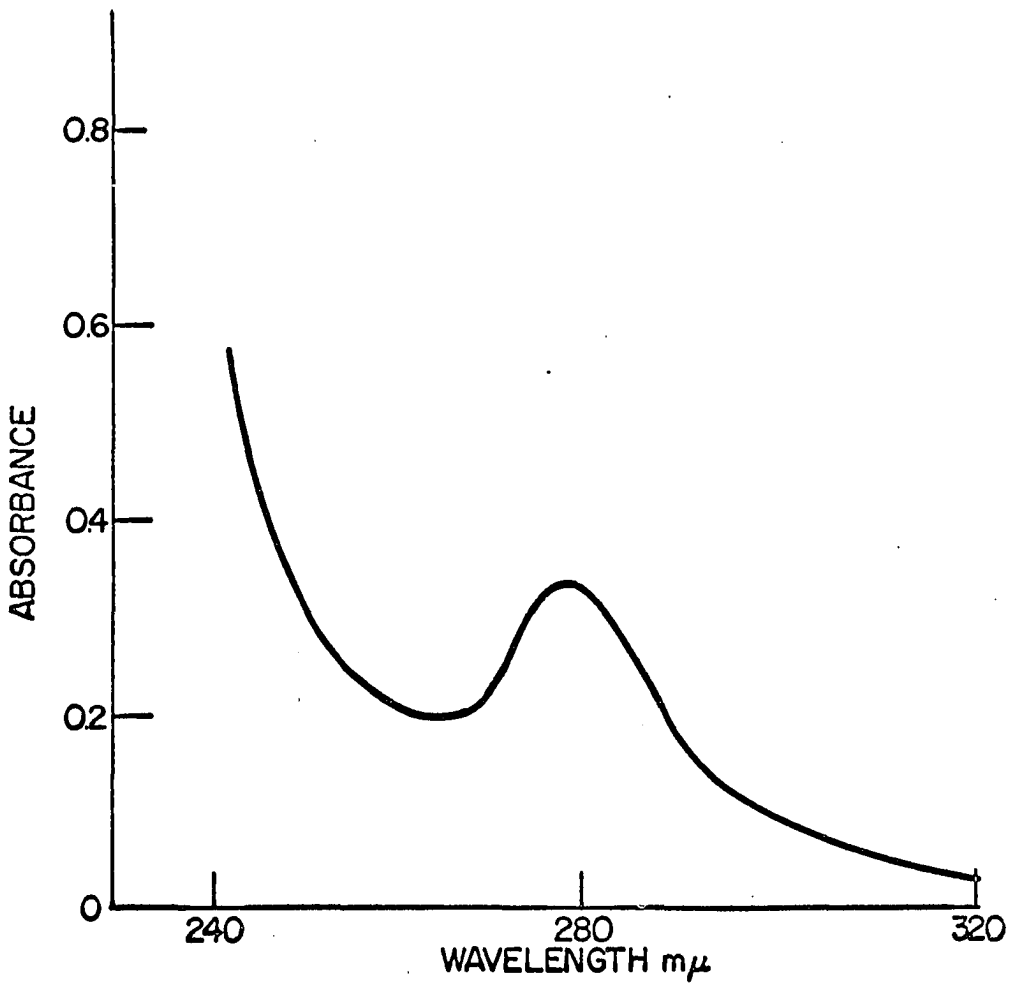
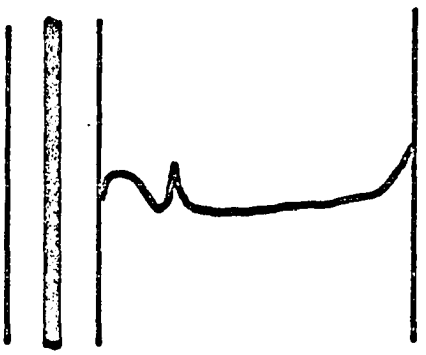
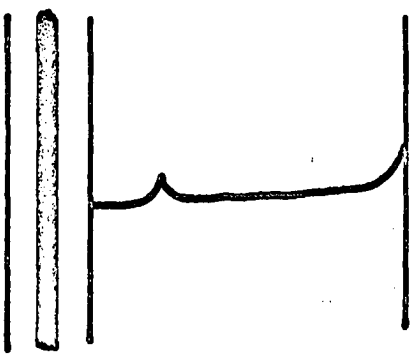

0.8
0.6
0.4
0.2
0
ABSORBANCE
240
280
320
WAVELENGTH mμ

Figure 4. Ultra-centrifugal pattern (after 20 minutes at 44,700 rpm) of a soluble chloroplastic protein extracted for 16 hours in a sodium borate buffer, pH 8.3

Figure 5. Ultra-centrifugal pattern (after 20 minutes at 44,700 rpm) of a Fraction I chloroplastic protein separated by gel filtration



SOLUBLE CHLOROPLASTIC PROTEIN, SODIUM BORATE BUFFER, pH 8.3.



FRACTION I OF SOLUBLE CHLOROPLASTIC PROTEIN, SODIUM BORATE BUFFER, pH 8.3, AFTER ELUTION THRU SEPHADEX G-50.

Amino acid analyses A comparison of the amino acid composition of total soluble and Fraction I chloroplastic protein in high-bloat versus non-bloat samples is shown in Table 11. The mean values reported represent the average of two high-bloat and two non-bloat samples is shown in Table 11. The mean values reported represent the average of two high-bloat and two non-bloat samples collected in 1965. Fifteen amino acids were identified in each sample. Two additional amino acids, methionine and cysteine, were also found, but only in trace quantities in two samples. Approximately half of the total amino acids were uniformly composed of aspartic acid, glutamic acid, glucine, leucine and alanine.

No major quantitative differences were noted in the amino acid composition of high-bloat versus non-bloat soluble chloroplastic proteins. Percentage wise, threonine and lysine were 25 and 13% higher, respectively, in the high-bloat samples. Isoleucine, arginine and histidine were 12, 17 and 33% higher, respectively, in the non-bloat samples. No other amino acids varied by more than 10%.

In the Fraction I protein, serine, aspartic acid and glutamic acid were markedly higher in the high-bloat samples, exhibiting 22, 22 and 17% increases, respectively. Threonine, proline and histidine were 29, 52 and 53% lower, respectively, in the high-bloat samples. The variation in the other amino acids was less than 15%.

Table 11. Comparison of the amino acid composition of total soluble and Fraction I chloroplastic protein in high-bloat versus non-bloat samples, 1965

Amino acid	PERCENT TOTAL AMINO ACIDS					
	Soluble chloroplastic protein			Fraction I protein		
	High-bloat	Non-bloat	% Change[a]	High-bloat	Non-bloat	% Change[a]
Aspartic acid	10.92	10.62	+ 2.9	11.85	9.70	+22.0
Threonine	4.81	3.82	+25.8	4.88	5.49	-29.0
Serine	5.29	5.70	- 7.1	6.31	5.18	+21.8
Glutamic Acid	10.53	11.05	- 4.7	12.81	10.46	+17.3
Proline	6.45	6.42	+ 0.4	4.02	8.46	-52.5
Glycine	11.27	12.31	- 8.4	10.98	10.36	+ 5.9
Alanine	9.20	8.91	+ 3.3	8.86	8.90	- 0.3
Valine	6.88	6.56	+ 4.9	6.41	7.32	-12.4
Cysteine	0.49	trace	---	2.86	1.68	---
Methionine	0.65	trace	---	trace	0.38	---
Isoleucine	4.91	5.62	-12.6	4.60	5.01	- 8.1
Leucine	8.62	8.70	- 0.8	7.93	8.15	- 2.6
Tyrosine	3.27	3.28	- 0.3	2.89	3.36	-14.0
Phenylalanine	4.77	4.53	+ 5.2	3.77	4.04	- 6.5
Lysine	6.52	5.75	+13.3	7.40	6.95	+ 6.4
Histidine	1.12	1.68	-33.4	0.87	1.88	-53.6
Arginine	4.22	5.08	-16.9	3.94	3.87	+ 1.8

[a] Percent change = $\frac{(\text{bloat} - \text{non-bloat})}{(\text{non-bloat})} \times 100.$

Extent of calcium and magnesium binding to Fraction I protein The relationship between the extent of calcium and magnesium binding to Fraction I chloroplastic protein and bloat is shown in Table 12. Calcium and magnesium binding at pH 5.5 were both significantly ($P < .01$) correlated with bloat severity, with correlation coefficients ranging from 0.76 to 0.99. In 1966, the correlation coefficients for both species were virtually one.

Table 12. Relationships between calcium and magnesium binding to Fraction I chloroplastic protein and bloat

Chemical component		Bloat score range				r	
		0	0 to 0.5	0.5 to 1.0	>1.0	Sheep	Cattle
Calcium binding to Fraction I chloroplastic protein, pH 5.5[a]	1965	0.53	0.81	1.31	1.89	0.93**	0.76**
	1966	0.34	0.86	1.94	4.35	0.99**	0.99**
Magnesium binding with Fraction I chloroplastic protein, pH 5.5[a]	1965	0.53	0.82	1.37	2.92	0.85**	0.96**
	1966	0.36	0.89	1.99	4.48	0.99**	0.99**

[a]Binding expressed as 1×10^{-5} moles of calcium or magnesium per g of Fraction I chloroplastic protein.

**$P < .01$.

Expressed as 10^{-5} moles per g of Fraction I chloroplastic protein, calcium binding ranged from 0.53 (non-bloat) to 1.89 (high-bloat) in 1965 and 0.34 (non-bloat) to 4.35

(high-bloat) in 1966. The extent of both calcium and magnesium binding was markedly higher in the high-bloat samples of 1966. The magnesium binding was very similar to that for calcium, with the exception of the greater magnesium high-bloat mean for 1965.

Apparent calcium and magnesium stability constants

The relationship between the log K_M of calcium and magnesium binding to Fraction I chloroplastic protein and bloat is shown in Table 13. The stability constants (log K_M) for calcium and magnesium were significantly correlated ($P < .01$) with bloat severity, with correlation coefficients ranging from 0.78 to 0.92. The correlation coefficients for cattle were higher than those for sheep.

Table 13. Relationship between the log K_M of calcium and magnesium binding to Fraction I chloroplastic protein and bloat, 1966

Item	Bloat score range				r	
	0	0 to 0.5	0.5 to 1.0	>1.0	Sheep	Cattle
	Log $K_M(M^{-1})$					
Calcium binding	3.22	3.63	3.96	4.34	+0.78**	+0.91**
Magnesium binding	3.24	3.64	3.97	4.35	+0.78**	+0.92**

**$P < .01$.

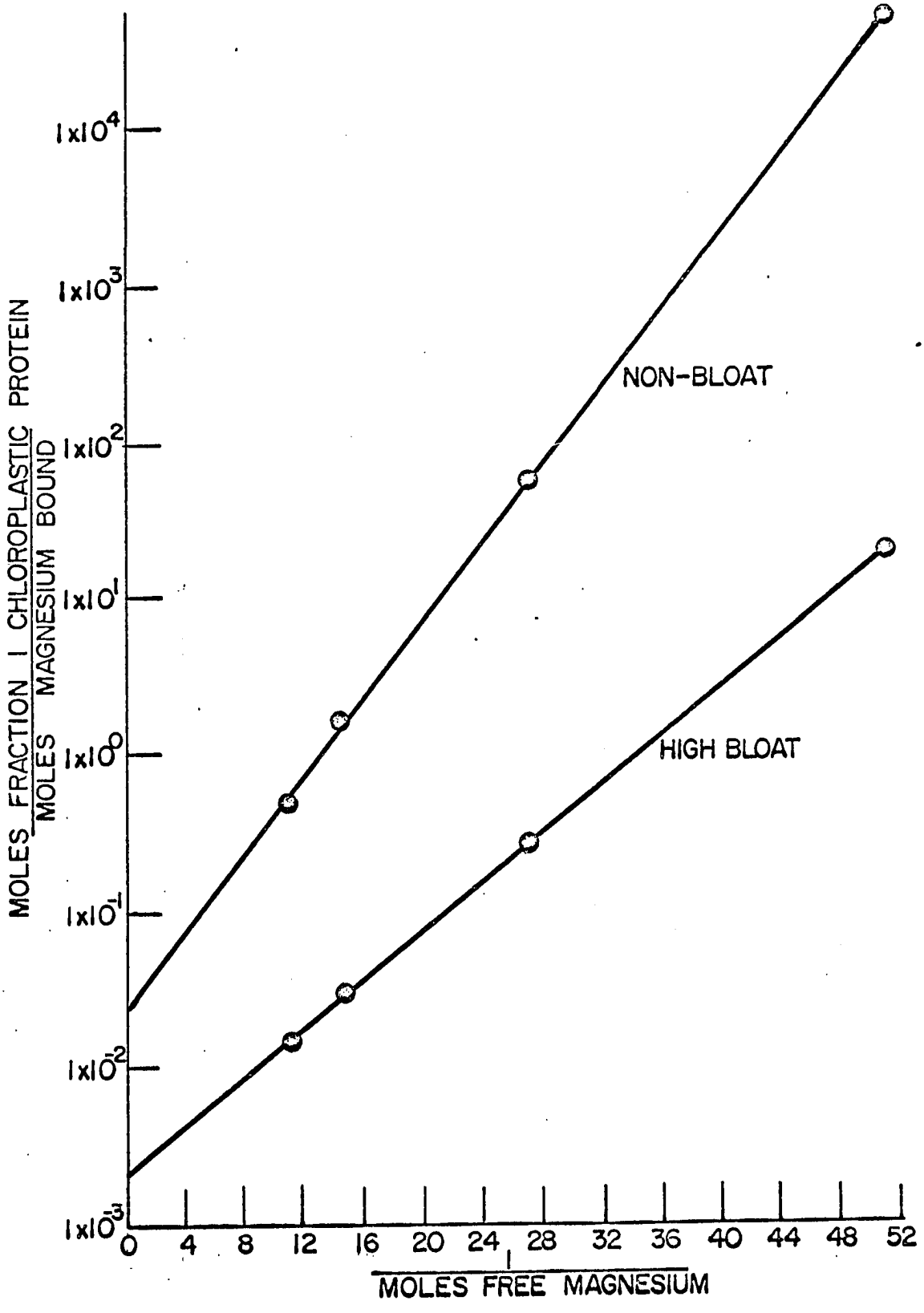
For both calcium and magnesium, the mean values for each bloat range were virtually identical. The values for the log K_M of calcium and magnesium increased uniformly with increasing bloat from 3.22 to 4.34 and 3.24 to 4.35, respectively.

Effect of salt concentration upon binding The effect of four different magnesium concentrations upon metal binding by Fraction I chloroplastic protein is shown in Figure 6. One non-bloat and one high-bloat sample were used for comparisons. As the free magnesium concentration increased from 0.02M to 0.10M so did the extent of binding with both samples.

By making three assumptions, it is possible to calculate the number of moles of aspartic acid and glutamic acid necessary for maximum binding. The three assumptions are: (1) The protein is pure Fraction I protein with a molecular weight to 5×10^5; (2) At pH 5.5, the two acidic amino acids, aspartic acid and glutomic acid, constitute the primary binding sites and (3) The Fraction I protein from non-bloat and high-bloat samples contains 10 and 12%, respectively, of both aspartic and glutamic acid, with an average molecular weight of 140.

The point where the plotted line crosses the ordinate equals 1/n, where n equals the maximum number of binding sites available to the metal on the protein moiety.

Figure 6. Comparison of the effect of four magnesium concentrations upon the extent of binding of magnesium to Fraction I chloroplastic protein from high-bloat and non-bloat samples


MOLES FRACTION I CHLOROPLASTIC PROTEIN / MOLES MAGNESIUM BOUND
1x10^4
1x10^3
1x10^2
1x10^1
1x10^0
1x10^-1
1x10^-2
1x10^-3
NON-BLOAT
HIGH BLOAT
0 4 8 12 16 20 24 28 32 36 40 44 48 52
MOLES FREE MAGNESIUM

Utilizing this information, it is found that the non-bloat and high-bloat proteins possess 56 and 446 maximum binding sites, respectively. The eightfold difference in binding sites corresponds closely with a similar difference in the actual extent of binding noted with the two proteins (Table 12).

The estimated number of moles of the two acidic amino acids varied from 714 to 857 moles of the non-bloat and high-bloat proteins, respectively. If aspartic and glutamic acids constitute the only binding sites for magnesium, then we can estimate the theoretical number of acidic amino acids required to bind each magnesium molecule. By comparison we find that two amino acids are required to bind each magnesium in the high-bloat sample. The non-bloat sample requires approximately twelve amino acids.

Effects of lecithin and fatty acid additions on calcium binding The effects of lecithin and fatty acid additions upon calcium binding to Fraction I chloroplastic protein are shown in Tables 14, 15 and 16. The lipid classes examined were dipalmitoyl phosphatidylcholine (lecithin), palmitic acid and linolenic acid. All three of these lipids are primary chloroplastic components with surface-active properties. One sample from each bloat range was studied for each lipid addition. Five lipid concentrations were prepared giving the following mole ratios of lipid to Fraction I protein: 4, 8, 16, 32 and 64.

The addition of lecithin to the dialysis medium of the non-bloat sample rapidly depressed calcium binding as seen in Table 14. At the four mole ratio, binding was depressed 13.5%. The decrease in binding was further stimulated with greater lecithin additions, until the binding was virtually eliminated at the 64 mole ratio. With the low-bloat sample, lecithin did not markedly depress calcium binding until the 16 mole ratio when a 14.9% decline was noted. The 64 mole ratio depressed binding 34.5%. With the medium-bloat protein, lecithin did not severely decrease the binding until the 32 and 64 mole ratios when depressions of 13.9 and 17.5%, respectively, were observed. Lecithin additions to the high-bloat protein failed to alter the binding appreciably at any mole ratio tested.

At all bloat levels, the addition of palmitic acid inhibited calcium binding considerably less than lecithin as seen in Table 15. In the non-bloat sample, binding was not markedly depressed until the 16 mole ratio when a 16.3% decline was noted. At the 64 mole ratio, binding was lowered 45.2%. In the remaining three bloat samples, palmitic acid exerted little influence upon the calcium binding. The slight depression in binding which did occur at the three bloat stages was progressively less as the bloat severity increased. At the 64 mole ratios, the percent decreases in binding were as follows: low-bloat, 6.2; medium-bloat, 1.4 and high-bloat, 0.3.

Table 14. Effect of lecithin upon the extent of calcium binding to Fraction I chloroplastic protein

Molar ratio of lecithin to Fraction I	Non-bloat		Low-bloat		Medium-bloat		High-bloat	
	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease
0	0.318	----	0.837	----	1.572	----	4.181	----
4	0.275	-13.5%	0.812	- 2.8%	1.552	- 1.2%	4.179	-0.04%
8	0.213	-36.1%	0.767	- 8.3%	1.517	- 3.5%	4.176	-0.12%
16	0.145	-54.5%	0.712	-14.9%	1.462	- 7.0%	4.165	-0.38%
32	0.054	-83.0%	0.620	-25.9%	1.353	-13.9%	4.132	-1.17%
64	0.012	-96.2%	0.548	-34.5%	1.296	-17.5%	4.103	-1.84%

[a]Binding expressed as 1 x 10^{-5} moles of calcium per g of Fraction I chloroplastic protein.

Table 15. Effect of palmitic acid upon the extent of calcium binding to Fraction I chloroplastic protein

Molar ratio of palmitic acid to Fraction I	Non-bloat		Low-bloat		Medium-bloat		High-bloat	
	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease
0	0.318	----	0.837	----	1.572	----	4.181	----
4	0.312	- 1.8%	0.835	-0.2%	1.573	+0.1%	4.185	+0.1%
8	0.297	- 6.5%	0.827	-1.1%	1.569	-0.2%	4.180	0.0%
16	0.266	-16.3%	0.825	-1.4%	1.564	-0.5%	4.177	-0.1%
32	0.213	-33.0%	0.815	-2.6%	1.558	-0.9%	4.178	-0.1%
64	0.174	-45.2%	0.785	-6.2%	1.550	-1.4%	4.170	-0.3%

[a]Binding expressed as 1×10^{-5} moles of calcium for g of Fraction I chloroplastic protein.

Table 16 shows that linolenic acid additions exerted considerably less influence upon calcium binding than either lecithin or palmitic acid. As with palmitic acid, the only noticeable depression occurred with the non-bloat sample at the higher mole ratio. A 64 lipid/protein mole ratio decreased binding 23.0% in the non-bloat sample. No appreciable depression was noted in binding with any of the bloat samples even at the 64 mole ratios.

Relationships Between Diurnal Changes in Alfalfa Leaf Constituents and Bloat

The significant correlations noted in 1965 between various leaf and chloroplastic constituents and bloat prompted an investigation into diurnal changes in the same plant components. Large diurnal changes were observed in most chemical constituents suggesting that a periodic alteration in the balance of certain plant factors might account for the marked daily changes observed in bloating patterns.

Five diurnal samples were collected at 3-hour intervals from 4 AM to 7 PM. The diurnal samples are classified as non-bloat, low-bloat, medium-bloat, high-bloat and highest-bloat on the basis of the bloat severity observed in cattle and sheep fed similar forage harvested at 7 AM.

Table 16. Effect of liolenic acid upon the extent of calcium binding to Fraction I chloroplastic protein

Molar ratio of linolenic acid to Fraction I	Non-bloat		Low-bloat		Medium-bloat		High-bloat	
	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease	Calcium bound[a]	Binding decrease
0	0.318	----	0.837	----	1.572	----	4.181	----
4	0.320	+ 0.6%	0.837	----	1.575	+0.2%	4.189	+0.2%
8	0.315	- 1.0%	0.838	+0.1%	1.573	+0.1%	4.185	+0.1%
16	0.294	- 7.6%	0.831	-0.7%	1.570	-0.1%	4.182	0.0%
32	0.273	-14.2%	0.823	- 1.7%	1.560	-0.8%	4.180	0.0%
64	0.245	-23.0%	0.809	-3.3%	1.548	-1.5%	4.178	-0.1%

[a]Binding expressed as 1 x 10^{-5} moles of calcium per g of Fraction I chloroplastic protein.

Percent leaves

The diurnal changes in percent leaves are shown in Figure 7. No consistent diurnal pattern was noted among the five bloat samples. However, all four bloat samples (low-, medium-, high- and highest-bloat) did show a general decreasing trend throughout the day. The percent leaves in the highest- and medium-bloat samples were consistently higher than the low- and high-bloat samples. Both pairs appeared to follow similar patterns.

The percent leaves in all four bloat samples were uniformly higher than that of the non-bloat. The percent leaves of the non-bloat samples showed the greatest variation, declining markedly from 7 AM to 1 PM and increasing in the late afternoon.

Leaf dry matter

The diurnal changes in leaf dry matter are shown in Figure 8. The dry matter content of all five diurnal samples tended to increase throughout the day, with the greatest increase usually occurring between 7 AM and 10 PM. The percent dry matter of the non- and medium-bloat samples was consistently the highest, ranging from 29 to 34 and 31 to 42%, respectively. These two samples also showed the greatest change in dry matter during the day.

With the exception of the final two values, the percent dry matter of the high- and highest-bloat samples were

Figure 7. Diurnal changes in percent alfalfa leaves (fresh-weight basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples

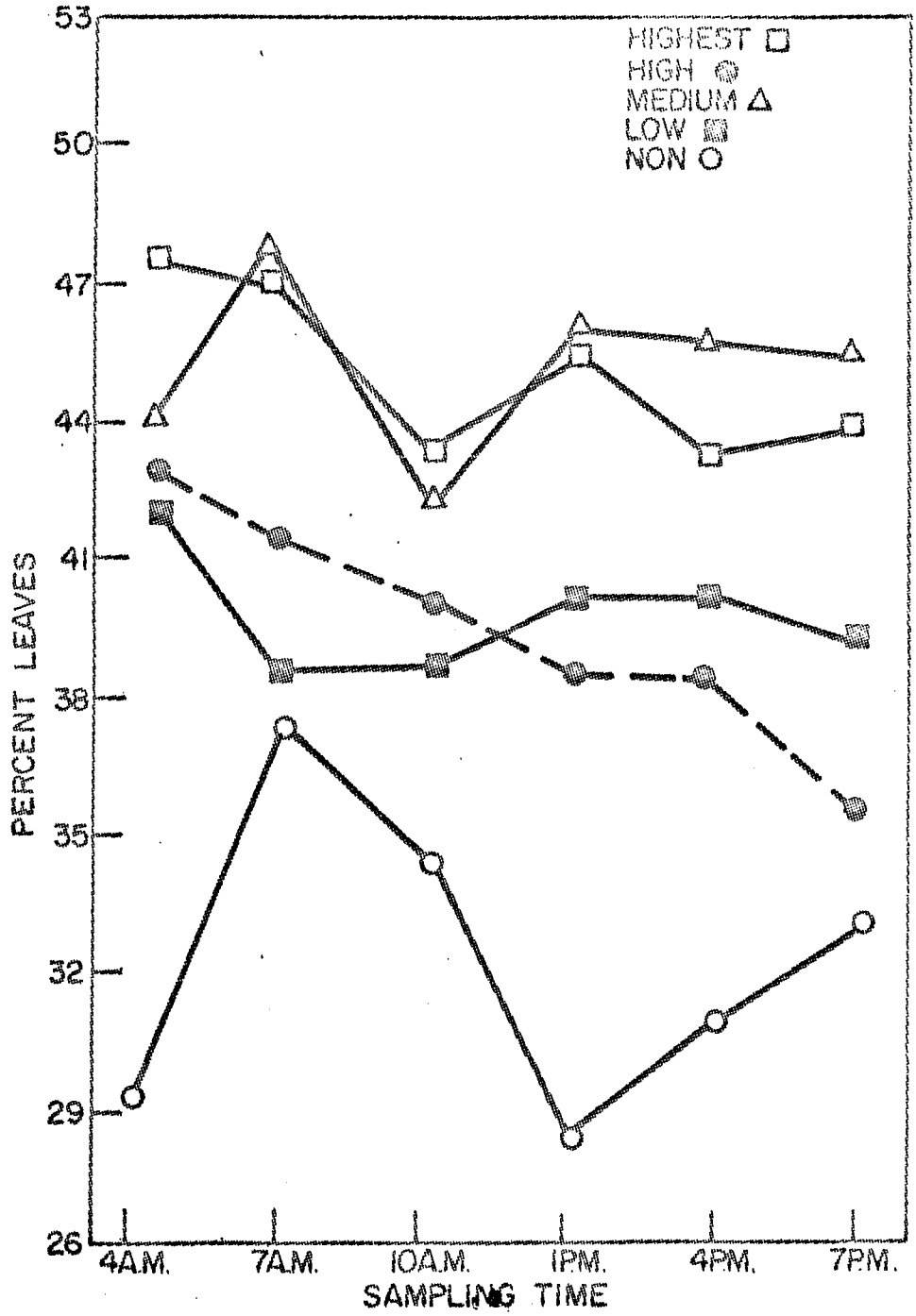
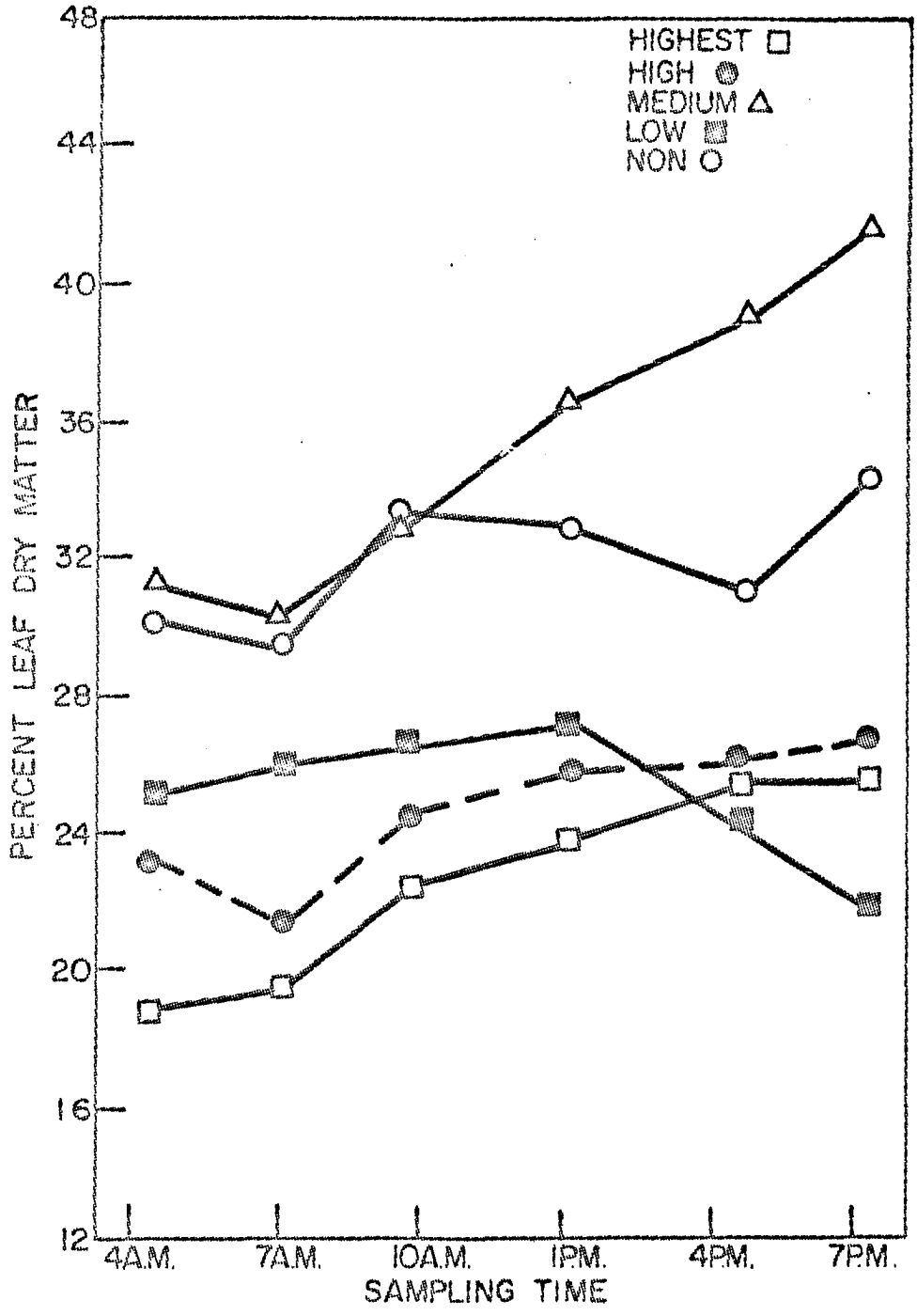

HIGHEST
HIGH
MEDIUM
LOW
NON
53
50
47
44
41
38
35
32
29
26
PERCENT LEAVES
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Figure 8. Diurnal changes in percent leaf dry matter from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
MEDIUM
LOW
NON
48
44
40
36
32
28
24
20
16
12
PERCENT LEAF DRY MATTER
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

uniformly the lowest, with ranges from 18 to 24 and 21 to 25%, respectively. At the 7 AM feeding, the dry matter content of the leaves increased progressively as bloat decreased with the exception of the medium-bloat sample.

Leaf and chloroplastic protein

The diurnal changes in total leaf and chloroplastic proteins are shown in Figures 9, 10 and 11.

The total leaf protein content of all five samples tended to decrease during the mid-day and then increase in late afternoon. The minimum protein content generally occurred about 1 PM. In all four bloat samples, the percent leaf protein increased markedly from 4 AM to 7 AM. A similar, but faster rise in protein content occurred in three bloat samples from 1 PM to 4 PM.

The leaf protein content of all four bloat samples was consistently higher than the non-bloat samples. The greatest daily change in percent leaf protein content occurred in the four bloat samples with the following ranges: highest-bloat, 26 to 37; high-bloat, 30 to 38; medium-bloat, 18 to 32 and low-bloat, 24 to 35. The non-bloat sample ranged from 15 to 19%. At the 7 AM feeding the leaf protein values were ranked in the same order as bloat severity, with the exception of the high- and highest-bloat.

Figure 10 shows the diurnal changes in percent chloroplastic protein. Like total leaf protein, the chloroplastic

Figure 9. Diurnal changes in percent leaf protein (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM sample

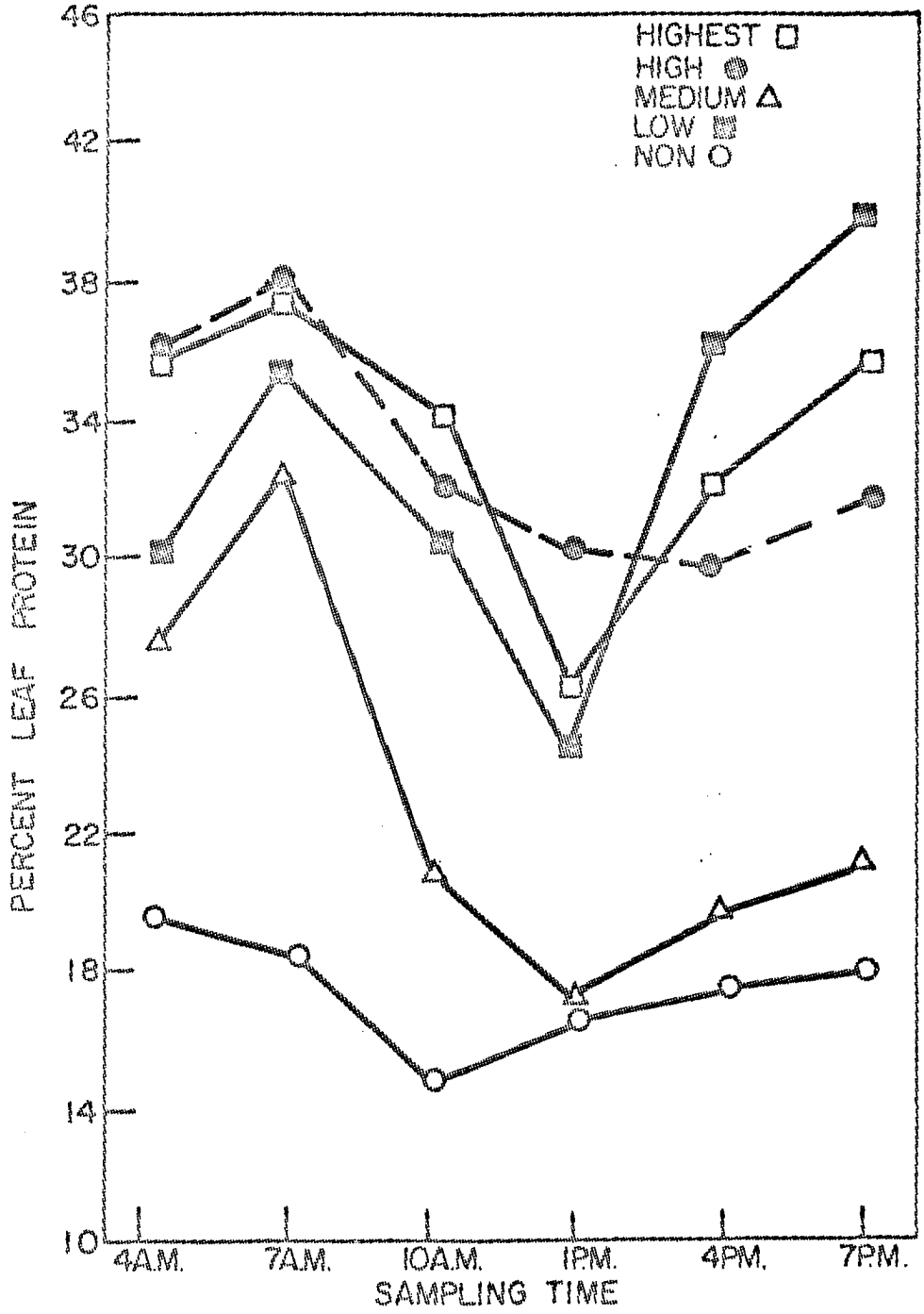

HIGHEST
HIGH
MEDIUM
LOW
NON
46
42
38
34
30
26
22
18
14
10
PERCENT LEAF PROTEIN
4A.M.
7A.M.
10A.M.
1P.M.
4PM.
7P.M.
SAMPLING TIME

Figure 10. Diurnal changes in percent chloroplastic protein (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples

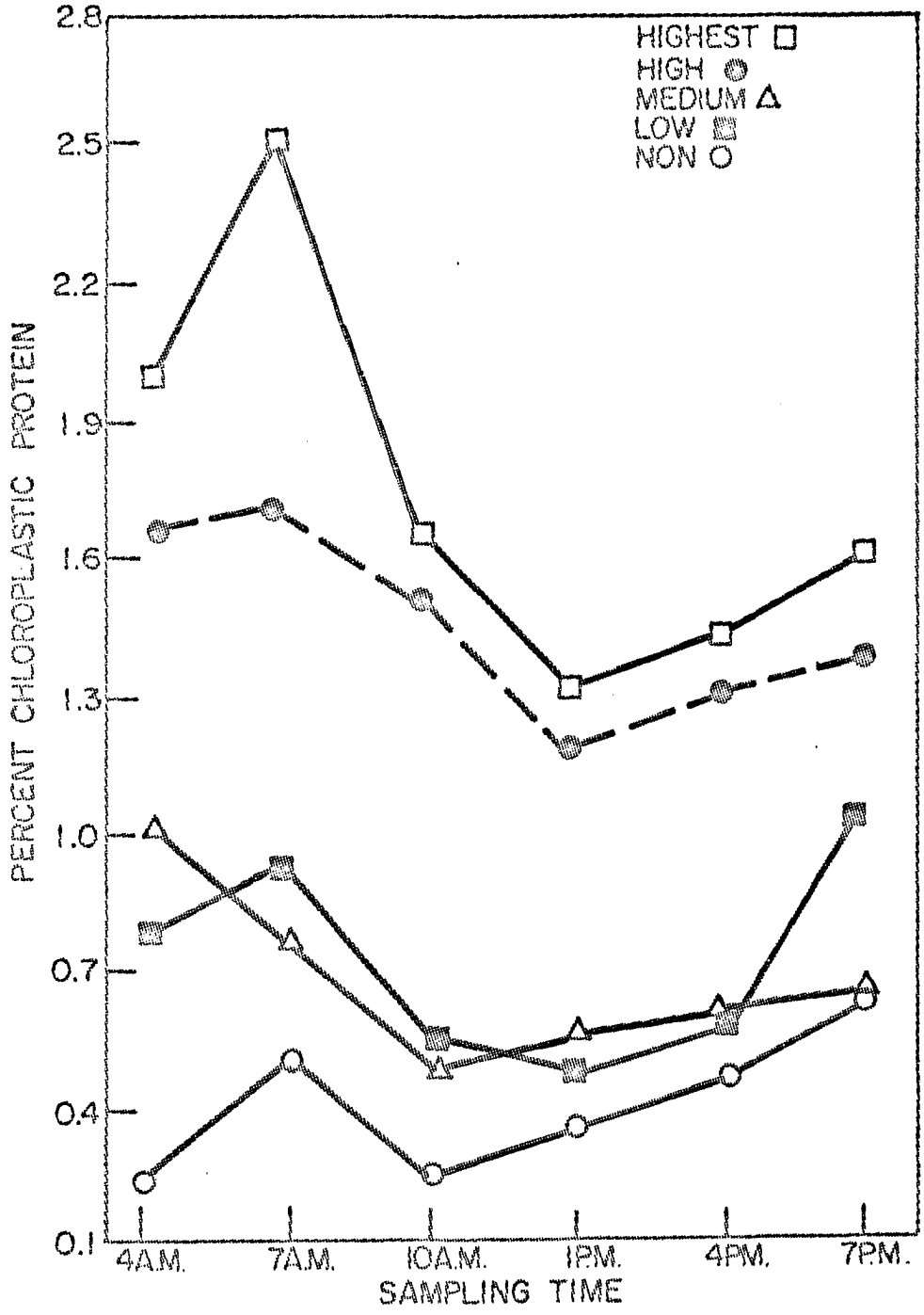
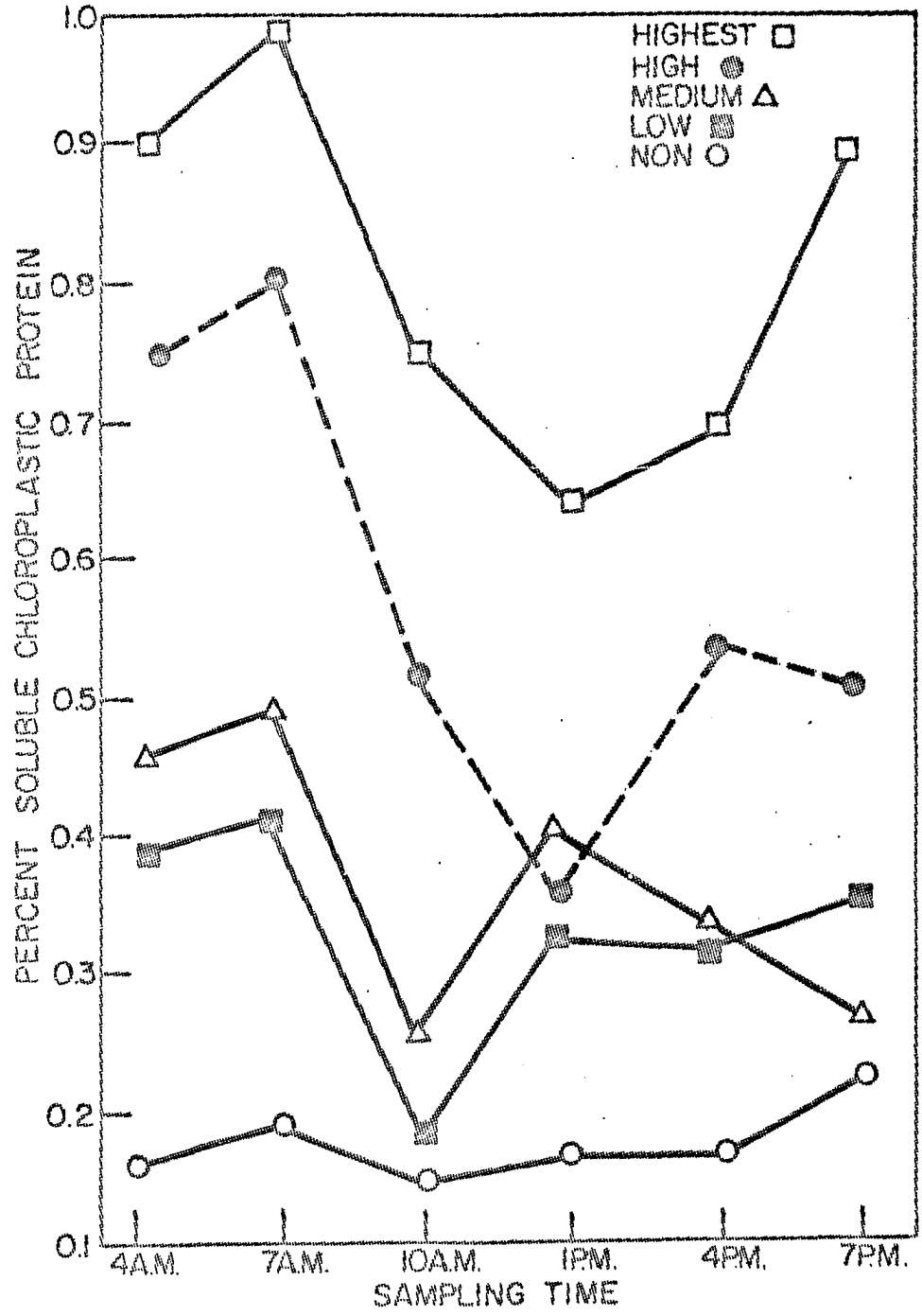

HIGHEST
HIGH
MEDIUM
LOW
NON
PERCENT CHLOROPLASTIC PROTEIN
2.8
2.5
2.2
1.9
1.6
1.3
1.0
0.7
0.4
0.1
4A.M.
7A.M.
10A.M.
1P.M.
4PM.
7P.M.
SAMPLING TIME

Figure 11. Diurnal changes in percent soluble chloroplastic protein (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
MEDIUM
LOW
NON
PERCENT SOLUBLE CHLOROPLASTIC PROTEIN
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

protein decreased during the early morning to a minimum at 10 AM to 1 PM and then increased during the afternoon. Chloroplastic protein levels were generally highest at 7 AM.

The chloroplastic protein patterns for the different bloat stages show considerably more variation in distribution than leaf protein values. The chloroplastic protein of the highest- and high-bloat samples are grouped markedly higher than the other three samples. The medium- and low-bloat samples are similarly grouped above the non-bloat sample. At the 7 AM feeding, the chloroplastic protein components are ranked in the same order as their bloat severity with the exception of the low- and medium-bloat samples. Ranges in percent chloroplastic protein were as follows: highest-bloat, 1.3 to 2.5; high-bloat, 1.2 to 1.7; medium-bloat, 0.5 to 1.0; low-bloat, 0.5 to 1.1, and non-bloat, 0.2 to 0.6.

Diurnal changes in percent soluble chloroplastic protein are shown in Figure 11. The soluble chloroplastic protein patterns were very similar to those for total chloroplastic protein. Soluble chloroplastic proteins were highest in the early morning, decreased to a minimum from 10 AM to 1 PM and then increased in the afternoon. Ranges in percent soluble chloroplastic protein were as follows: highest-bloat, 0.65 to 1.00; high-bloat, 0.35 to 0.80; medium-bloat, 0.25 to 0.50; low-bloat, 0.20 to 0.40, and non-bloat, 0.15 to 0.20. A striking difference observed between total chloroplastic and soluble chloroplastic protein patterns was the great

range in soluble chloroplastic protein bloat samples as compared with the extremely narrow range found in the non-bloat samples.

Like total chloroplastic protein, the diurnal patterns of the five samples were arranged into three groups: the highest- and high-bloat, medium- and low-bloat and non-bloat. One exception noted in the groupings was the overlap of the high-bloat sample by the 1 PM medium-bloat sample. At the 7 AM feeding, the soluble chloroplastic protein levels were ranked in the identical order as the bloat severity.

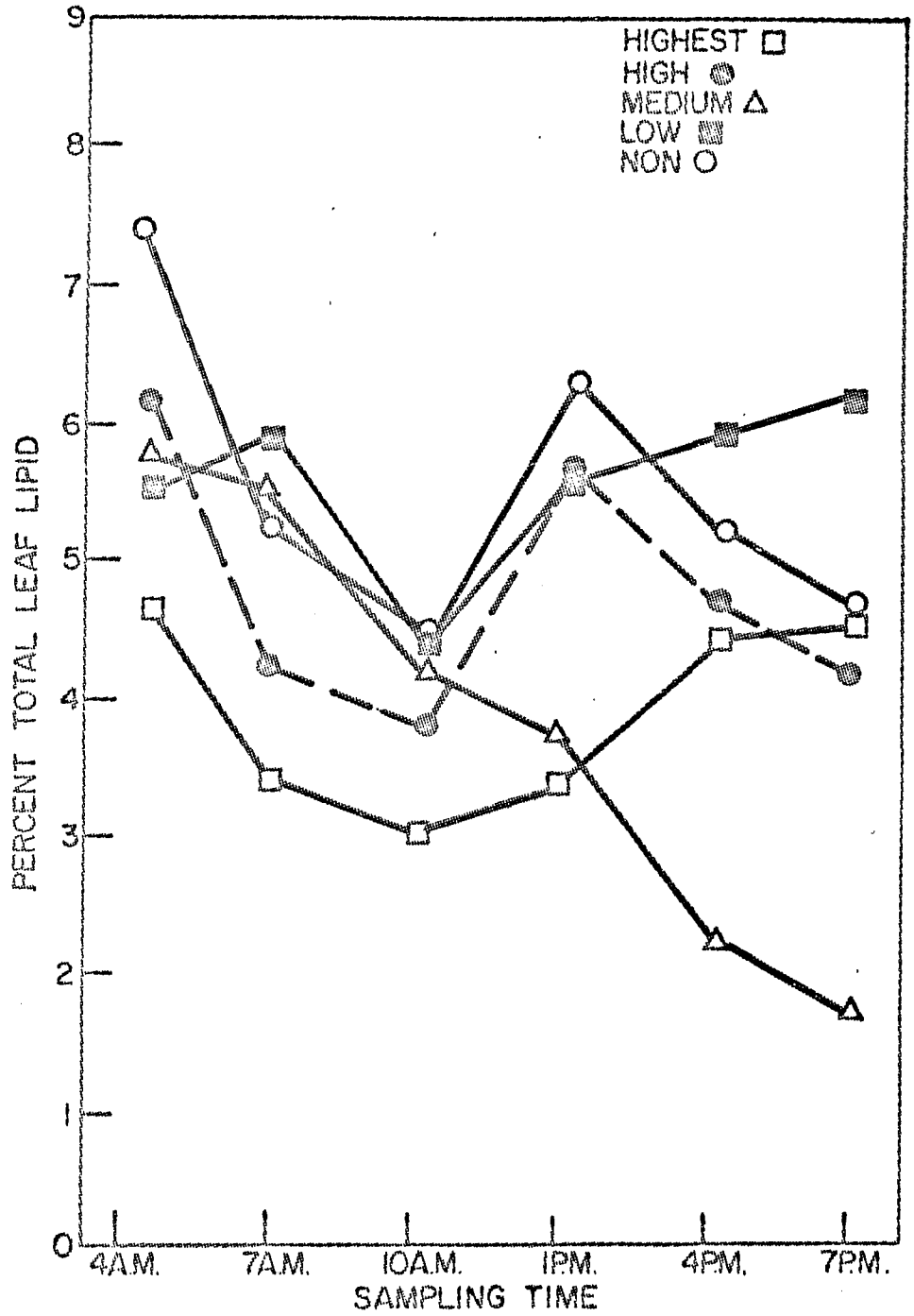
Leaf and chloroplastic lipids

The diurnal changes in leaf and chloroplastic lipid values are shown in Figures 12, 13 and 14.

Figure 12 shows the diurnal changes in percent leaf lipids. In four of the five samples, the percent leaf lipids decreased in the morning to a minimum at 10 AM and then increased abruptly in the early afternoon except for the medium-bloat which showed a steady decline throughout the day. In the non-bloat and high-bloat samples the leaf lipid values declined after 1 PM. In the low- and highest-bloat samples, the percent leaf lipids continued to increase until 7 PM.

The daily variation among individual samples was not very great. In general the non-bloat lipids were highest and the high- and highest-bloat lipids lowest throughout the day. Ranges in percent leaf lipids were as follows: highest-bloat,

Figure 12. Diurnal changes in percent leaf lipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
MEDIUM
LOW
NON
PERCENT TOTAL LEAF LIPID
0
1
2
3
4
5
6
7
8
9
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

3.0 to 4.6; high-bloat, 3.8 to 6.2; medium-bloat, 1.9 to 6.0; low-bloat, 4.2 to 6.2 and non-bloat, 4.5 to 7.4. At the 7 AM feeding the low-, medium- and non-bloat lipids were grouped together above the high- and highest-bloat samples.

The diurnal changes in total chloroplastic lipids are seen in Figure 13. In the non-, low- and medium-bloat samples chloroplastic lipids generally decreased throughout the day. In the high- and highest-bloat samples, chloroplastic lipids declined to a minimum at 7 AM to 10 AM.

Contrasted with total leaf lipid patterns, considerably more scatter was seen in the chloroplastic lipids of the different samples. The non-bloat chloroplastic lipids are uniformly higher than all other samples throughout the day. From 4 AM to 10 AM the chloroplastic lipid patterns follow the bloating patterns of the respective samples. Only in the late morning and afternoon do any chloroplastic lipids overlap. The ranges in percent chloroplastic lipids were as follows: highest-bloat, 1.1 to 2.4; high-bloat, 1.8 to 3.4; medium-bloat, 1.5 to 3.8; low-bloat, 2.7 to 4.3, and non-bloat, 3.6 to 5.4.

Figure 14 shows the diurnal changes in soluble chloroplastic lipids. The diurnal patterns for soluble chloroplastic lipids are very similar to those observed for total chloroplastic lipids. In all samples soluble chloroplastic lipids declined rapidly in the morning to a minimum at 10 AM.

Figure 13. Diurnal changes in percent chloroplastic lipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples

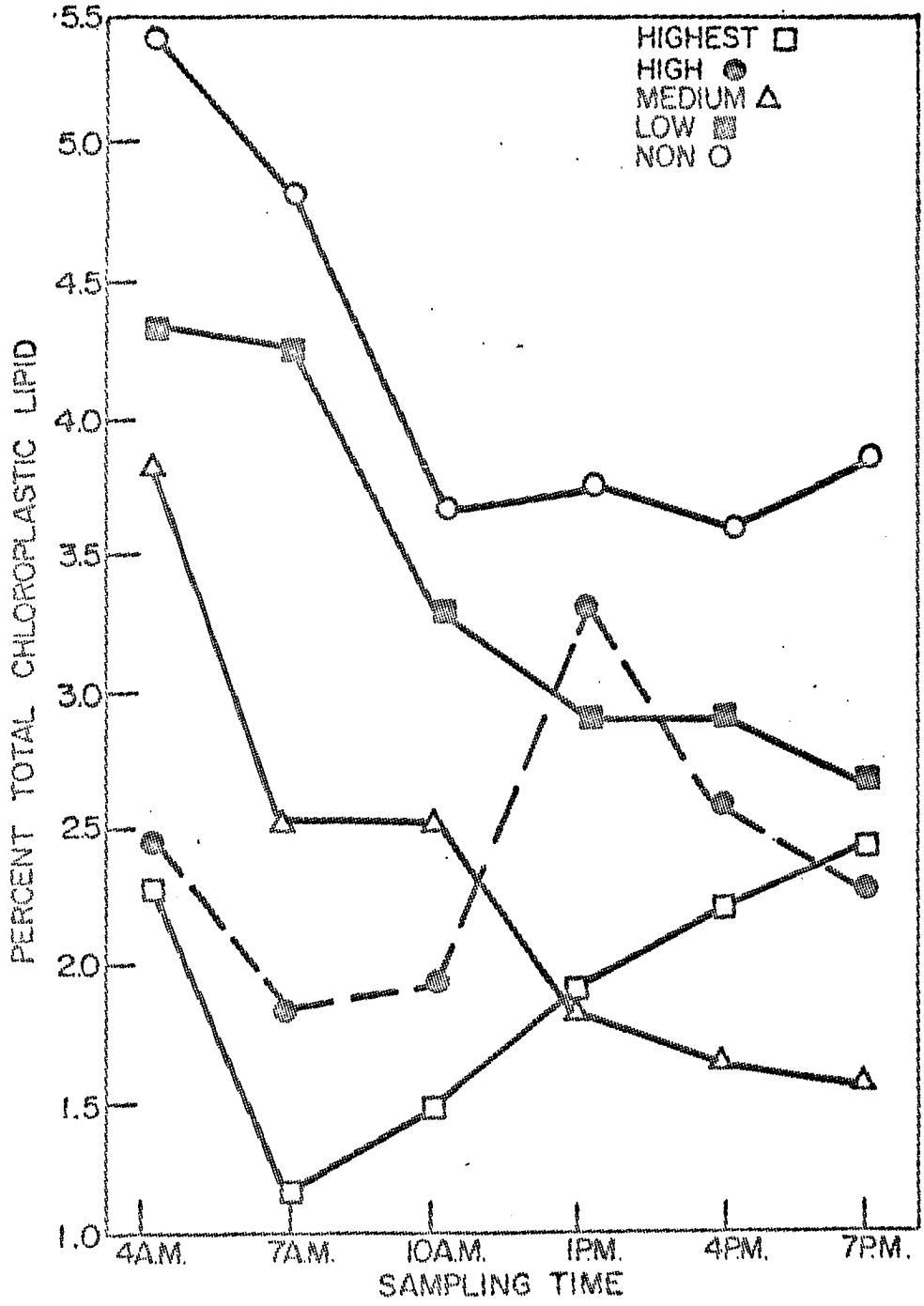
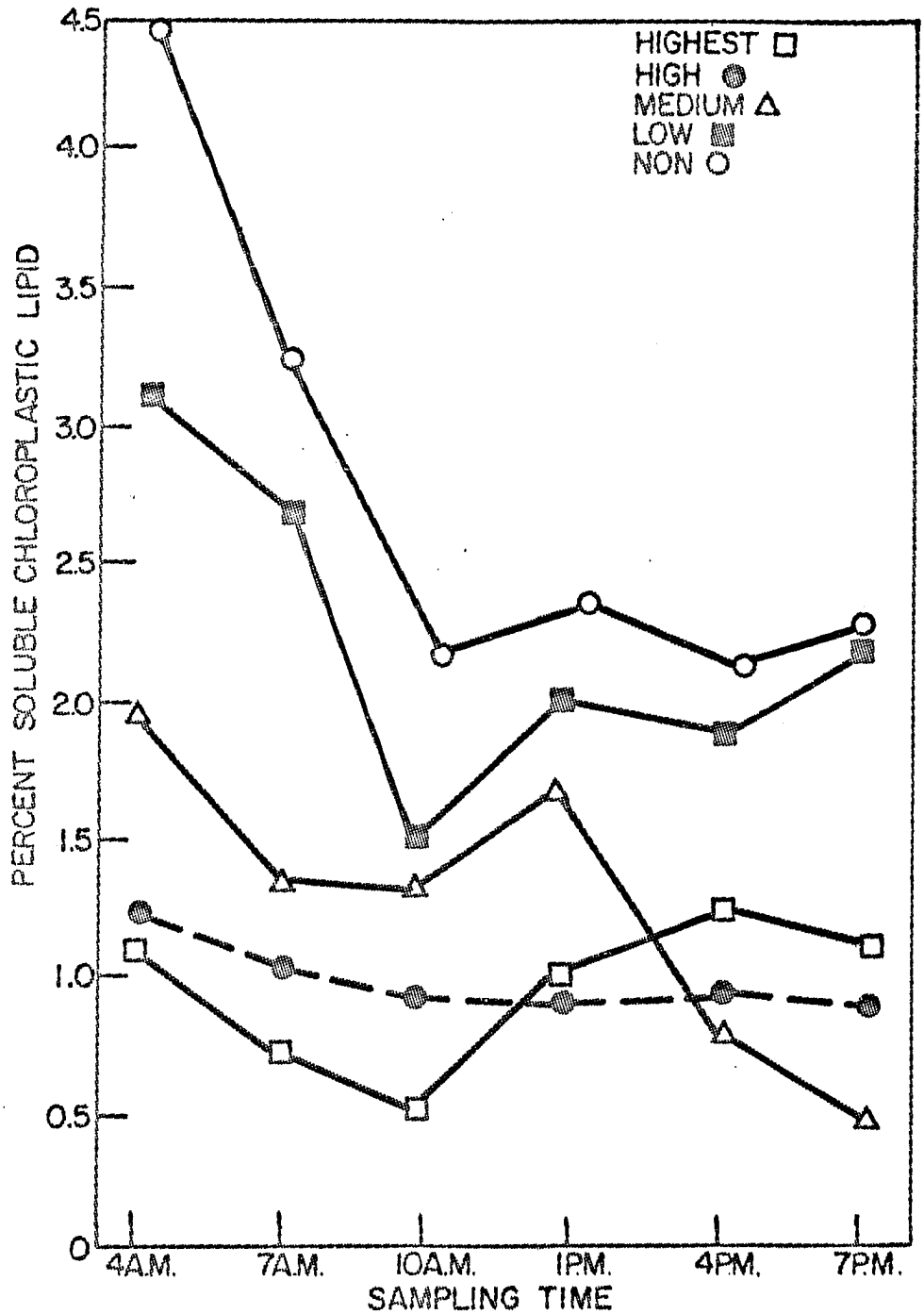

HIGHEST
HIGH
MEDIUM
LOW
NON
PERCENT TOTAL CHLOROPLASTIC LIPID
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Figure 14. Diurnal changes in percent soluble chloroplastic lipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
MEDIUM
LOW
NON
PERCENT SOLUBLE CHLOROPLASTIC LIPID
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

A gradual rise in soluble chloroplastic lipids was generally observed in the afternoon. However, in one sample, the soluble chloroplastic lipids declined markedly after a 1 PM peak.

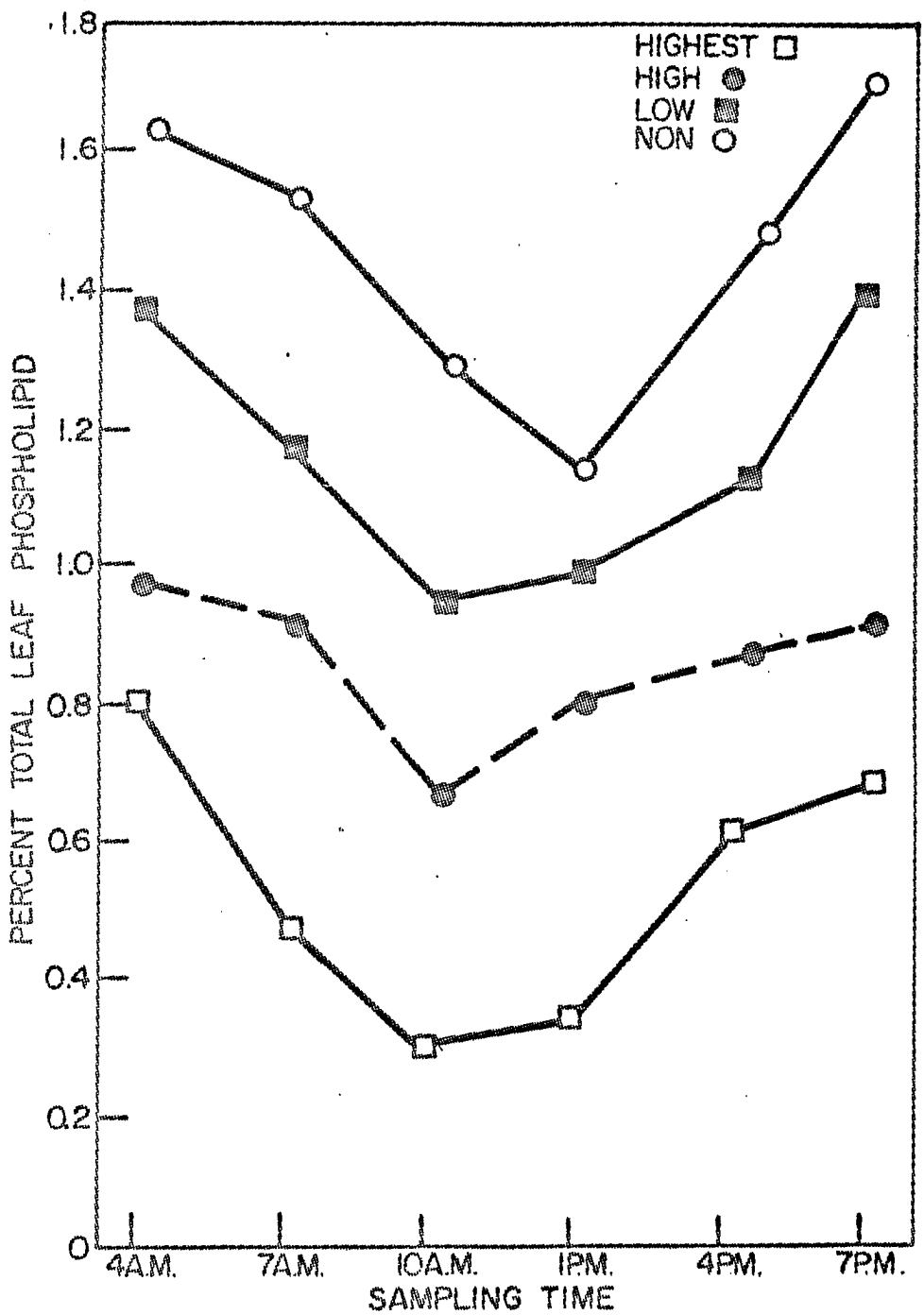
The variation in soluble chloroplastic lipids for different bloat samples was similar to that observed with total chloroplastic lipids. The non-bloat and low-bloat samples were consistently higher than the others throughout the day. The highest- and high-bloat lipids were generally lowest. From 4 AM to 10 AM, the soluble chloroplastic lipids were ranked in the same order as the bloat severity. The ranges in percent soluble chloroplastic lipids were as follows: highest-bloat, 0.5 to 1.2; high-bloat, 1.0 to 1.2; medium-bloat, 0.5 to 2.0; low-bloat, 1.6 to 3.1, and non-bloat, 2.2 to 4.5.

Leaf and chloroplastic phospholipids

The diurnal changes in leaf and chloroplastic phospholipids are shown in Figures 15 and 16. Phospholipid analyses were conducted on only four samples; none were made on the medium-bloat sample.

As seen in Figure 15, the diurnal patterns in leaf phospholipids are very similar. The leaf phospholipids decreased during the morning to a minimum at 10 AM to 1 PM and then increased progressively in the afternoon. In each sample the leaf phospholipids were virtually the same at 4 AM and 7 PM.

Figure 15. Diurnal changes in percent leaf phospholipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
LOW
NON
PERCENT TOTAL LEAF PHOSPHOLIPID
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
4A.M.
7A.M.
10A.M.
1P.M.
4PM.
7P.M.
SAMPLING TIME

The diurnal changes in leaf phospholipids followed their respective 7 AM bloating patterns and did not overlap. The ranges in percent leaf phospholipids were as follows: highest-bloat, 0.3 to 0.8; high-bloat, 0.7 to 1.0; low-bloat, 1.0 to 1.4, and non-bloat, 1.2 to 1.7.

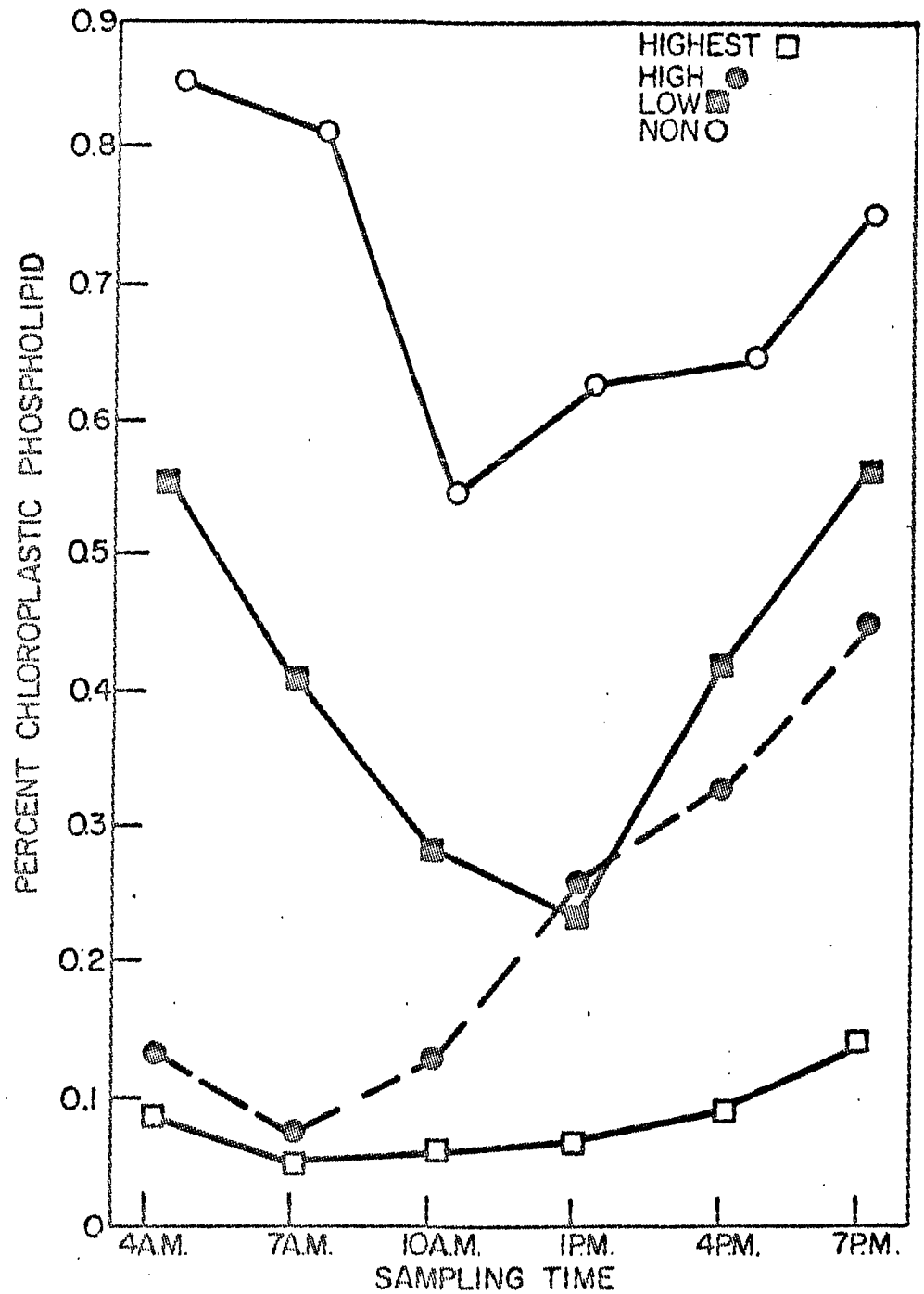
Figure 16 shows the diurnal changes in chloroplastic phospholipids. In the non- and low-bloat samples, chloroplastic phospholipids followed similar trends to their total leaf phospholipids, decreasing in the morning to approximately noon and then increasing in the afternoon. In the other two samples, chloroplastic phospholipids declined slightly at 7 AM and then increased throughout the remainder of the day. The high-bloat sample increased markedly in the afternoon while the highest-bloat sample increased only slightly.

The non-bloat chloroplastic phospholipids were consistently higher than the other samples. The highest-bloat chloroplastic phospholipids were uniformly the lowest. At the 7 AM feeding, the chloroplastic phospholipids were ranked in the same order as the bloat severities. The ranges in percent chloroplastic phospholipids were as follows: highest-bloat, 0.05 to 0.12; high-bloat, 0.08 to 0.42; low-bloat, 0.22 to 0.56, and non-bloat, 0.54 to 0.84.

Protein to lipid ratios

The diurnal changes in protein to lipid ratios are shown in Figures 17, 18 and 19.

Figure 16. Diurnal changes in percent chloroplastic phospholipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
LOW
NON
PERCENT CHLOROPLASTIC PHOSPHOLIPID
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

The ratios of percent leaf protein to percent leaf lipid for the five samples followed similar patterns (Figure 17). In general the ratio increased abruptly at 7 AM, declined somewhat (except for the high-bloat sample) until 10 AM, decreased markedly to a minimum at 1 PM and then increased during the remainder of the afternoon.

The greatest variation in diurnal changes was generally seen in the highest- and high-bloat ratios. The low-bloat ratio showed the least variation and was consistently the lowest. From 4 AM to 1 PM the leaf protein/ leaf lipid ratios followed their respective bloat classifications. The ranges in leaf protein/leaf lipid ratios were as follows: highest-bloat, 7.2 to 10.8; high-bloat, 5.2 to 8.8; medium-bloat, 4.4 to 10.5; low-bloat, 4.5 to 7.0, and non-bloat, 2.5 to 3.6.

The diurnal changes in the chloroplastic protein/ chloroplastic lipid ratios are similar to those observed for the leaf protein/leaf lipid ratios (Figure 18). However, following the increase at 7 AM, the chloroplastic protein/ chloroplastic lipid ratio generally decreased to a minimum from 10 AM to 1 PM and then gradually increased.

Little variation was observed in diurnal changes of the medium-, low- and non-bloat samples. The chloroplastic protein/chloroplastic lipid ratios of the highest- and high-bloat samples were uniformly higher and exhibited the most variation. All chloroplastic protein/lipid ratios were ranked in the same order as the bloat severities throughout

Figure 17. Diurnal changes in the ratio of total leaf protein to total leaf lipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM sample

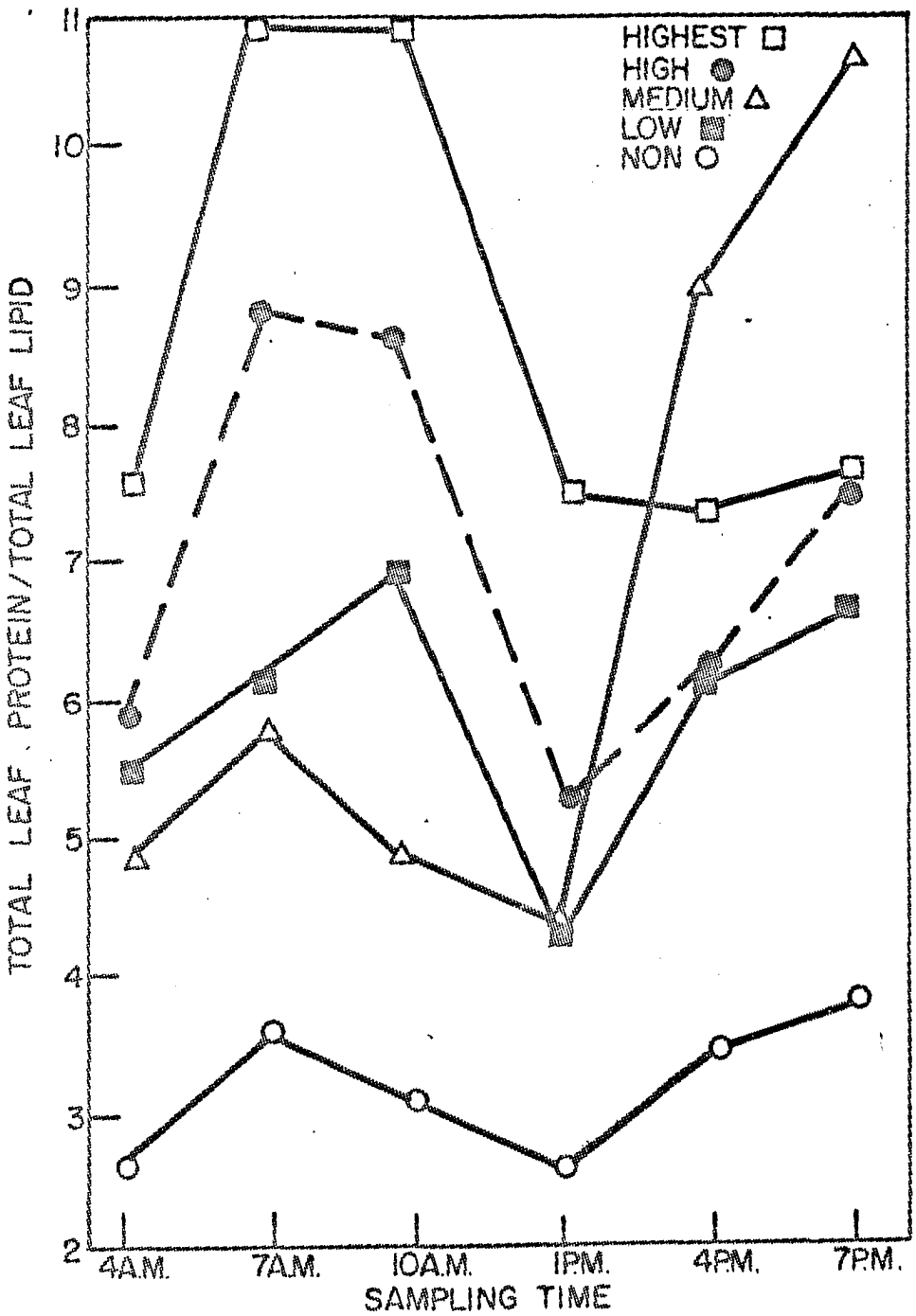
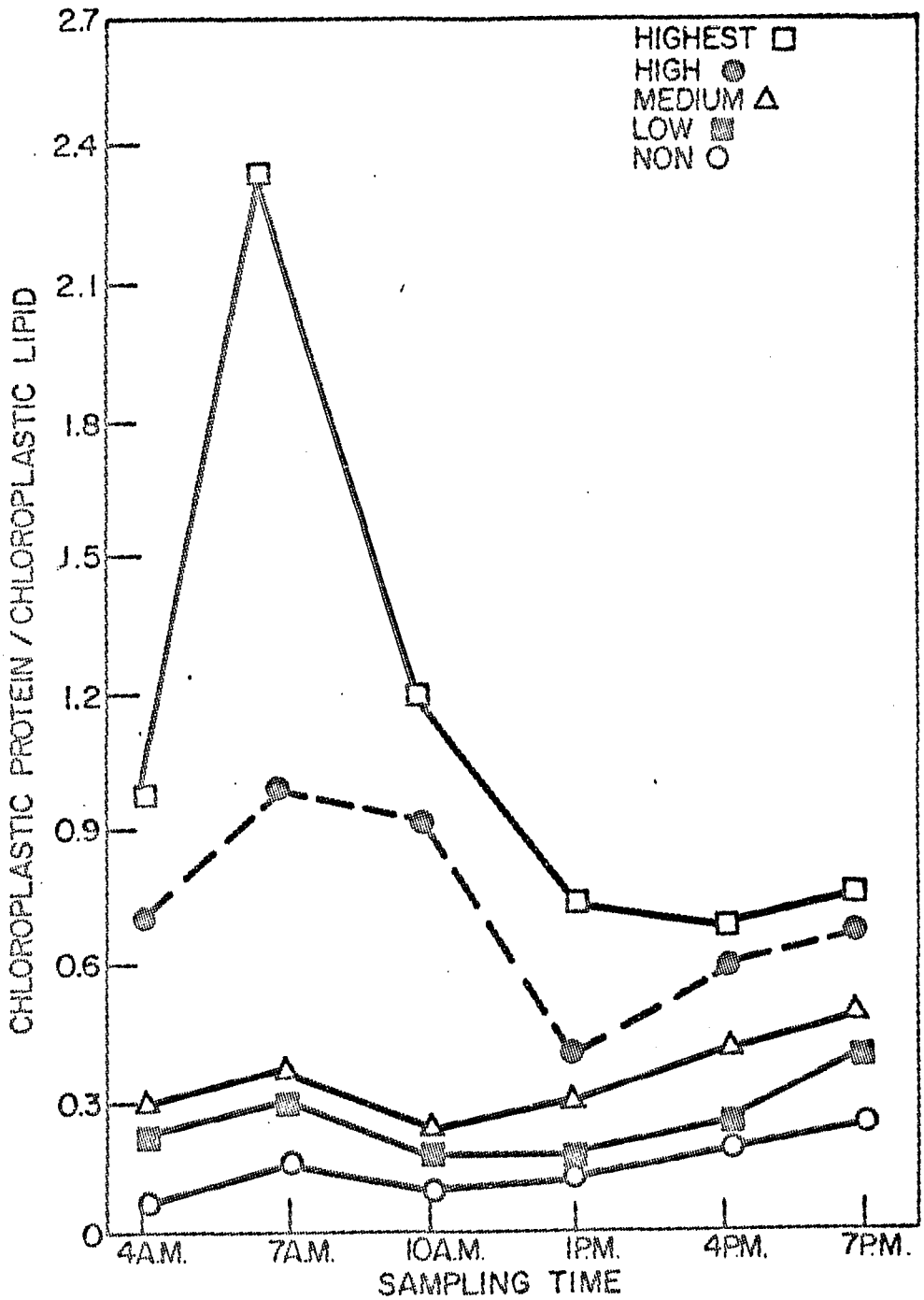

HIGHEST
HIGH
MEDIUM
LOW
NON
TOTAL LEAF PROTEIN/TOTAL LEAF LIPID
11
10
9
8
7
6
5
4
3
2
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Figure 18. Diurnal changes in the ratio of chloroplastic protein to chloroplastic lipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
MEDIUM
LOW
NON
CHLOROPLASTIC PROTEIN / CHLOROPLASTIC LIPID
2.7
2.4
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

the day. The ranges in the chloroplastic protein/chloroplastic lipid ratios were as follows: highest-bloat, 0.7 to 2.3; high-bloat, 0.4 to 1.0; medium-bloat, 0.2 to 0.5; low-bloat, 0.2 to 0.4, and non-bloat, 0.1 to 0.2.

As shown in Figure 19, the diurnal changes in soluble chloroplastic protein/soluble chloroplastic lipid ratios were very similar to the trends observed in the chloroplastic protein/chloroplastic lipid ratios (Figure 18). Following an increase at 7 AM, the soluble chloroplastic protein/soluble chloroplastic lipid ratios generally decreased to a minimum at 10 AM to 1 PM and the increased throughout the afternoon.

The variation in the soluble chloroplastic protein/soluble chloroplastic lipid ratios was greatest for the highest-bloat sample. The range in the medium- and high-bloat ratios was greater than that for the low- and non-bloat samples. The soluble chloroplastic protein/soluble chloroplastic lipid ratios were ranked in the same order as the bloat severities. The ranges in soluble chloroplastic protein/soluble chloroplastic lipid ratios were as follows: highest-bloat, 0.5 to 1.7; high-bloat, 0.4 to 0.7; medium-bloat, 0.2 to 0.5; low-bloat, 0.1 to 0.2, and non-bloat, 0.05 to 0.10.

Chloroplastic protein to phospholipid ratios

As shown in both Figures 20 and 21, the diurnal changes in total chloroplastic and soluble chloroplastic

Figure 19. Diurnal changes in the ratio of soluble chloroplastic protein to soluble chloroplastic lipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM sample

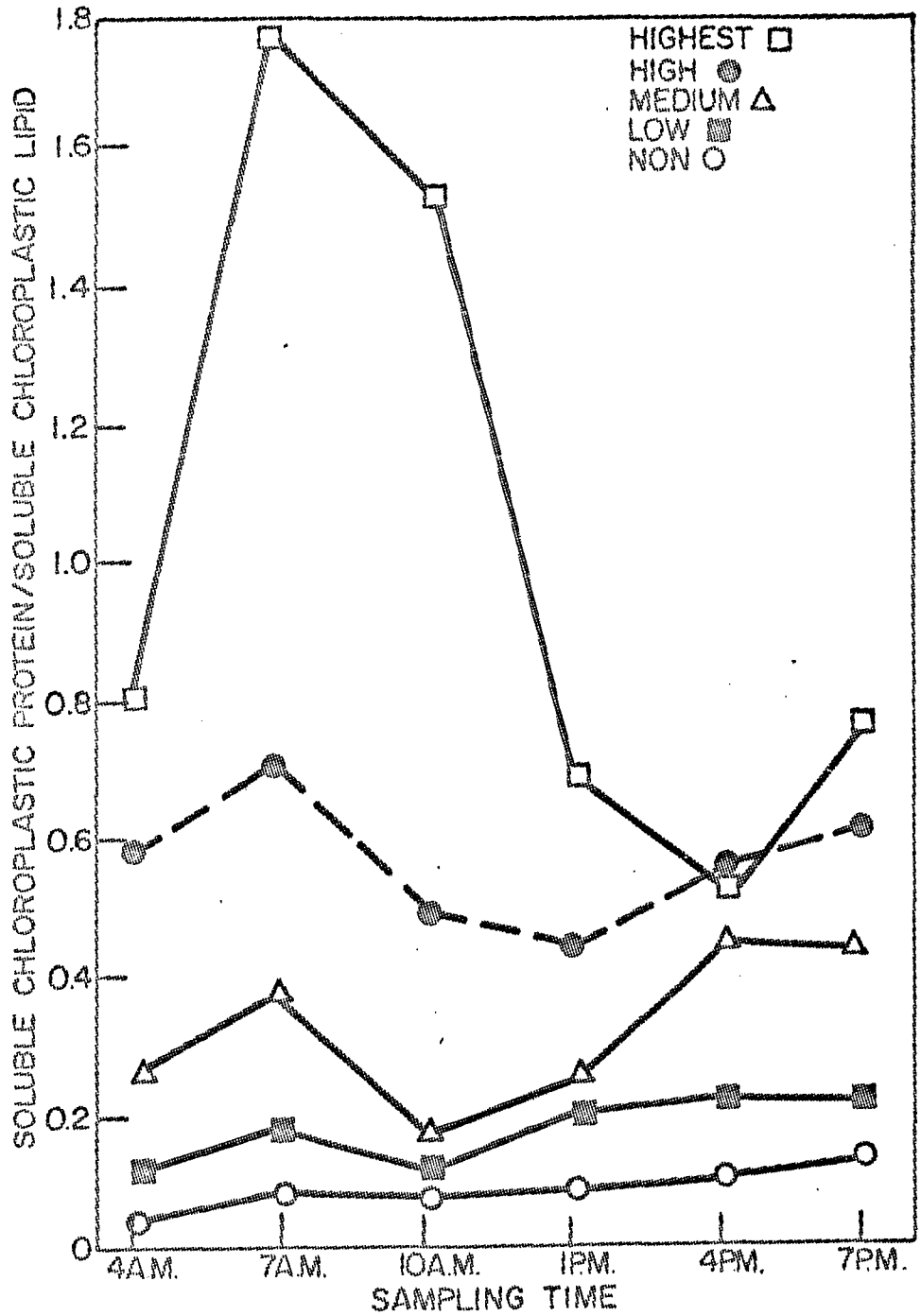

HIGHEST
HIGH
MEDIUM
LOW
NON
SOLUBLE CHLOROPLASTIC PROTEIN/SOLUBLE CHLOROPLASTIC LIPID
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Figure 20. Diurnal changes in the ratio of chloroplastic protein to chloroplastic phospholipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples

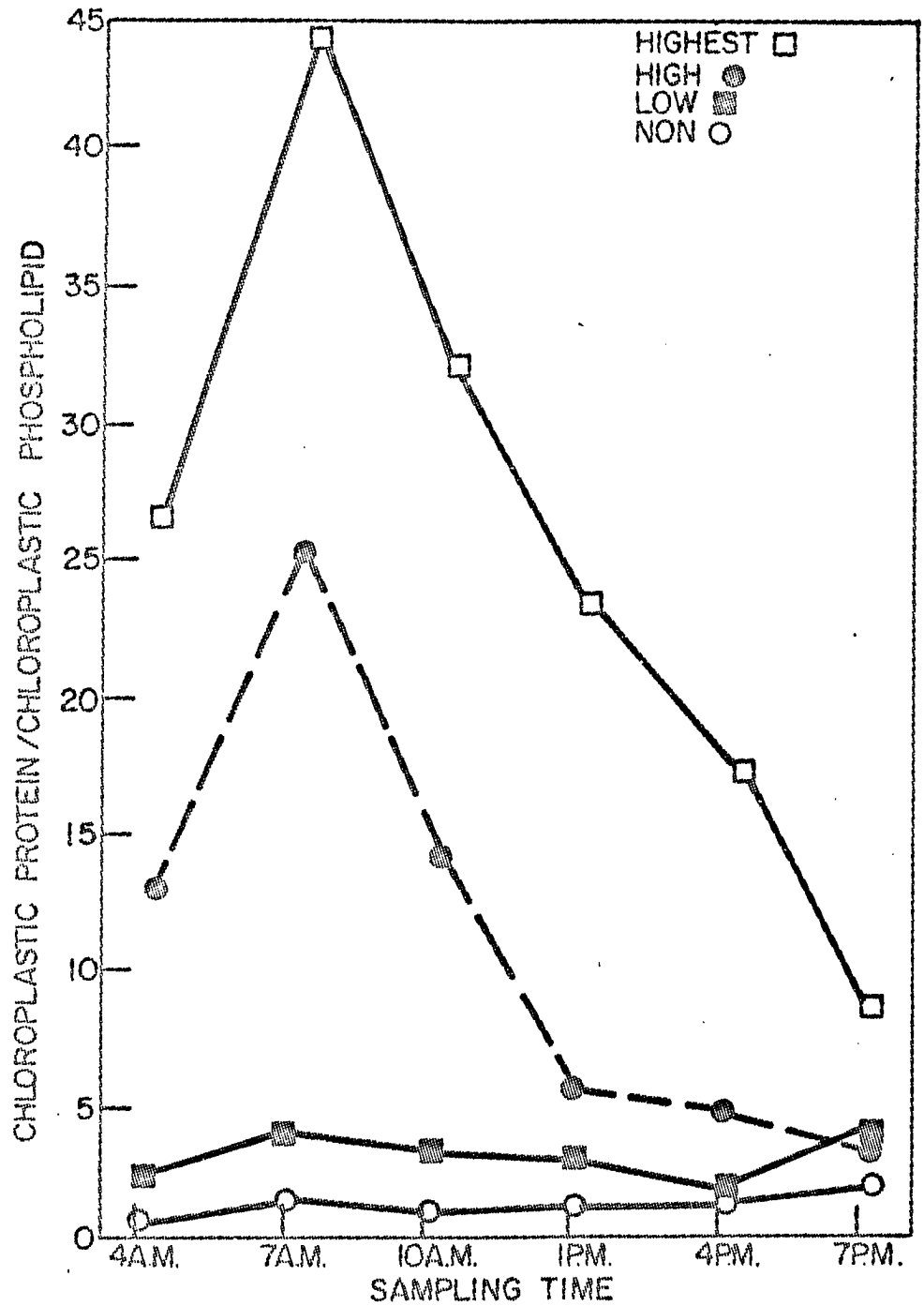
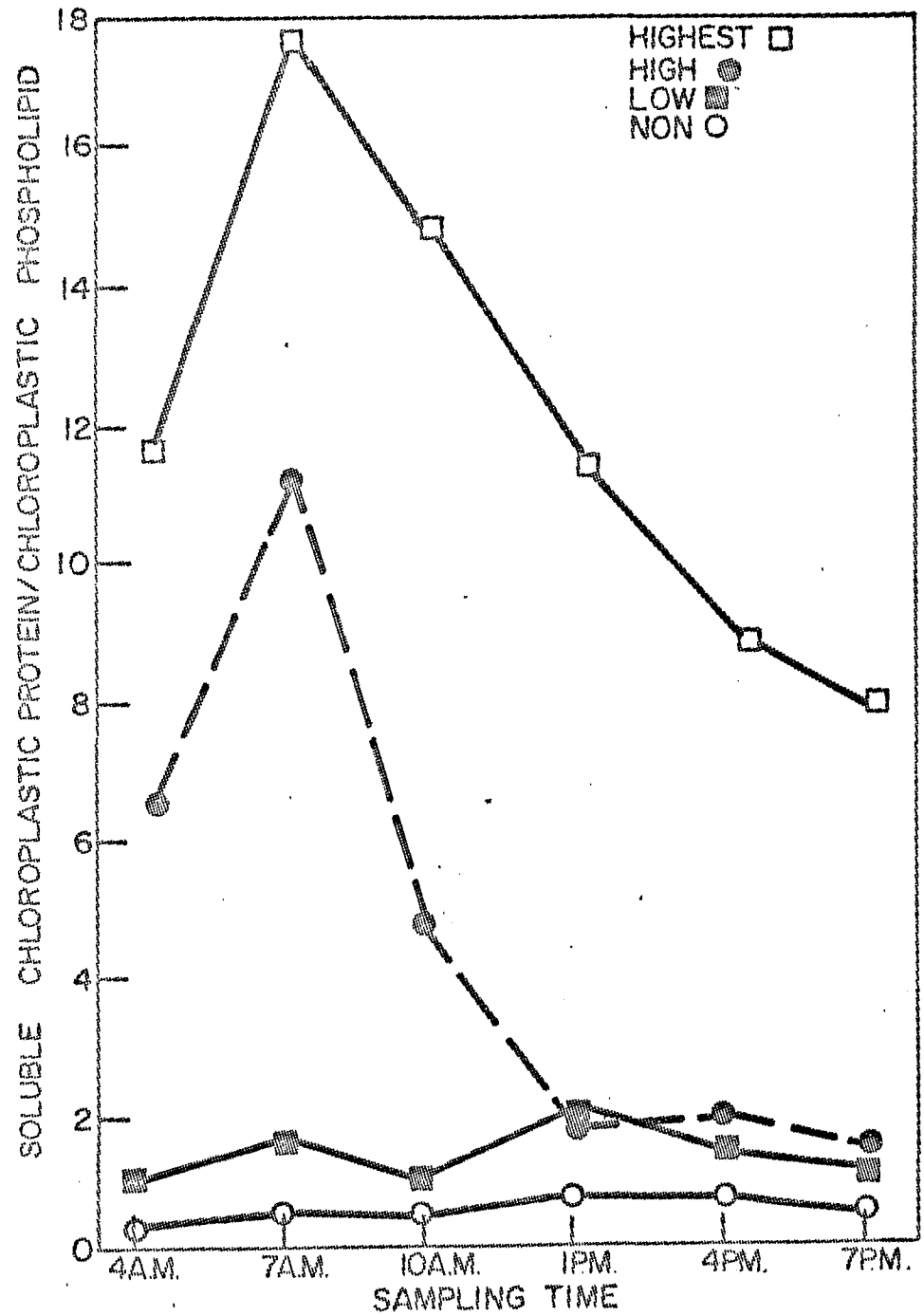

HIGHEST
HIGH
LOW
NON
CHLOROPLASTIC PROTEIN/CHLOROPLASTIC PHOSPHOLIPID
45
40
35
30
25
20
15
10
5
0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Figure 21. Diurnal changes in the ratio of soluble chloroplastic protein to chloroplastic phospholipids (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
LOW
NON O
SOLUBLE CHLOROPLASTIC PROTEIN/CHLOROPLASTIC PHOSPHOLIPID
0
2
4
6
8
10
12
14
16
18
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

protein/chloroplastic phospholipid ratios are very similar to the patterns observed for soluble chloroplastic protein/soluble chloroplastic lipid ratios (Figure 19). Following an increase at 7 AM, the chloroplastic protein/chloroplastic phospholipid and soluble chloroplastic protein/chloroplastic phospholipid ratios for the high- and highest-bloat samples declined throughout the remainder of the day.

The highest- and high-bloat ratios were consistently higher than the low- and non-bloat ratios. The non-bloat ratios were uniformly low. In general the chloroplastic protein/chloroplastic phospholipid and soluble chloroplastic protein/chloroplastic phospholipid ratios were ranked in the same order as the bloat severities throughout the day.

The diurnal variations in both the high- and highest-bloat ratios were the greatest of any of the calculated ratios. The low- and non-bloat ratios were virtually constant throughout the day. The ranges in the chloroplastic protein/chloroplastic phospholipid and soluble chloroplastic protein/chloroplastic phospholipid ratios were, respectively, as follows: highest-bloat, 7 to 44 and 8 to 17; high-bloat, 3 to 25 and 2 to 11; low-bloat, 2 to 4 and 1 to 2, and non-bloat, 0 to 1 and 0 to 1.

Leaf and chloroplastic calcium

Diurnal changes in leaf and chloroplastic calcium are presented in Figures 22 and 23. Chloroplastic calcium values

were not obtained for the medium-bloat sample.

Figure 22 shows that leaf calcium values generally decreased in the morning to a minimum at 7 AM to 10 AM and then increased during the remainder of the afternoon. However, in one sample the percent leaf calcium rose progressively all day.

The leaf calcium of the non-bloat sample was generally the highest; the highest-bloat sample was consistently the lowest. At the 7 AM sampling, the leaf calcium values followed inversely the bloat severities with the exception of the medium-bloat sample. The ranges of percent leaf calcium were as follows: highest-bloat, 0.83 to 0.96; high-bloat, 0.90 to 1.25; medium-bloat, 0.85 to 1.08; low, 1.12 to 1.40, and non-bloat, 1.05 to 1.68.

As seen in Figure 23, the diurnal changes in chloroplastic calcium are similar to those for total leaf calcium. Chloroplastic calcium declined rapidly to a minimum at 7 AM to 1 PM and then increased abruptly in the afternoon.

The highest- and high-bloat chloroplastic calcium levels were uniformly lower than the other two samples. Very little difference was seen in the overall non- and low-bloat levels. At the 7 AM feeding, the chloroplastic calcium levels were ranked in the same order as the bloat severities. The ranges in chloroplastic calcium levels were as follows: highest-bloat, 0.13 to 0.22; high-bloat, 0.13 to 0.26; low-bloat, 0.21 to 0.34, and non-bloat, 0.21 to 0.35.

Figure 22. Diurnal changes in percent leaf calcium (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples

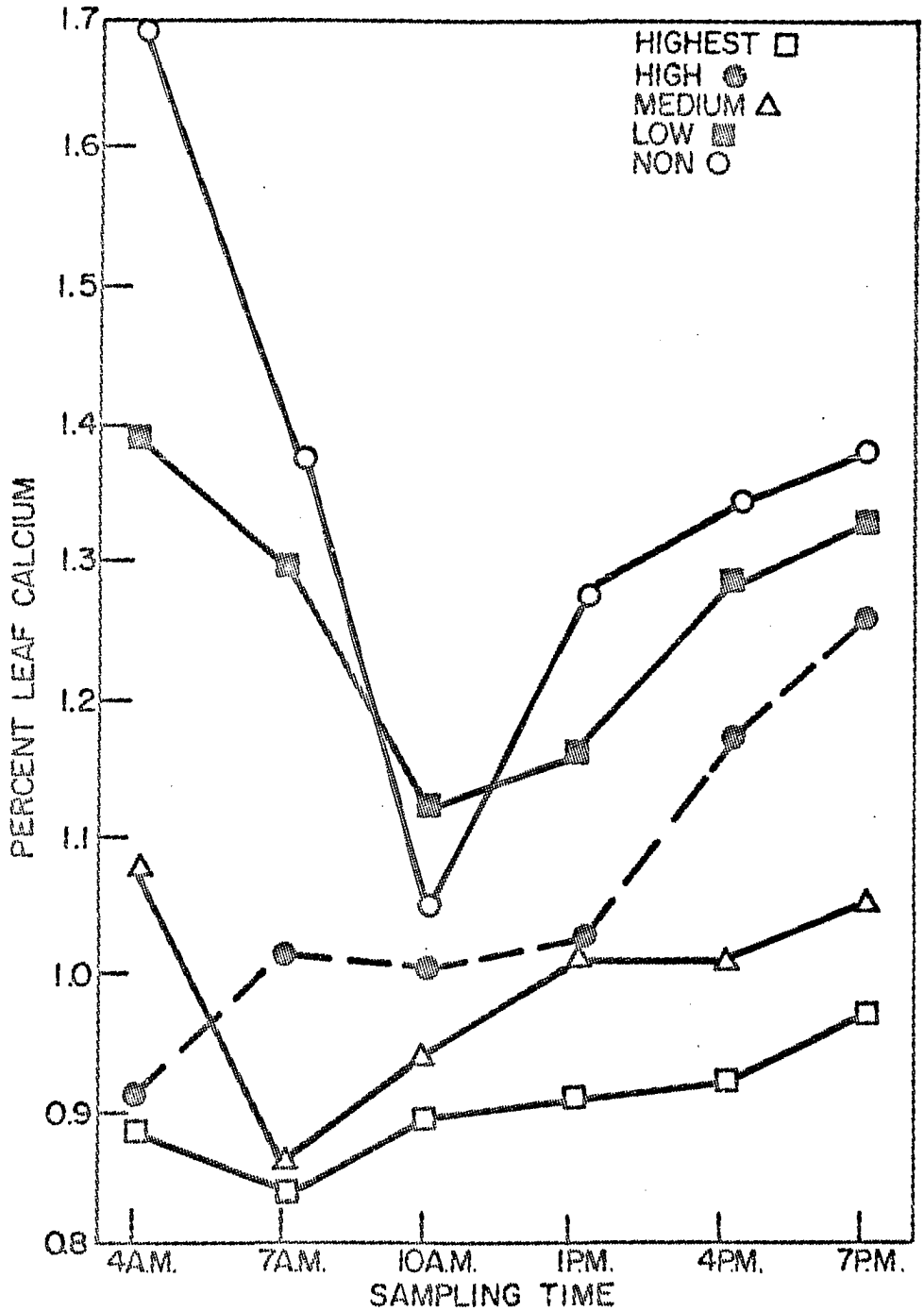
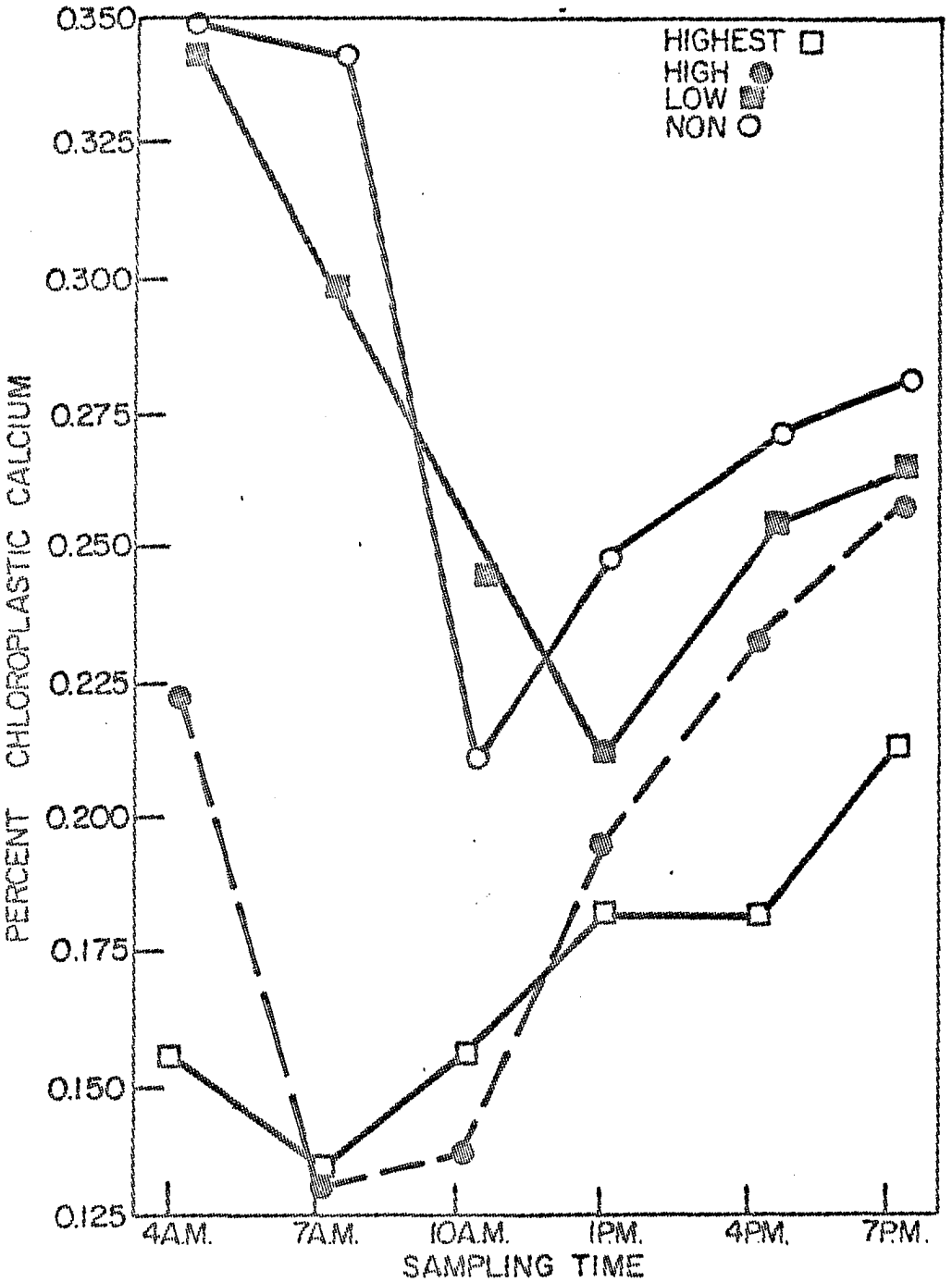

HIGHEST
HIGH
MEDIUM
LOW
NON
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
PERCENT LEAF CALCIUM
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Figure 23. Diurnal changes in percent chloroplastic calcium (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
LOW
NON
0.350
0.325
0.300
0.275
0.250
0.225
0.200
0.175
0.150
0.125
PERCENT CHLOROPLASTIC CALCIUM
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Leaf and chloroplastic magnesium

The diurnal changes in leaf and chloroplastic magnesium levels are shown in Figures 24 and 25. Chloroplastic magnesium values were not obtained for the medium-bloat sample.

As seen in Figures 24 and 25, the diurnal patterns in both leaf and chloroplastic magnesium were similar. Following a rise at 7 AM, the leaf and chloroplastic magnesium generally declined to a minimum at 10 AM to 1 PM and then increased in the late afternoon. In one sample, however, the total leaf magnesium continued to decline from 10 AM the rest of the day.

The total leaf magnesium of the four bloat samples exhibited considerably more diurnal variation and was uniformly higher than the non-bloat sample. The chloroplastic magnesium levels of the highest- and high-bloat samples were consistently greater than the low- and non-bloat samples. For both total leaf and chloroplastic values, the magnesium contents at 7 AM followed the bloat severities. The ranges for percent leaf and chloroplastic magnesium were, respectively, as follows: highest-bloat, 0.54 to 0.84 and 0.20 to 0.34; high-bloat, 0.30 to 0.70 and 0.14 to 0.24; medium-bloat, 0.30 to 0.55; low-bloat, 0.20 to 0.40 and 0.02 to 0.15 and non-bloat, 0.10 to 0.16 and 0.02 to 0.12.

Figure 24. Diurnal changes in percent leaf magnesium (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples

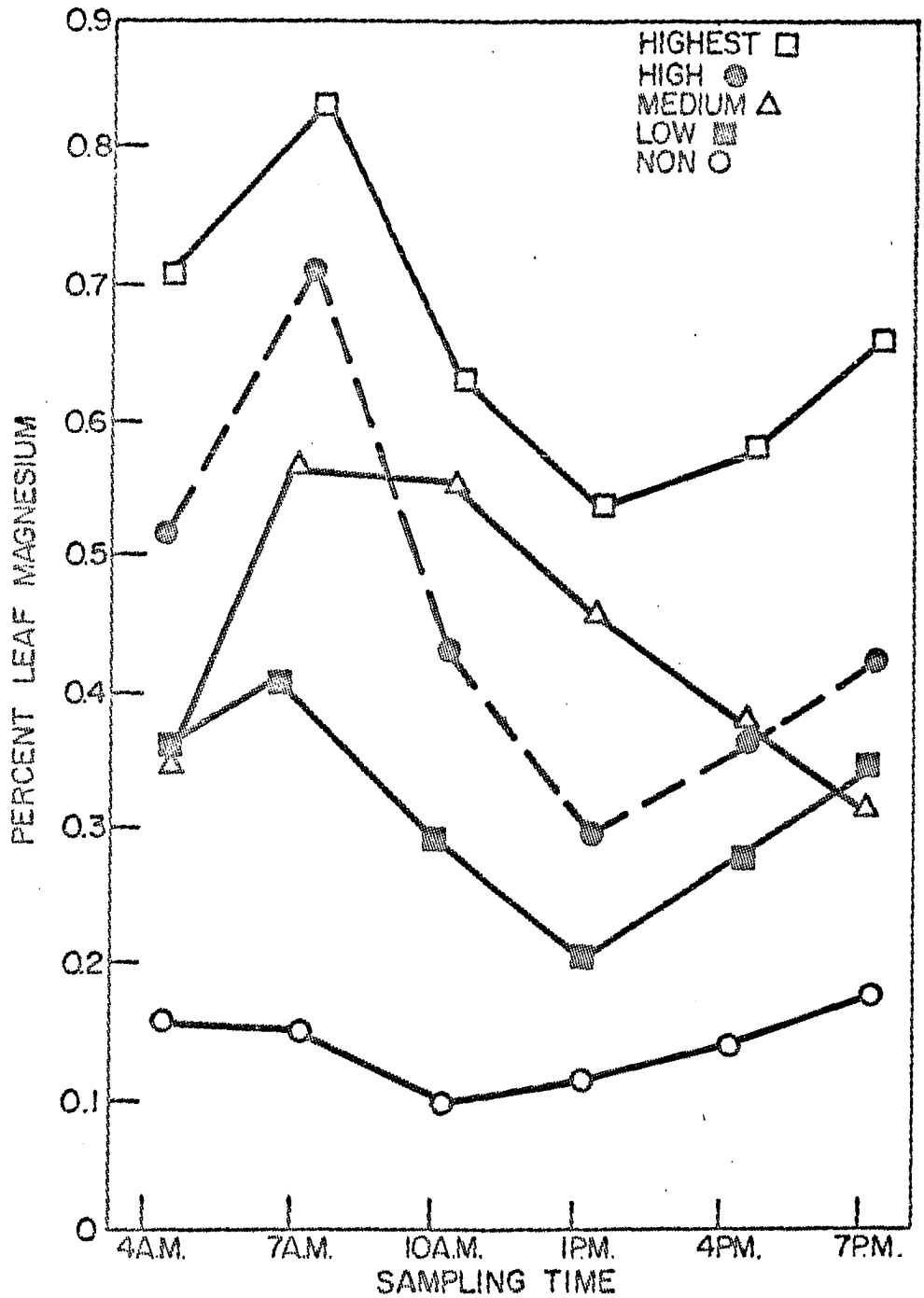
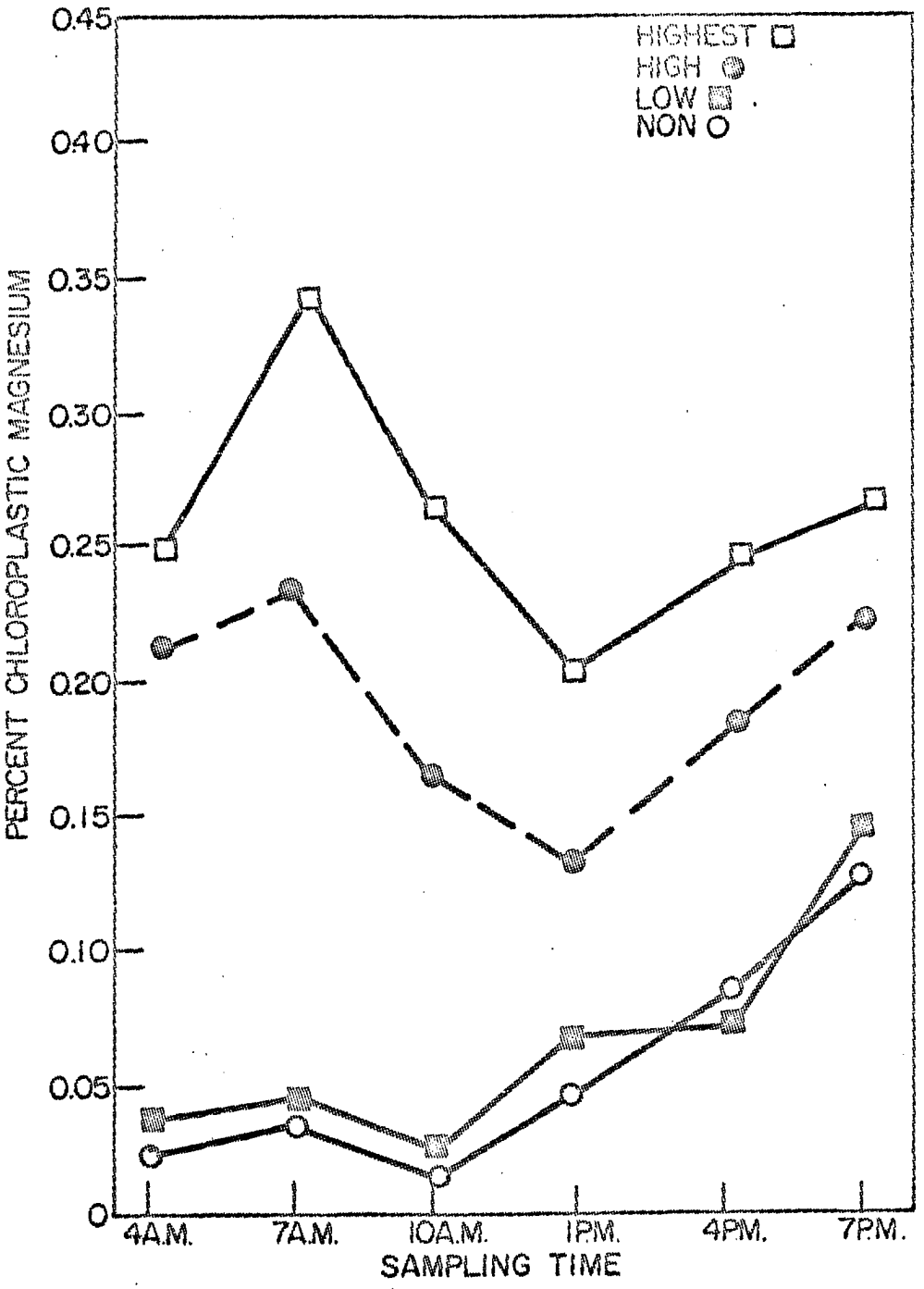

HIGHEST
HIGH
MEDIUM
LOW
NON
PERCENT LEAF MAGNESIUM
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Figure 25. Diurnal changes in percent chloroplastic magnesium (dry matter basis) from 4 AM to 7 PM based upon the bloat severity of the 7 AM samples


HIGHEST
HIGH
LOW
NON O
PERCENT CHLOROPLASTIC MAGNESIUM
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
4A.M.
7A.M.
10A.M.
1P.M.
4P.M.
7P.M.
SAMPLING TIME

Relationship Between Chloroplast Fragility and Bloat

The relationship between leaf chlorophyll and bloat is shown in Table 17. The total leaf content of both chlorophyll a and b was determined for three samples in each bloat range. Following a 2-minute homogenization in a 0.15M sucrose buffer, total chlorophyll was measured as a quantitative indication of chloroplastic fragility.

Table 17. Relationship between chlorophyll content, an indicator of chloroplastic fragility, and bloat, 1966

Component value[a]	Bloat score range			
	0	0 to 0.5	0.5 to 1.0	> 1.0
Chlorophyll a, mg/l	7.1	8.8	10.1	9.0
Chlorophyll a/total chlorophyll, %	42.0	42.9	42.6	42.2
Chlorophyll b, mg/l	9.8	11.7	13.6	12.4
Chlorophyll b/total chlorophyll, %	57.9	57.0	57.3	57.7
(1) Total leaf chlorophyll, a + b,mg/l	16.9	20.6	23.7	21.4
(2) Chlorophyll following homogenization, mg/l	5.3	7.0	9.2	7.5
(2)/(1), %	31.2	34.3	38.8	35.3
% Change against non-bloat control	---	+9.4%	+23.8%	+12.7%

[a]Values within a given bloat range represent the mean value for three samples.

As seen in Table 17, both chlorophyll a and b generally increased quantitatively with increasing bloat, ranging, respectively, from 7.1 to 10.1 mg/l and 9.8 to 13.6 mg/l. Expressed as percent total leaf chlorophyll, virtually no difference in either chlorophyll a or b concentration was observed over the different bloat ranges.

Comparisons of the chlorophyll homogenate with total leaf chlorophyll showed only slight differences in chloroplastic fragility ranging from 31.2 (non-bloat) to 38.8% (medium-bloat). The percent increases in chloroplastic fragility above the non-bloat control were as follows: low-bloat, 9.4; medium-bloat, 23.8, and high-bloat, 12.7.

Electron Microscopy

Chloroplast pellet

The nature of the nonaqueously isolated pellet is shown in Figure 26. Organelles from partially disrupted chloroplasts were apparent throughout the sections. Starch grains, osmiophilic granules, stroma and grana lamellae were present in great abundance. No other cellular bodies, such as nuclei or mitochondria, were detected in the electron micrographs. These observations demonstrate that the chloroplasts were effectively isolated using nonaqueous isolation procedures.

Non-bloat and high-bloat whole leaves

Figures 27 and 28 show typical cells from non-bloat and high-bloat whole alfalfa leaves. Leaf cells are bounded by a bimolecular membrane which encloses the cytoplasm, nucleus, vacuole, mitochondria and chloroplasts. Intercellular air spaces separate the individual cells. The only noticeable differences between non-bloat and high-bloat leaf cells are found in the chloroplasts. The non-bloat chloroplasts contain many large osmiophilic lipid granules, while no granules are apparent in high-bloat chloroplasts. The stroma appears more dense in the non-bloat chloroplasts. No consistent differences were noted in number and size of chloroplasts per cell in high-bloat versus non-bloat leaf cells.

Higher magnification micrographs of non-bloat and high-bloat chloroplasts are seen in Figures 29 and 30. Each chloroplast possesses a bimolecular membrane similar to that enclosing the cytoplasm. A marked difference in the osmiophilic lipid granules is readily apparent between the two different bloating conditions. The non-bloat chloroplasts are engorged with numerous large osmiophilic granules, but the high-bloat chloroplasts contain only a few small granules. The stroma in the non-bloat chloroplasts is more dense, while more grana are present in the high-bloat chloroplast. No apparent differences are noted in the degree of differentiation of the lamellae in the two bloat

stages nor are any consistent differences seen in the number and size of the starch grains.

Diurnal changes in chloroplast ultrastructure

Diurnal changes in the ultrastructure of non-bloat, low-bloat and high-bloat chloroplasts are shown in Figures 31 through 48. Diurnal changes in individual chloroplasts were observed at 3-hour intervals from 4 AM to 7 PM.

The most striking chloroplast differences occur in the osmiophilic granules. The non-bloat chloroplasts contain many large osmiophilic granules at each sampling time (Figures 31-36). The low-bloat chloroplasts (Figures 37-42) generally possess granules larger than the high-bloat chloroplasts (Figures 43-48) but considerably smaller and fewer than the non-bloat chloroplasts at each hour sampled.

To quantitate diurnal changes in the ultrastructure of chloroplast lipids, both the number and the size of the osmiophilic lipid granules were determined at each sampling time. Table 18 shows the relationships between the diurnal changes in the osmiophilic granules and bloat; the mean values represent from three to five chloroplasts per time period. In non- and low-bloat stages, the number of osmiophilic granules per chloroplast was greatest in the early morning and late evening. In these two samples, the least number of osmiophilic granules occurred at 10 AM and 1 PM. Very little change occurred in the number of osmiophilic

Table 18. Relationships between bloat and diurnal changes in the number and size of chloroplastic osmiophilic granules, 1966.

Bloat severity	Number and size of osmiophilic granules					
	4 AM	7 AM	10 AM	1 PM	4 PM	7 PM
Non-bloat	22[a]-large[b] (110)[c]	10[a]-large[b] (50)[c]	9[a]-medium (36)[c]	6[a]-medium large[b] (24)[c]	13[a]-medium large[b] (52)[c]	12[a]-large[b] (60)[c]
Low-bloat	7[a]-medium[b] (21)[c]	7[a]-medium large[b] (28)[c]	5[a]-medium large[b] (20)[c]	5[a]-medium[b] (15)[c]	8[a]-small[b] (16)[c]	18[a]-small[b] (36)[c]
High-bloat	8[a]-small[b] (16)[c]	9[a]-tiny[b] (9)[c]	9[a]-small[b] (18)[c]	9[a]-small[b] (18)[c]	13[a]-small[b] (26)[c]	22[a]-small[b] (44)[c]

[a]The average number of osmiophilic granules per chlorplast.

[b]The size of the granules represents the average diameter of the granules at a magnification of 38,000 using the following scale:

Large (5 points)	- > 0.31 inches
Medium large (4 points)	- 0.25 to 0.31 inches
Medium (3 points)	- 0.19 to 0.25 inches
Small (2 points)	- 0.13 to 0.19 inches
Tiny (1 point)	- < 0.13 inches

[c](Number of granules) x (Numerical score used to indicate the average diameter of granules).

granules in high-bloat chloroplasts until 4 PM and 7 PM when a marked increase was noted. The size of the osmiophilic granules in non-bloat and high-bloat chloroplasts varied little during the day while that of the low-bloat granules decreased progressively from 10 AM to 4 PM.

The product of the number of granules and the average diameter of the granules (expressed as a numerical score) was used as a crude quantitative measure of the chloroplastic lipids. Correlation coefficients between this quantitative ultrastructural measurement of chloroplastic lipids and actual chemical analyses were as follows: chloroplastic lipids, 0.78; soluble chloroplastic lipids, 0.83, and chloroplastic phospholipids, 0.82. The diurnal ultrastructural changes in the osmiophilic granules agreed closely with the diurnal changes observed in chloroplastic phospholipids. In particular, the osmiophilic granules increased markedly from 7 AM to 7 PM in the high-bloat chloroplasts. This observation parallels changes in the chloroplastic phospholipids, but not those of either total or soluble chloroplastic lipids.

Diurnal changes in the lamellar membranes in the chloroplasts from the different bloat stages were not readily apparent. Table 19 shows values for the two criteria which were used to determine crude quantitative diurnal changes in the lamellar membranes; i.e., the number of grana

Table 19. Relationship between bloat and diurnal changes in the number of grana per chloroplast and lamella per granum, 1966.

Bloat severity	Number of grana per chloroplast and lamella per granum					
	4 AM	7 AM	10 AM	1 PM	4 PM	7 PM
Non-bloat	18[a]- (4)[b]	22[a]- (6)[b]	20[a]- (3)[b]	16[a]- (5)[b]	24[a]- (4)[b]	29[a]- (6)[b]
	(72)[c]	(132)[c]	(60)[c]	(80)[c]	(106)[c]	(174)[c]
Low-bloat	29[a]- (7)[b]	30[a]- (6)[b]	22[a]- (6)[b]	19[a]- (8)[b]	43[a]- (4)[b]	29[a]- (8)[b]
	(203)[c]	(180)[c]	(132)[c]	(132)[c]	(172)[c]	(232)[c]
High-bloat	58[a]- (4)[b]	49[a]- (7)[b]	52[a]- (4)[b]	24[a]- (7)[b]	39[a]- (5)[b]	57[a]- (5)[b]
	(232)[c]	(343)[c]	(208)[c]	(168)[c]	(195)[c]	(285)[c]

[a]The number of grana per chloroplast.

[b]The average number of lamella per granum.

[c](Grana per chloroplast) x (Lamella per granum).

per chloroplast and the number of lamella per granum. Both criteria were used in an attempt to correlate ultrastructural changes in the chloroplastic lamellae with those in the chloroplastic proteins. The results shown in Table 19 are the mean values obtained from three to five chloroplasts per sampling time. In all three stages, the number of grana per chloroplast was greatest in the early morning and late afternoon. The minimum value occurred at 1 PM in each group of diurnal samples. No consistent changes were observed in the number of lamella per granum in the three samples.

The product of the number of grana per chloroplast and the number of lamella per granum was used as a crude quantitative measure of the chloroplastic proteins. Correlation coefficients between this quantitative ultrastructural measurement of chloroplastic protein and actual chemical analyses were as follows: chloroplastic protein, 0.96, and soluble chloroplastic protein, 0.98. The diurnal ultrastructural changes in the chloroplastic lamellae agreed closely with diurnal changes observed in total and soluble chloroplastic proteins. As noted for the chloroplastic protein values, the chloroplastic lamellae decreased from a peak at 4 AM and 7 AM to a minimum at 10 AM and 1 PM and then increased in the late afternoon.

Table 20 shows the relationship between diurnal ultrastructural changes in the ratio of chloroplastic lamellae to osmiophilic granules and bloat. No consistent changes

Table 20. Relationship between bloat and diurnal changes in the chloroplastic ratio of grana lamellae to osmiophilic granules, 1966

Bloat severity	Ratio of grana lamellae to osmiophilic granules[a]					
	4 AM	7 AM	10 AM	1 PM	4 PM	7 PM
Non-bloat	0.65	2.64	1.67	3.33	2.04	2.90
Low-bloat	9.67	6.43	6.60	8.80	10.75	6.44
High-bloat	14.50	38.11	11.56	9.33	7.50	6.48

[a] $$\frac{\text{(Number of grana/chloroplast) x (Number of lamella/gramum)}}{\text{(Number of osmiophilic granules) x (Average diameter of osmiophilic granules)}}$$.

were observed in the non- and low-bloat chloroplasts. However, in the high-bloat chloroplasts the ratio of lamellae to osmiophilic granules peaked at 7 AM and then declined throughout the remainder of the day. The changes in the high-bloat ultrastructural ratios agree closely with those observed in the ratios of total and soluble chloroplastic protein to chloroplastic phospholipid. The difference in the ultrastructural ratios between the low- and high-bloat chloroplasts began to narrow at 10 AM; this was also true of the chloroplastic protein to chloroplastic phospholipid ratios for the same two diurnal samples. However, no such agreement between ultrastructural and chemical ratios was noted with the non- and low-bloat chloroplasts.

Starch grains were present in all chloroplasts. The number of starch grains per chloroplast appeared to increase

from 4 AM to 7 AM in each case. However, no other major diurnal changes were observed in the chloroplastic starch grains in the different bloat stages.

In vitro incubation of whole, flailed and bolus leaves

Whole, flailed and bolus leaves from non- and low-bloat alfalfa were incubated in rumen fluid for 1 and 2 hours while leaves from high-bloat alfalfa were incubated for 1 hour only. No differences were observed in the chloroplastic ultrastructure of whole, flailed and bolus leaves in each bloat stage prior to incubation. Following incubation in rumen fluid for 1 hour, non- and low-bloat chloroplasts from whole and flailed leaves remained unchanged. Incubation for 1 hour in rumen fluid also failed to alter the chloroplasts in non-bloat bolus leaves. However, chloroplasts in low-bloat bolus leaves were disrupted as shown in Figure 49. The bimolecular chloroplastic membranes were missing and a portion of the stroma was dispersed. The other chloroplastic components, the starch grains, osmiophilic granules and grana lamellae, appeared unchanged.

Figure 50 shows the extreme state of chloroplastic disruption in high-bloat whole leaves following a 1-hour incubation in rumen fluid. The bimolecular chloroplastic membrane had disappeared, the lamellar strands were highly disorganized and the stroma was completely dispersed. Only the starch grains and osmiophilic granules appeared unchanged.

Following a 2-hour incubation in rumen fluid, dramatic changes were noted in the non- and low-bloat chloroplasts from whole, flailed and bolus leaves. Figure 51 shows the disrupted state of the non-bloat chloroplasts in bolus leaves. The chloroplastic membrane was gone, the lamellar membranes were in a state of disarray and the stroma was partially dispersed. These changes also typified the conditions noted in the non-bloat flailed and whole leaves following a 2-hour incubation in rumen fluid. A chloroplast from a low-bloat bolus leaf is shown in Figure 52 after a 2-hour incubation in rumen fluid. Only the skeleton of a chloroplast remains, etched with lamellar membranes and osmiophilic granules. The starch grains have disappeared and the stroma was totally dispersed. This micrograph (Figure 52) typified the condition of the low-bloat chloroplasts in whole and flailed leaves following a 2-hour incubation in rumen fluid.

Bolus juices

Electron micrographs of bolus juice collected at the cardia of the rumen are shown in Figures 53 and 54. Figure 53 shows the various components present in the juice. Two chloroplastic components (starch grains and lamellar membranes) are readily identifiable in the bolus juice. No intact chloroplasts were found in any micrographs of the bolus juice. Many different species of bacteria were seen throughout the field.

Figure 54 depicts a higher magnification of the chloroplastic fragments in bolus juice. The lamellar skeleton of a chloroplast is seen enclosing a single starch grain. Several osmiophilic granules appear enmeshed in the lamellar framework.

Rumen fluid 20 minutes after feeding

Electron micrographs of rumen fluid collected 20 minutes after feeding are shown in Figures 55 and 56. Chloroplastic components in the rumen fluid were similar to those seen in bolus juice, except for the absence of stroma and starch grains. The osmiophilic lipid granules appeared to coalesce within the chloroplastic framework. Several species of bacteria containing numerous gas vacuoles were seen.

Numerous bacteria and a few protozoa were present in the rumen fluid. Figure 57 shows a micrograph of a protozoan, complete with cilia, basal bodies and gullet. In a high magnification micrograph (Figure 58) chloroplastic lamellae are shown in the interior of a protozoan following ingestion through its gullet. Starch grains were also found within the protozoan (Figure 59).

Ruminal foam 20 minutes after feeding

Figures 60 and 61 show ruminal foam collected from a fistulated steer 20 minutes after feeding. An extensive network of chloroplastic lamellar membranes containing osmiophilic granules was found in the foam (Figures 57 and 58).

The lamellae appeared in various states of disorganization. Some lamellae were completely uncoiled as individual strands of unit membranes while other lamellae existed within the usual grana framework. One chloroplastic component, the stroma, was not present in the foam while another, the starch grains, was found only inside protozoa. The only plant cellular components identified in the foam were chloroplastic in nature. No other plant cellular organelles were present, such as mitochondria and nuclei.

GENERAL DISCUSSION

The presently accepted theory concerning the etiology of bloat involves the concept of a delicate ruminal balance between foam-stabilizing and foam-inhibiting factors. Both the foaming agents (soluble proteins, minerals, saponins, pectic materials) and antifoaming agents (chloroplastic lipids, polyphenols) are rapidly released into the rumen environment following ingestion. The relative percentages of the various factors released into the rumen affect the extent of both gas production and foam formation. If the release of the factors favors a stable foam, the balance is shifted to favor a bloating condition.

The plant is generally accepted as the primary source of foam-stabilizing and foam-inhibiting factors. Since the balance of these factors is critical, it is important to identify their distribution within the plant. The chloroplast has been identified as the plant organelle containing the highest percentages of three important chemical constituents - protein, lipids and minerals.

In the results reported herein, various alfalfa leaf protein values were significantly correlated with the incidence of bloat. Both total and soluble chloroplastic protein had higher positive correlations with bloat severity than did total leaf protein. The Fraction I chloroplastic protein had the highest positive correlation coefficients

ranging from 0.97 to 0.99.

All of the chloroplastic protein components increased percentagewise with increasing bloat. Protein to chlorophyll ratios of both the total leaf and leaf homogenates demonstrated a preferential synthesis of chloroplastic proteins during increasing bloat. The Fraction I component of the chloroplastic protein increased sevenfold from non-bloat to high-bloat forage.

Smillie (1963) and Zucker and Stinson (1962) identified the chloroplast as the major site of leaf protein, containing up to 75% of the total leaf protein. Amino acid activating enzymes have been identified in isolated chloroplasts by numerous workers, including Bove and Raacke (1959) and Heber (1962). It appears that the chloroplast is quantitatively the most important site of protein synthesis in the plant. Several pathways may be operating to account for the observed shift in chloroplastic protein synthesis. Either the amino acids are being synthesized as metabolic products of the photosynthetic carbon-reduction cycle or they (or their precursors) are being mobilized more efficiently from the cytoplasm into the chloroplasts.

Bassham and Calvin (1962) observed that $C^{14}O_2$ was rapidly fixed into alanine, aspartate, serine and glutamate in the chloroplasts. The amino acids were directly formed from intermediates of the carbon-reduction cycle. Other

pools of amino acids were found to exist inside the chloroplasts. These authors concluded that rapid protein synthesis occurs in chloroplasts either directly from the carbon-reduction cycle or from free amino acids. Smith et al. (1961) concluded that the independent amino acid pool in the chloroplasts resulting from the photosynthetic reduction cycle accounted for more than 60% of cellular protein synthesis.

The possibility of amino acid mobilization into the chloroplasts is suggested by Parthier (1963) who found that amino acids can be quickly transported through the chloroplastic membrane.

A third mechanism for chloroplastic protein synthesis involves the transport of metabolic intermediates and amino acid precursors into the chloroplast. Mudd and McManus (1962, 1964) found that chloroplastic preparations do incorporate acetate into non-volatile, water-soluble compounds including aspartic and glutamic acids. Adenosine triphosphate, coenzyme A and magnesium are required cofactors. Oxaloacetic acid was observed to favor incorporation into glutamic acid and inhibit incorporation into fatty acids.

Undoubtedly all three pathways contribute to the shifts observed in chloroplastic protein synthesis. To gain a better understanding of the pathways, let us consider the relationship of protein synthesis to plant maturity.

As the alfalfa plant matures, the dry matter content rises and the percent fresh leaf weight declines markedly. Both of these factors are significantly correlated with bloat severity.

The percent leaf protein decreases with advancing plant maturity. Balaza (1961) observed that protein content of whole alfalfa plants decreased with age from 25 to 8%. The decrease was most rapid after the onset of flowering. Smillie and Krotkov (1961) demonstrated that soluble leaf protein in peas decreased 25% within 11 days after germination. Granick (1938) found a 30% decrease in the total nitrogen of chloroplasts per g fresh leaf material in mature spinach leaves versus young leaves. The chloroplastic nitrogen content of old yellow leaves declined 53%. The greatest decline in protein synthesis occurred in the chloroplasts.

Spencer (1965) observed that the amino acid incorporating ability decreased sharply with age. A 90% decline in incorporation of amino acids occurred from 3- to 5-week old chloroplasts. Bassham and Calvin (1962) found that the chloroplastic formation of amino acids directly from intermediates of the carbon-reduction cycle decreased with age of the plant. These observations suggest that a chloroplastic protein synthesis is probably depressed most by a decrease in the amino acid formation by the photosynthetic carbon-reduction cycle.

Fraction I or 18S protein has been identified as the primary proteinaceous foaming-agent in alfalfa responsible for foam formation in the rumen in pasture bloat (McArthur and Miltimore, 1964). However, they did not report any direct correlations of protein values with bloat. Lyttleton and T'so (1958) found that 65 to 70% of the Fraction I protein in green spinach leaves was associated with the chloroplasts. Both the grana lamellae and stroma have been identified as chloroplastic sites of Fraction I protein (Park and Pon, 1961; Lichtenthalter and Park, 1963).

Francki et al. (1965) found that removal of the amino acid incorporating ability was highly correlated with the loss of mobile stroma from the chloroplasts. These authors concluded that the materials responsible for in vitro protein synthesis by chloroplasts are located primarily in the mobile phase of the chloroplasts.

As shown by electron microscopy, some of the grana lamellae persist for at least 2 hours in the rumen following ingestion. This suggests that the lamellae do not contribute directly to a bloating condition. However, the chloroplastic lamellae were unmistakably identified in the ruminal foam. In addition, the bolus juices contained chloroplastic fragments with only partial lamellar networks and no intact chloroplasts. In all cases, the chloroplastic membranes in bolus leaves were disrupted after in vitro incubation and the

stroma material readily dispersed. Due to the speed with which bloat occurs following ingestion of alfalfa, one would expect the stroma to be the likely site of chloroplastic Fraction I protein. However, no direct evidence is given to substantiate this hypothesis.

The Fraction I chloroplastic protein isolated by gel-filtration using Sephadex G-50 was highly correlated with bloat. Following exhaustive foaming, Fraction I chloroplastic protein was completely denatured and removed by filtering while the concentration of Fraction II chloroplastic protein was unchanged. McArthur and Miltimore (1964) observed a similar effect of foaming on the optical density of the surface-denatured 18S protein solution. They suggested that the 18S protein stabilizes the liquid films in the foam only after surface-denaturation.

The highly significant ($P < .01$) correlation coefficients between calcium and magnesium binding to Fraction I chloroplastic protein at pH 5.5 partially confirm the surface-denaturation theory of McArthur and Miltimore. Through a salting-out process with calcium and magnesium, the 18S protein may be surface denatured and surface activated. Further comments regarding the observed binding phenomenon will be reviewed later following a discussion of the relaionship of chloroplastic lipids and minerals to bloat.

Our results showed a significant ($P < .01$) negative

correlation between total leaf lipids and bloat. Total and soluble chloroplastic lipids were also inversely related to bloat severity. The importance of the chloroplastic lipids was first suggested by Mangan (1958) when he identified the chloroplastic lipids as vigorous anti-foaming agents.

Mangan's observations with chloroplastic lipids are not surprising due to presence of large amounts of surface-active components within the chloroplastic lipids. Allen et al. (1966) found that the surface-active lipids accounted for more than 60% of the total chloroplastic lipids. The distribution of surface-active chloroplastic lipids was as follows: monogalactosyl diglycerides > digalactosyl diglycerides > phosphatidyl glycerol > sulpholipid > lecithin > phosphatidyl inositol.

The percentage of leaf lipids, both total and soluble chloroplastic lipids, decreased with increasing bloat. Total and soluble chloroplastic lipids composed as much as 70 and 55% of the total leaf lipids, respectively, in non-bloat forage. These percentages decreased as low as 19% in high-bloat samples. The soluble chloroplastic lipids composed from 41 to 86% of the total chloroplastic lipids.

Electron micrographs of alfalfa leaf chloroplasts demonstrated a definite relationship between the osmiophilic lipid granules and bloat. Both the number and size of the osmiophilic granules increased with decreasing bloat. Chloroplasts in high-bloat alfalfa were virtually devoid of

the osmiophilic granules, while chloroplasts from non-bloat forage were densely populated with large granules.

Murakami and Takamiya (1962) isolated the osmiophilic granules from spinach chloroplasts with lipid solvents such as chloroform, methanol and chloroform-methanol (3:1). The insolubility of the granules in acetone or benzene indicated that they were not composed of neutral fat. However, the exact lipid composition was not determined. Menke and Jacob (1942) identified the phosphatides as the primary component of the acetone-insoluble fraction from spinach chloroplastic lipids.

Greenwood et al. (1963) found that spheroidal osmiophilic granules, ranging from 100 to 5000 Å in diameter, were characteristic components of higher plant chloroplasts. Small dense globules were characteristic of young chloroplants and large osmiophilic granules of older chloroplasts. Silica gel chromatography of lipid extracts indicated that the globules contained galactolipids, phosphatides and plastoquinones; other less polar lipids were also present. The globules contained no β-carotene or chlorophyll.

The distribution of the chloroplastic lipids and the osmiophilic granules suggest a preferential synthesis of chloroplastic lipids in non-bloat forage. Significant negative correlation coefficients between chloroplastic protein and chloroplastic lipids suggest further that the

lipids are being synthesized in preference to protein in non-bloating forage. One thinks immediately of a probable common pathway for the synthesis of both lipids and protein--the photosynthetic carbon-reduction cycle. Jolchine (1962, 1963) demonstrated that acetate-2-C^{14} is rapidly incorporated into the chloroplastic phospholipids, glycolipids, carotenoids and fatty acids. Beta-hydroxybutyrate and glutamate were also radioactive.

Both total leaf and chloroplastic phospholipids were negatively correlated with bloat severity. The percentage of total leaf lipids as chloroplastic phospholipids decreased from 8.49 (non-bloat) to 1.24% (high-bloat). When the chloroplastic phospholipids were expressed as a percent of leaf phospholipids, an even greater decline was noted from 54.4 (non-bloat) to 18.5% (high-bloat). This suggests that the phospholipids are a major component of those chloroplastic lipids which increase as a forage decreases in its ability to produce bloat. This idea is supported by the observation of Eberhardt and Kates (1960) that the phosphatide content of runner bean leaves increased as the plant matured.

Phospholipids have been identified as important factors in controlling bloat. Fraser (1961) observed that the phospholipid levels were 80% higher in non-bloat than in bloat-provoking clover. Nichols *et al*. (1957) found that

granular plant lecithin consistently reduced paunch surface tension and viscosity 7%, resulting in excellent control of mechanical foaming.

The research reported herein demonstrated a preferential synthesis of phospholipids in chloroplasts of non-bloat alfalfa. Benson and Maruo (1958) found that the chloroplasts were the primary sites of phospholipid synthesis in sweet clover and barley. Among the respective phospholipid fractions showing the greatest variation, lecithin was 22% higher in barley than in sweet clover. Allen et al. (1966) and Wintermans (1960) observed that lecithin and phosphatidyl glycerol are the major chloroplastic phospholipids. The further importance of lecithin in controlling bloat is shown in our data by its ability at low mole ratios to competitively inhibit calcium binding to Fraction I chloroplastic protein. The relationship of lipid additions to metal-binding will be discussed later in more detail.

The surface activity of lecithin and other phospholipids enables the ingested chloroplastic lipids to act as efficient anti-foaming agents. However, once degraded, the phospholipids appear to lose their native anti-foaming potency. Thus the presence of catabolic enzymes in either the plant or the rumen would greatly influence the foam-inhibiting activity of the phospholipids. Lecithinases have been identified in both plant juices and rumen fluid.

Kates (1953) found that plant lecithinases are localized in the chloroplasts and rapidly liberate choline. Dawson (1959) observed a rapid release of P^{32} from lecithin in washed suspensions of rumen microorganisms. However, no attempt has been made to correlate the level or relative activity of lecithinases with bloat.

The sulpholipids are another primary chloroplastic lipid fraction with strong surface activity. Benson et al. (1959) first identified the chloroplastic sulpholipid as 1-O-oleoyl-3-(beta-galactopyranosyl-6-sulphate)-1-glycerol. The sulpholipid was rapidly labeled with either S^{35} or C^{14} during photosynthesis by Chlorella algae. Zill and Harmon (1962) attributed a primary structural role to the sulpholipid involving the formation of an interfacial linkage between layers containing water-soluble and lipid-soluble materials. The structural role was confirmed by Shibuya et al. (1965) who demonstrated that the sulpholipids are localized in lamellar fragments or lipoprotein subunits of similar size.

The strongly hydrophilic sulphonic group of the chloroplastic sulpholipid suggests a possible role for this surface active agent in bloat. Russell and Bailey (1966) observed a rapid release of lipid-bound sulphoquinovose into the rumen within 2 hours after eating. The sulpholipid concentration in the rumen remained extremely high for 5 hours post-feeding.

These workers found a 14% variation in sulpholipid concentrations during a single growing season. O'Brien and Benson (1964) observed a 96% variation in sulpholipid content of alfalfa leaves during a two-month period.

The galactolipids are a third primary class of chloroplastic lipids. Neufield and Hall (1964) found a rapid incorporation of UDP-D-galactose into chloroplastic galactolipids. Benson et al. (1958) also observed a rapid accumulation of label from $C^{14}O_2$ into galactolipids. In a 5-minute incubation period, over half of the C^{14} in the chloroplastic lipid was found in the galactose moieties. However, no workers have been able to directly correlate chloroplastic galactolipids with bloat. Bailey (1964) did observe a rapid release and degradation of galactolipids in the rumen. Although a 40% variation in lipid-bound galactose was shown during a single season, no evidence was obtained which implicated these compounds in bloat.

An unusual feature of the chloroplastic lipids is their extremely high percentages of unsaturated fatty acids. Wolf et al. (1962) found that 89% of the fatty acids in spinach chloroplasts were unsaturated, 75% of which were in the carbon 18 series. Linolenic acid and palmitic acid were the main unsaturated and saturated fatty acids, respectively.

The pronounced differences we observed in the ability of palmitic and linolenic acid to inhibit calcium binding to Fraction I chloroplastic protein suggest a relationship

between the chloroplastic fatty acids and bloat. Palmitic acid was seen to inhibit binding more effectively at lower concentrations than linolenic acid.

The possible importance of the chloroplastic fatty acids is further shown by Klopfenstein (1965) who observed considerable daily variation in the fatty acid content of chloroplastic phospholipids and sulpholipids. The linolenic acid content was highest in the young, immature alfalfa, while palmitic acid increased with advancing maturity. Newman (1962) also found that plastids contain less unsaturated fatty acids in mature leaf tissue. Shah and Schulman (1965) observed that saturated fatty acids in synthetic lecithin enhanced calcium binding, while unsaturated fatty acids depressed the binding. These observations confirm the binding studies reported herein and suggest that diurnal variations in the ratio of saturated to unsaturated fatty acids may influence bloat.

It is interesting to note that saturated and unsaturated fatty acids are synthesized by different pathways in higher plants. Barron and Stumpf (1962) and Stumpf and James (1962) observed that oleic acid is synthesized from acetate, in the presence of biotin, but not from palmitate or stearate. James (1963) proposed the presence of two pools of long-chain fatty acids in leaves. The first pool involves the conversion of myristic to palmitate and stearate, which are then

esterified to give phospho and sulpholipids. In the second pool the conversion proceeds from myristic to palmitate to stearate, which is then esterified or converted further to linoleic or linolenic acid.

Despite the probable relationship of chloroplastic lipids to bloat, correlation coefficients between these two factors were disappointingly low. Because of the importance of both chloroplastic protein and lipids upon foam stability, it was thought that the ratios of these components might give extremely high correlations with bloat. This was found to be true. The correlation coefficients between chloroplastic protein/chloroplastic lipids and soluble chloroplastic protein/soluble chloroplastic lipid ratios and bloat ranged from 0.76 to 0.99. The ratios of total and soluble chloroplastic protein/chloroplastic phospholipids gave correlation coefficients consistently above 0.97.

High negative correlations were shown between chloroplastic proteins and chloroplastic lipids. This suggests that when chloroplastic metabolism favors protein synthesis, chloroplastic foam-stabilizing factors predominate and bloat occurs. When the chloroplastic metabolism shifts to favor lipid synthesis, presumably phospholipids or sulpholipids, foam-inhibiting factors predominante and bloat is prevented.

In addition to protein and lipids, calcium and magnesium also appear to play a vital role in the etiology of bloat.

As reported herein, significant negative correlations were shown between total leaf and chloroplastic calcium and bloat ranging from -.46 to -.73. As bloat severity increased the percentage of total leaf calcium in the chloroplasts decreased from 23.7 to 12.3. Total leaf and chloroplastic magnesium were directly correlated with bloat with correlation coefficients ranging from 0.27 to 0.97. As bloat severity increased, the percent of total leaf magnesium in the chloroplasts rose from 20 to 60%.

Previous research studying the relationships of calcium and magnesium to bloat has been confined to observations of the mineral content of the whole plant. Johnson (1959) found both a negative and positive correlation of calcium with bloat severity in two consecutive grazing seasons. Jackson et al. (1962) observed no significant correlation between cattle bloat on Ladino clover and the potassium, phosphorus and calcium levels in the plant. Smith (1963) reported no significant correlation between plant calcium and magnesium and the bloat severity in lambs grazing alfalfa pasture.

The general low correlations between bloat and the total plant content of calcium and magnesium contrasts markedly with observations that salt additions enhance bloat. Reid et al. (1961) reported that slaked lime additions to drinking water increased the incidence of bloat. Warner et al. (1962)

demonstrated that spraying alfalfa with calcium carbonate enhanced bloat severity in lambs. Both the foliar application and the drenching of lambs with calcium and magnesium salts significantly increased bloat (Smith, 1963).

The observations reported above suggest that calcium and magnesium may influence bloat in two different ways. The first role involves the relationship of calcium and magnesium upon plant metabolism. The second role concerns the direct effect of the salts within the rumen.

Magnesium is primarily involved in plant metabolism as a major factor in protein synthesis. Chloroplastic magnesium levels were found to have a significant ($P < .01$) positive correlation coefficient (0.90) with chloroplastic protein levels. Lindahl et al. (1966) proposed that magnesium functions in protein synthesis through specific site binding and renaturation of transfer ribonucleic acids.

Calcium functions primarily in plant metabolism as an essential cofactor in phospholipid synthesis. A significant positive correlation of 0.95 was found between chloroplastic calcium and chloroplastic phospholipids. Hubscher (1962) observed that calcium increased the incorporation of L-serine into phosphatidyl serine in microsomal subfractions. Of the various bivalent cations, only calcium was effective. Similar observations were made by Dils and Hubscher (1961) with choline incorporation into lecithin.

A consideration of their primary roles in plant metabolism suggests that calcium and magnesium would exert different effects upon bloat. Due strictly to their major metabolic plant functions as defined above, magnesium would be expected to enhance and calcium to depress bloat. To completely understand the metabolic role of calcium and magnesium, it is essential to examine their interrelationships concerning ion mobilization, absorption, transport and distribution in plants.

Plant nutrients in the soil reach the root in three ways--root extension, mass flow and diffusion. Barber et al. (1963) showed that plant roots extend to less than 3% of the total available nutrients in the soil. However, it was observed that plant roots acquire a majority of the plant's calcium and magnesium requirement through diffusion. Mass flow generally supplies the root with the remainder of the need for calcium and magnesium.

Once the minerals contact the root hairs, absorption may occur. The ratio of cations to anions absorbed by a plant is relatively constant provided the anion concentration does not vary. De Witt et al. (1963) found that one main system of cation competition occurs between calcium and magnesium. Moore et al. (1961) observed that small amounts of calcium effectively block the absorption of large amounts of magnesium. They proposed that calcium acts by altering the

selective permeability properties of the cell surface region. Magnesium absorption from single salt solutions paralleled that of alkali cations.

Competition by different cations for absorption may be due to specific binding sites on carrier molecules which transport the molecules across cell membranes. Examples of mutually competing cations are calcium, magnesium and strontium. In barley roots, Epstein (1960) found that lithium severely inhibits calcium absorption due to competition for binding sites. Avdonin and Milovidova (1961) observed that aluminum inhibits nitrogen and magnesium absorption by plants, but has no effect upon calcium. Calcium was shown to increase the assimilation of phosphorus.

Fertilization and liming also influence the availability of cations for plant absorption. MacLean (1956) observed that liming decreased the magnesium and increased the calcium content of alfalfa grown in greenhouse soil. Nitrate and urea fertilizer increased magnesium uptake in grass (Olofsson, 1964), while calcium levels decreased with growth.

Calcium appears to be transported in three main forms: salts of organic acids, free calcium ions and calcium-proteinate (Petrov-Spiridonov, 1965). Barley was shown to contain no ionic calcium. The bulk of the calcium was present in aqueous fractions as proteinate and citrate. Approximately 3% of the calcium was absorbed on lipids, lipoproteins and steroids.

The mechanism regulating cation transport is unknown. It has been suggested that cation transport is dependent upon cellular respiration. Grodzinskii (1960) observed that illumination increased the entrance of Ca^{45} into leaves threefold. When carbon dioxide was removed from the air, calcium transport to the leaves was retarded. Carles (1963) found that calcium, magnesium and soluble nitrogen are concentrated in the leaf petiole. He suggested that the petiole controls the movement of the elements.

Pireyre (1963) showed that Ca^{45} is deposited very early in chloroplasts and is strongly bound within the chloroplasts. Gonzales-Silicia de Juan and Mosse (1963) observed that the calcium leaf content increases with age. Spinach chloroplasts were shown by Nobel and Packer (1964) to actively accumulate calcium. Maximum calcium uptake required ATP, the hydrolysis of which required magnesium.

The chloroplasts appear to be the major sites of accumulation of most plant cations, including calcium and magnesium. Stocking and Ongun (1962) demonstrated that nonaqueously isolated bean and tobacco chloroplasts contained from 40 to 75% of the total leaf calcium, magnesium and potassium. The results reported herein show that alfalfa chloroplasts contained from 20 to 60% of the total leaf magnesium and from 14 to 23% of the total leaf calcium.

The importance of minerals to chloroplastic development

is shown by the changes observed in the ultrastructure of chloroplasts from mineral-deficient leaves (Thompson and Weier, 1962). With a lack of nitrogen and magnesium, chloroplasts changed before full development. In the case of a magnesium deficiency, the grana became disorganized. In the case of nitrogen, the stroma were greatly diminished and the grana compartments were swollen and reduced in number.

Before we can fully assess the role of calcium and magnesium upon bloat, we must also consider their roles upon foam-stability in the rumen. Salts have been shown to have a marked influence on maintaining a stable foam. Mangan (1959) found that the salt concentration altered the foaming properties of legume forages. Following exhaustive dialysis, cytoplasmic protein solutions produced small amounts of an unstable foam. The addition of as little as 0.063M NaCl increased the foam capacity tenfold and resulted in maximum foam stability. A linear increase in foam strength was observed with salt additions up to 0.33M. On a molar basis, calcium was found to be twice as effective as sodium in enhancing foam stability. Divalent cations did not significantly stabilize foam until reaching a minimum concentration of 0.1M. Perri and Hazel (1947) observed that calcium and sodium additions up to 0.5M increased the foaming capacity of α-soybean protein. Other researchers have

demonstrated that calcium is essential for maximum foam stability with saponins (Mangan, 1959) and pectic substances (Gupta et al., 1962).

The importance of calcium in maintaining foam stability suggests that the role of minerals may be mediated through binding phenomena with plant foaming constituents. As the primary foaming constituent of alfalfa, Fraction I protein merited particular attention as a metal-binding agent.

The results reported herein demonstrate highly significant positive correlation coefficients between calcium and magnesium binding to Fraction I chloroplastic protein and bloat. Both the extent and strength of binding were directly correlated with bloat severity. To account for the differences in binding, high-bloat and non-bloat Fraction I proteins were analyzed for amino acid content. Although aspartic and glutamic acids were 17 and 22% higher in the bloat samples, respectively, the quantitative differences in these two amino acids alone accounted for only 10% of the actual increase noted in binding. Data from tests with four different salt concentrations indicate that two acidic amino acids were required to bind each molecule of magnesium in the high-bloat sample. By contrast, the non-bloat sample required 12 acidic amino acids. These observations suggest that both the position of aspartic acid and glutamic acid in the polypeptide chains and the steric configuration of the

protein molecule are important factors contributing to the differences observed in protein binding.

The significance of the binding phenomenon upon bloat may be thought of in terms of the theory of McArthur and Miltimore (1964) on surface-denaturation. These authors assert that the Fraction I protein acts as a foam stabilizer only in the denatured state. If this is true, any factor which contributes to the denaturation of the protein would consequently enhance bloat. The metal-binding phenomenon reported herein may act to "salt-out" the Fraction I protein, thereby stabilizing the liquid films in the foam.

Factors actively competing with the protein as metal binding sites would decrease the surface denaturation of the protein and thus act as anti-foaming agents. This theory of foam inhibition has been confirmed by Ross and Haak (1958) who observed that anti-foamers act by effectively absorbing or binding the foaming agents from the surface of interlamellar bulk solutions.

Our results show that low mole ratios of lecithin to protein depress calcium binding to Fraction I chloroplastic protein in non-bloat samples. The inhibitory effect of lecithin upon metal to protein binding was diminished as bloat increased. The inability of lecithin to compete effectively in high-bloat samples is reflected by the higher log K_M values of Fraction I protein. The log K_M values for

calcium 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 calcium binding to phosphatidyl serine was 4.13 (Hendrickson and Fullington, 1965). If comparable stability constants occur between calcium and lecithin, one would expect competitive calcium binding by lecithin in the non-bloat forage.

Numerous workers have previously reported calcium binding to phospholipid films. Kimizuka and Koketsu (1962) observed calcium binding to lecithin monolayers at physiological pH values. These workers suggested that calcium binds with two anionic oxygen sites forming a bridge between radically oriented phospholipids. Sodium and potassium were weak competitors with calcium. Rojas et al. (1966) showed that calcium absorption to phosphatidyl serine monolayers depends upon the concentration of the cation present. Rosano et al. (1962) found that calcium injected under cephalin monolayers breaks the phosphoric acid amine polar group intramolecular association, permitting intermolecular associations among neighboring cephalin molecules. The intermolecular bonds allow surface lattice formation.

Both the free and bound fatty acid content of complex lipids appear to be important in the balance between calcium binding to Fraction I protein and bloat. The most common chloroplastic saturated fatty acid, palmitic acid, inhibited calcium to protein binding less than lecithin at all bloat

levels. In the non-bloat stage, palmitic acid did depress binding, but required four times the mole ratio as lecithin. In all three bloat samples, palmitic acid exerted little influence on calcium binding.

Linolenic acid, the major unsaturated chloroplastic fatty acid, exerted less influence on calcium to protein binding than either lecithin or palmitic acid. The only noticeable depression occurred with the non-bloat sample, where twice the mole ratio of palmitic acid was required. These observations suggest that the extent and rate of hydrolysis of the fatty acid ester linkages in phospholipids, sulpholipids and other complex lipids may be important in determining the anti-foaming capacity of the plant lipids. Another important factor would be the decreased water solubility of palmitic and linolenic acids as compared with the phospholipid.

In recent years workers have shown a relationship between the fatty acid content and calcium to phospholipid binding. Goddard and Ackilli (1963) observed that calcium and magnesium binding by stearic acid monolayers decreased surface tension and surface volume up to 75%. Deamer and Cornwell (1966) found that calcium additions to dipalmitoyl phosphatidyl choline significantly lowered surface viscosity. Calcium additions to oleic acid had no such effect.

Based upon the previous findings, we may hypothesize

that as the concentration of saturated fatty acids increases in the phospholipid fraction, calcium to phospholipid binding is enhanced at the expense of calcium binding to protein and bloat is prevented. This condition is favored in older, mature alfalfa. When the concentration of unsaturated fatty acids is high, as in young, immature alfalfa, calcium to phospholipid binding decreases, calcium binding to Fraction I protein increases, surface viscosity increases and bloat ensues. Any other factors, such as polyphenols, which competitively bind calcium and magnesium in preference to protein, would also function as anti-foaming agents.

In addition to calcium and magnesium, other trace-minerals may be quantitatively involved in the binding phenomenon. Harris and Sebba (1965) observed that nickel and zinc enhanced foam stability. During the first 10 minutes of foaming, the concentration of nickel in foam of young alfalfa was five times that of aged alfalfa; this coincides with the observations made herein with calcium and magnesium. Harris and Sebba identified protein as the surface active agent in foam which binds nickel ions on its negatively charged sites. The authors concluded that nickel was available for protein attachment in young alfalfa, but unavailable in aged plants. Another possibility involves changes in the protein configuration and composition.

If the interrelationships among proteins, lipids and minerals are meaningful in the etiology of bloat, then

changes in the various chemical constituents should help account for seasonal variations in bloating patterns. Definite changes do occur in plant composition with advancing maturity. These changes have been covered extensively before. The gross differences between the chemical composition of immature and mature plants do not explain the daily variations in bloat severity. However, these differences may be explained by daily diurnal variations in plant composition.

Diurnal changes in the protein, lipid and mineral content of alfalfa leaf chloroplasts were measured. The magnitude of the changes in the chloroplastic phospholipids was 500%. Three to four hundred percent changes were observed in chloroplastic magnesium and in chloroplastic protein/chloroplastic phospholipids and soluble chloroplastic protein/chloroplastic phospholipids ratios. Diurnal changes of 200 to 300% were seen in leaf phospholipids and in chloroplastic protein/chloroplastic lipids and soluble chloroplastic protein/soluble chloroplastic lipids ratios. Diurnal variations of 100 to 200% were found in leaf magnesium, chloroplastic calcium, leaf protein, total and soluble chloroplastic protein, leaf lipids, total and soluble chloroplastic lipids and in leaf protein/leaf lipids ratios.

The greatest diurnal variation in the various protein levels occurred in the medium- and high-bloat samples. Changes were quite small in the non-bloat samples for leaf, chloroplastic and soluble chloroplastic protein and all of

the protein/lipid ratios. The greatest variation in the non-bloat samples occurred in the various lipid and calcium levels.

Trends among the five diurnal samples were often quite uniform. In general, leaf dry matter increased progressively from 7 AM to 7 PM. Several criteria increased to a maximum at 7 AM, declined to a minimum at 10 AM to 1 PM and then increased progressively until 7 PM. These criteria included total and soluble chloroplastic protein and the following ratios: leaf protein/leaf lipids, chloroplastic protein/ chloroplastic lipids and soluble chloroplastic protein/ soluble chloroplastic lipids. Percent leaf phospholipids decreased from 4 AM to 10 AM and then increased slightly in the afternoon. Trends in chloroplastic phospholipids were similar to total leaf values for two samples, but the other two increased progressively from 4 AM. Both chloroplastic calcium and magnesium levels were highly correlated with chloroplastic phospholipid and chloroplastic protein changes, respectively.

Diurnal changes in the chloroplastic ultrastructure were observed by electron microscopy. Quantitative values for lipids and proteins were determined by counting the number and average diameter of the osmiophilic lipid granules and the grana per chloroplast and lamella per granum, respectively. Positive correlation coefficients between the osmiophilic

granules and both chloroplastic lipids and phospholipids ranged from 0.78 to 0.83. Those for grana lamella and chloroplastic protein ranged from 0.96 to 0.98.

The general trend in the number and size of the osmiophilic granules paralleled the diurnal changes in chloroplastic phospholipids most closely. Only in the chloroplastic phospholipids did the values increase in the afternoon in the high-bloat sample. In general the number and size of the osmiophilic granules decreased during the midday and then increased in the afternoon.

Changes in the number of grana per chloroplast corresponded closely to diurnal changes in total and soluble chloroplastic protein. The same pattern was observed at the three bloat levels studied. The quantitative value for the grana increased at 7 AM, decreased to a minimum at 10 AM or 1 PM and then rose in the afternoon. The greatest variation was observed in the high-bloat samples.

Only a limited amount of previous data is available concerning diurnal changes in plant constituents. Kupke (1962) found that the 18S protein increased rapidly following illumination. Noguchi and Tamaki (1962) studied the diurnal variation in amino acid composition of tobacco leaves. Total leaf nitrogen increased until early afternoon, declined at 4 PM and then increased at 6 PM. All amino acids except aspartic acid reached a maximum at 2 PM. Aspartic acid reached a maximum level during the night. Serine, glycine

and arginine increased most rapidly during the daytime. Allen et al. (1961) found that the ash, phosphorus and total nitrogen contents of alfalfa were lowest at 2 PM. Total nitrogen and phosphorus both increased in the late afternoon. Calcium levels were observed to increase slightly during the day. No definite correlations could be made between diurnal changes in alfalfa and bloat.

The observations reported herein show that major diurnal changes do occur in each of the protein, lipid and mineral values. The chloroplastic components show generally greater variation than the same leaf values. The changes in the protein/lipid ratios appear to reflect the general changes observed in bloat severity. It definitely appears that diurnal changes in the content of chloroplastic protein, lipids and minerals are of sufficient magnitude to help explain differences in bloating patterns. However additional research is needed in this area.

In the discussion it has been shown that the structure and chemical composition of alfalfa leaf chloroplasts are intimately associated with the bloat syndrome. Quantitatively the chloroplast is a major site of protein, lipid and mineral synthesis and metabolism in the alfalfa plant. Changes in the ratios of chloroplastic protein to chloroplastic lipids and phospholipids reflect variations in bloat severity. Calcium and magnesium binding to Fraction I chloroplastic protein is highly correlated with bloat. The extent to

which chloroplasts directly contribute to the structure of the foam is not entirely clear. However the presence of chloroplastic lamella and osmiophilic granules in the foam suggests that they may play a prominent structural role. The rapid dispersion of the stroma from disrupted chloroplasts suggest that this may be the major chloroplastic site of 18S protein.

SUMMARY

Chemical and electron microscopic studies were conducted for two consecutive summers to determine the relationship between the protein, lipid and mineral composition of alfalfa (leaf and leaf chloroplasts) and bloat. Leaves collected from alfalfa tops were analyzed for dry matter, protein, total and soluble lipids, phospholipids, calcium and magnesium. Leaf chloroplasts were analyzed for total and soluble protein, Fraction I protein, total and soluble lipids, phospholipids, calcium and magnesium. Diurnal samples of alfalfa tops were collected from 4 AM to 7 PM and analyzed for the above components. In addition, calcium and magnesium binding to Fraction I chloroplastic protein were measured. All of the chemical constituents were correlated with bloat severity in cattle and sheep. Electron microscopic studies were made of whole leaves, flailed leaves, bolus leaves, bolus juice, rumen fluid and ruminal foam.

All leaf protein values were significantly correlated with bloat. Both total and soluble chloroplastic proteins had higher positive correlation coefficients with bloat severity than did total leaf protein. Fraction I chloroplastic protein had the highest positive correlation coefficients (0.97 to 0.99). All chloroplastic protein components increased percentagewise as bloat increased. Protein to chlorophyll ratios of both total leaf and leaf

homogenates demonstrated a preferential synthesis of chloroplastic protein during increasing bloat. Electron micrographs showed that the number of grana per chloroplast and lamella per granum were highly correlated with diurnal changes in chloroplastic protein.

Negative correlation coefficients were found between leaf and chloroplastic lipids and bloat. The percentage of total leaf lipids found as chloroplastic lipids decreased with increasing bloat. Electron micrographs showed that high-bloat chloroplasts were virtually devoid of osmiophilic lipid granules, while non-bloat chloroplasts were densely populated with large granules. The number and size of osmiophilic granules correlated best with chloroplastic phospholipids. The distribution of chloroplastic lipids suggested that chloroplastic lipids were synthesized in preference to protein in non-bloat provoking forage.

Various protein/lipid ratios were highly correlated with bloat. The correlation coefficients between chloroplastic protein/lipids and soluble chloroplastic protein/lipids and bloat ranged from 0.76 to 0.99. The ratios of total and soluble chloroplastic protein/chloroplastic phospholipids gave correlation coefficients consistently above 0.97.

Leaf and chloroplastic calcium levels were negatively correlated with bloat and positively correlated with

chloroplastic phospholipids. Leaf and chloroplastic magnesium values were positively correlated with chloroplastic proteins and bloat.

Both the extent and strength of calcium and magnesium binding to Fraction I chloroplastic protein at pH 5.5 were directly correlated with bloat. Low mole ratios of lecithin to protein depressed calcium binding to Fraction I protein in non-bloat samples. The inhibitory effect of lecithin upon metal to protein binding was diminished as bloat increased. Palmitic acid inhibited calcium to protein binding less than lecithin at all bloat stages, but consistently more than linolenic acid.

Diurnal changes of considerable magnitude were noted in the protein, lipid and mineral content of alfalfa leaf chloroplasts. The greatest diurnal variation in the protein levels occurred in medium- and high-bloat samples. The greatest variation in non-bloat samples occurred in the various lipid and calcium levels. The chloroplastic components showed greater variation than the same whole leaf values.

Chloroplastic fragments were major components of bolus juices. No intact chloroplasts were found in the juices. Skeletons of chloroplastic lamellae were the major identifiable plant components in foam. Rumen microorganisms were shown to disrupt high-bloat chloroplasts into a mass of

disorganized lamellae within 1 hour; however, non-bloat chloroplasts showed no apparent disruption. In all observations, the stroma was rapidly dispersed from the disrupted chloroplasts.

These studies show the importance of chloroplasts in the etiology of bloat.

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APPENDIX

Table 21. Coefficients of variation for chemical analyses

Analysis[a]	Sample values					Mean	% CV[b]
	1	2	3	4	5		
			%				
Leaf dry matter	20.84	20.57	20.45	20.51	20.61	20.60	1.78
Leaf protein	36.16	36.33	36.25	36.19	36.09	36.20	0.67
Chloroplastic protein	2.13	2.21	2.17	2.08	2.17	2.16	5.91
Soluble chloroplastic protein	0.76	0.78	0.78	0.75	0.79	0.77	5.77
Leaf lipids	4.12	4.25	4.32	4.28	4.04	4.20	6.52
Chloroplastic lipids	1.56	1.47	1.47	1.42	1.55	1.48	8.99
Soluble chloroplastic lipids	0.49	0.44	0.49	0.49	0.47	0.47	9.28
Leaf phospholipids	1.28	1.31	1.32	1.30	1.30	1.30	2.84
Chloroplastic phospholipids	0.71	0.70	0.71	0.71	0.72	0.71	2.82
Leaf calcium	0.93	0.93	0.92	0.93	0.94	0.94	1.50
Chloroplastic calcium	0.21	0.21	0.20	0.21	0.21	0.21	0.87
Leaf magnesium	0.57	0.57	0.57	0.58	0.58	0.57	1.98
Chloroplastic magnesium	0.35	0.35	0.35	0.35	0.36	0.35	0.66

[a]Expressed as a percentage of leaf dry matter.

[b]Percent coefficient of variation.

Table 22. Coefficients of variation for binding studies

Analysis[a]	Sample values 1	2	3	Mean	% CV[b]
Calcium bound per g Fraction I chloroplastic protein	4.18	4.16	4.21	4.18	1.20
Magnesium bound per g Fraction I chloroplastic protein	4.31	4.25	4.29	4.28	1.56

[a]Expressed as 1×10^{-5} moles of calcium or magnesium bound per g Fraction I chloroplastic protein, pH 5.5.

[b]Percent coefficient of variation.

Figure 26. Nonaqueously isolated chloroplastic pellet showing starch grains (sg), osmiophilic granules (o) and grana (g).


sg
0.5

Figure 27. Mesophyll cells from non-bloat provoking whole alfalfa leaves showing the cell wall (cw), intercellular spaces (is), mitochondria (M), chloroplasts (c) and vacuole (v).

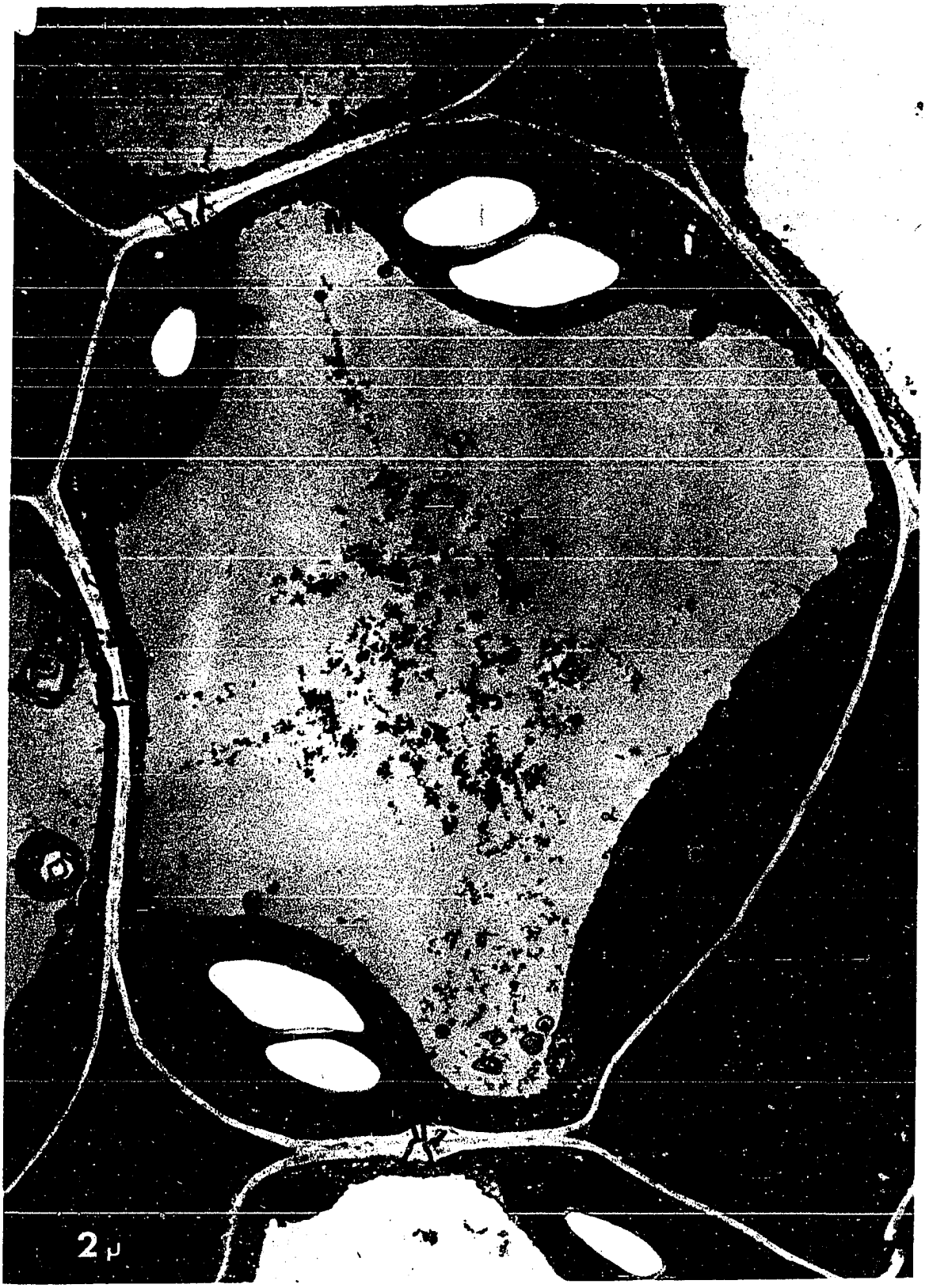

2 μ

Figure 28. Mesophyll cells from high-bloat provoking whole alfalfa leaves showing the cell wall (cw), intercellular spaces (is), nucleus (n), mitochondria (M), chloroplasts (c) and vacuole (v).


c
is
v
n
M
2 μ

Figure 29. Typical chloroplast from non-bloat provoking whole alfalfa leaf showing the cell wall (cw), intercellular spaces (is), chloroplast limiting membranes (cm), osmiophilic granules (o), stroma (s), starch grains (sg), grana (g) and vacuole (v).

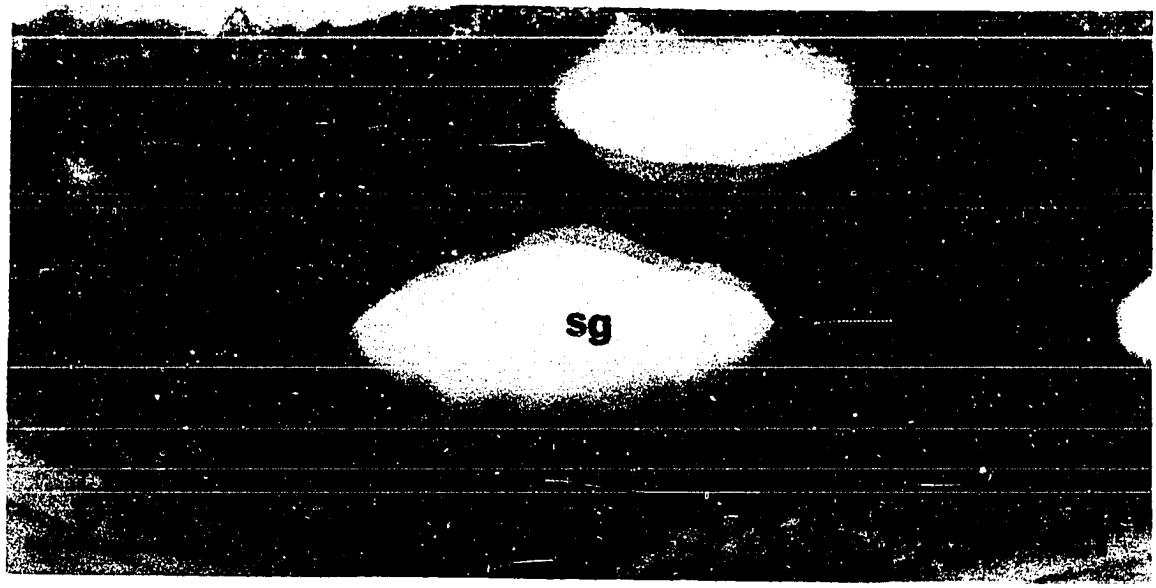

IS
g
sg
cm
s
o
v
0.5
29

Figure 30. Typical chloroplast from high-bloat provoking whole alfalfa leaf showing the cell wall (cw), intercellular spaces (is), chloroplast limiting membranes (cm), osmiophilic granules (o), stroma (s), grana (g) and vacuole (v).


CW
O
V
0.5μ
30

Figure 31. Chloroplast from non-bloat provoking alfalfa leaf collected at 4 AM showing starch grains (sg) and osmiophilic granules (o).

Figure 32. Chloroplast from non-bloat provoking alfalfa leaf collected at 7 AM.


sg

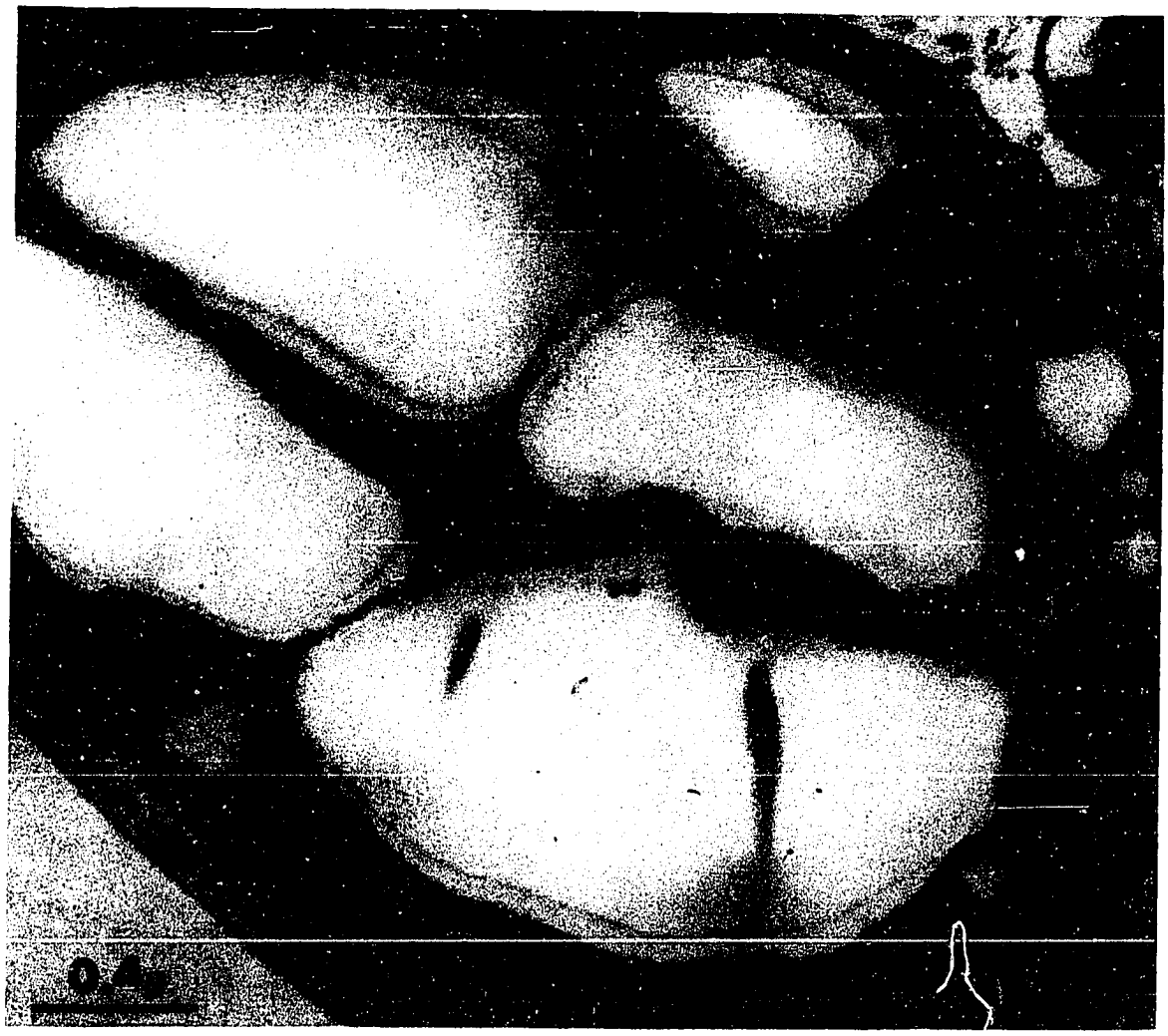


Figure 33. Chloroplast from non-bloat provoking alfalfa leaf collected at 10 AM showing the endoplasmic reticulum (er).

Figure 34. Chloroplast from non-bloat provoking alfalfa leaf collected at 1 PM showing mitochondria (M).


M
0.5μ

Figure 35. Chloroplast from non-bloat provoking alfalfa leaf collected at 4 PM.

Figure 36. Chloroplast from non-bloat provoking alfalfa leaf collected at 7 PM.

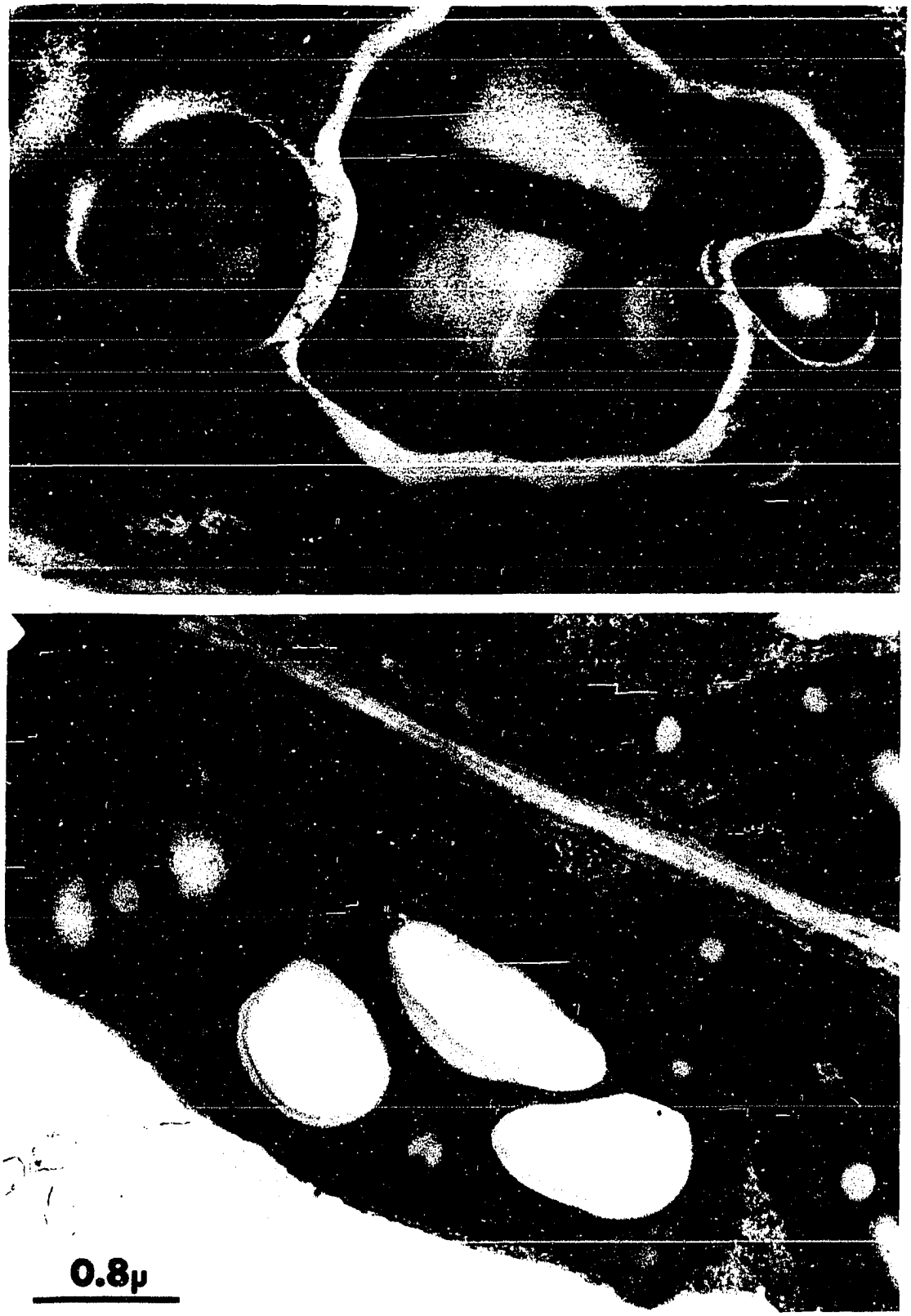

0.8μ

Figure 37. Chloroplast from low-bloat provoking alfalfa leaf collected at 4 AM showing starch grains (sg), stroma (s) and osmiophilic granules (o).

Figure 38. Chloroplast from low-bloat provoking alfalfa leaf collected at 7 AM.

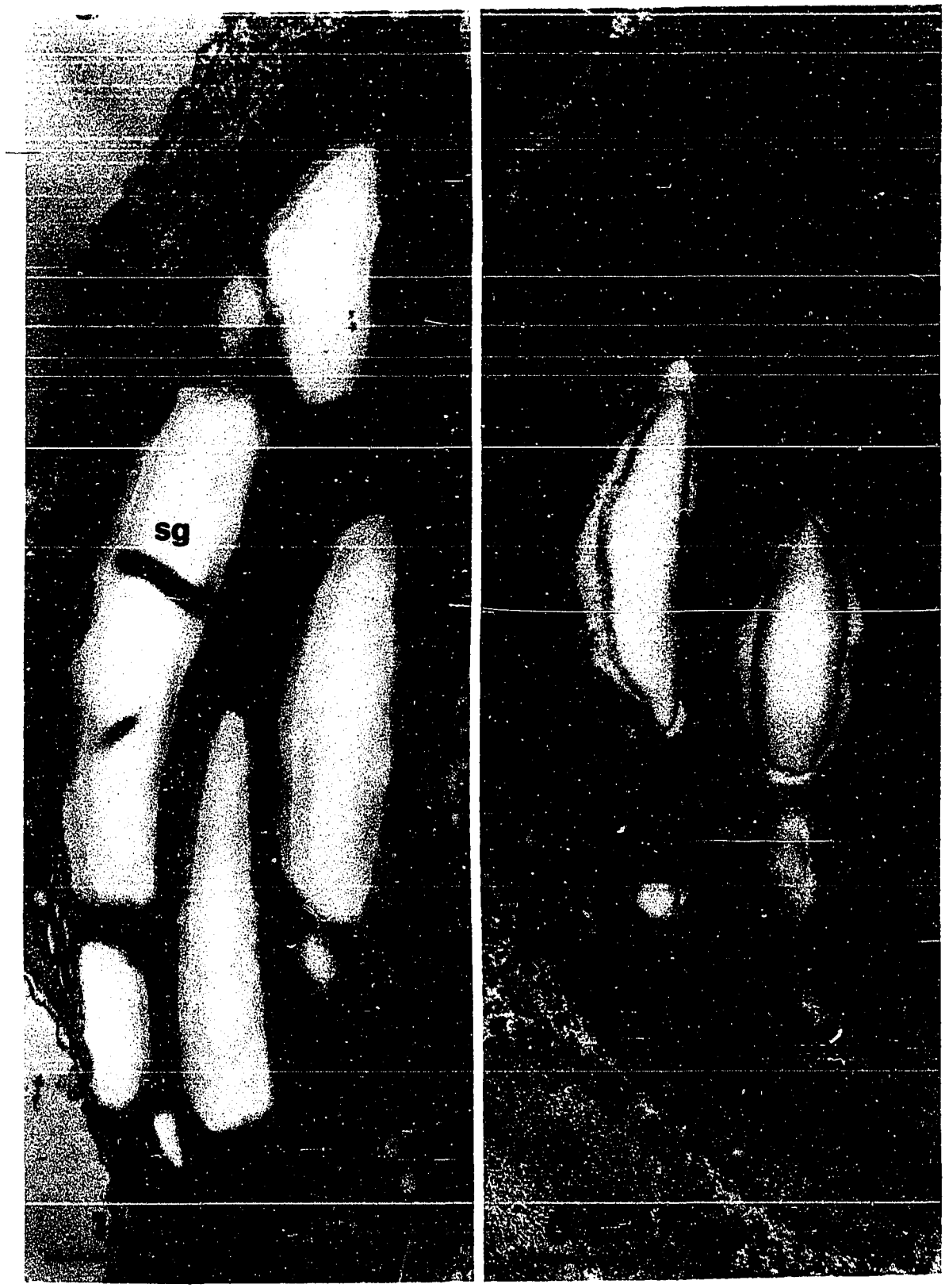

sg

Figure 39. Chloroplast from low-bloat provoking alfalfa leaf collected at 10 AM.

Figure 40. Chloroplast from low-bloat provoking alfalfa leaf collected at 1 PM.

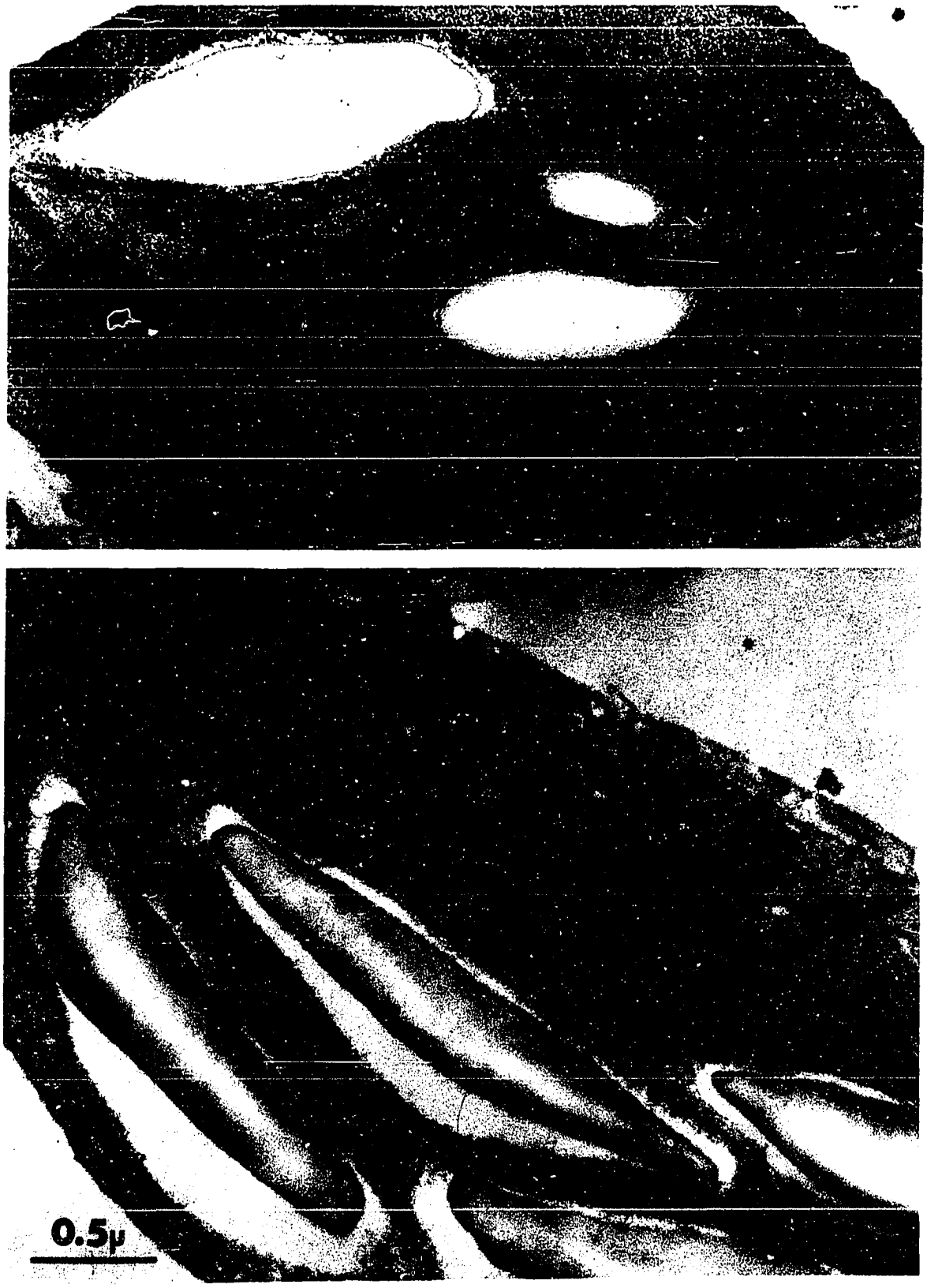

0.5μ

Figure 41. Chloroplast from low-bloat provoking alfalfa leaf collected at 4 PM.

Figure 42. Chloroplast from low-bloat provoking alfalfa leaf collected at 7 PM.

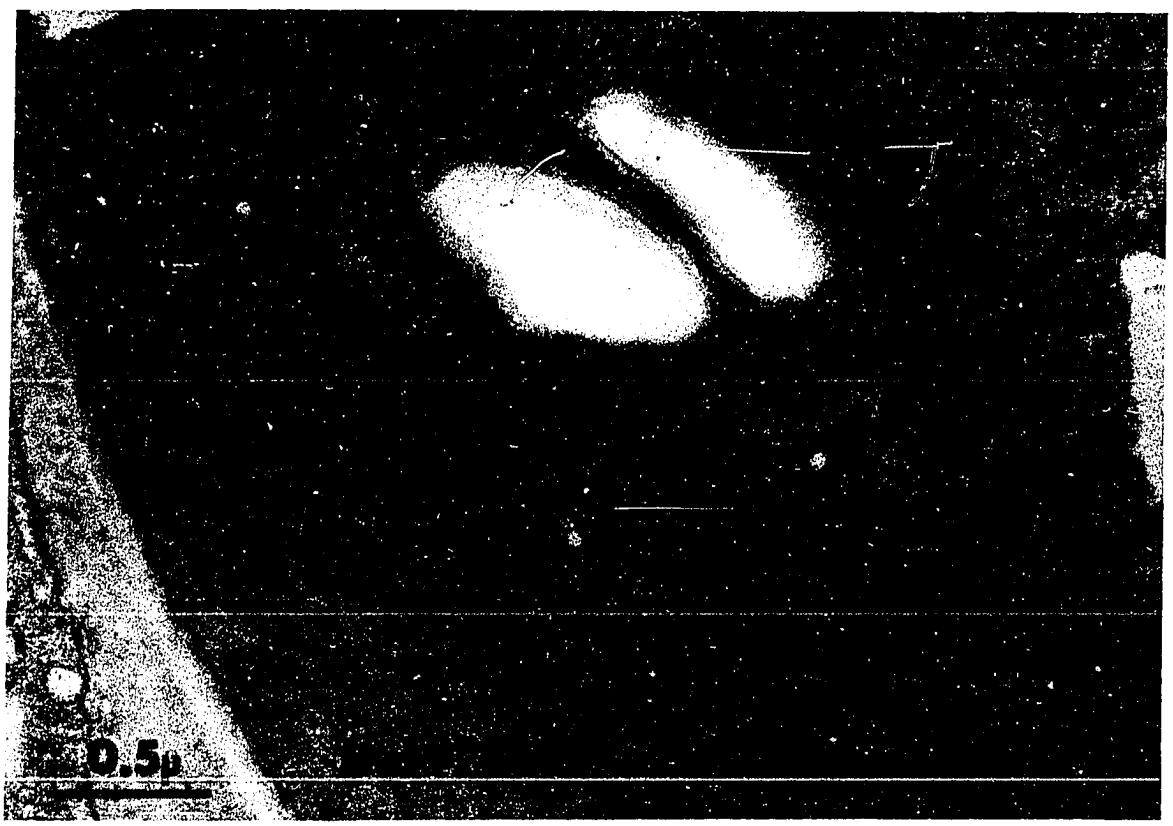
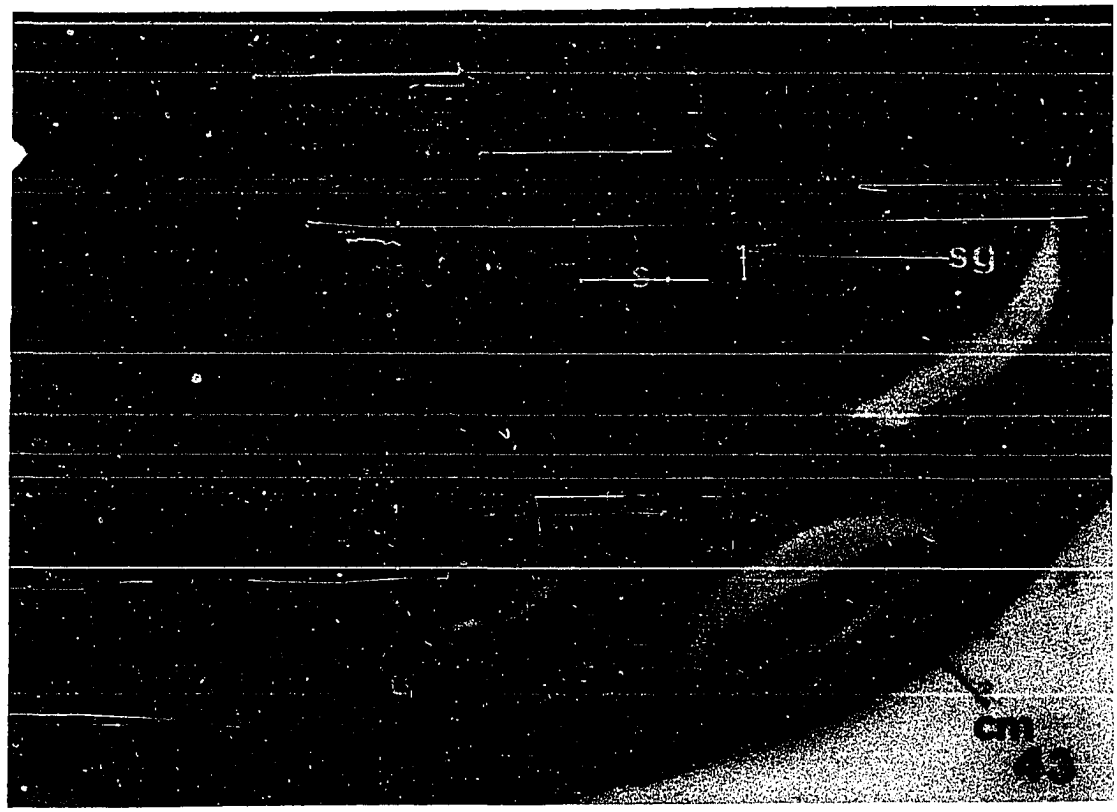

0.5μ

Figure 43. Chloroplast from high-bloat provoking alfalfa leaf collected at 4 AM showing chloroplast limiting membranes (cm), starch grains (sg), stroma (s) and osmiophilic granules (o).

Figure 44. Chloroplast from high-bloat provoking alfalfa leaf collected at 7 AM.


s
l
sg
cm

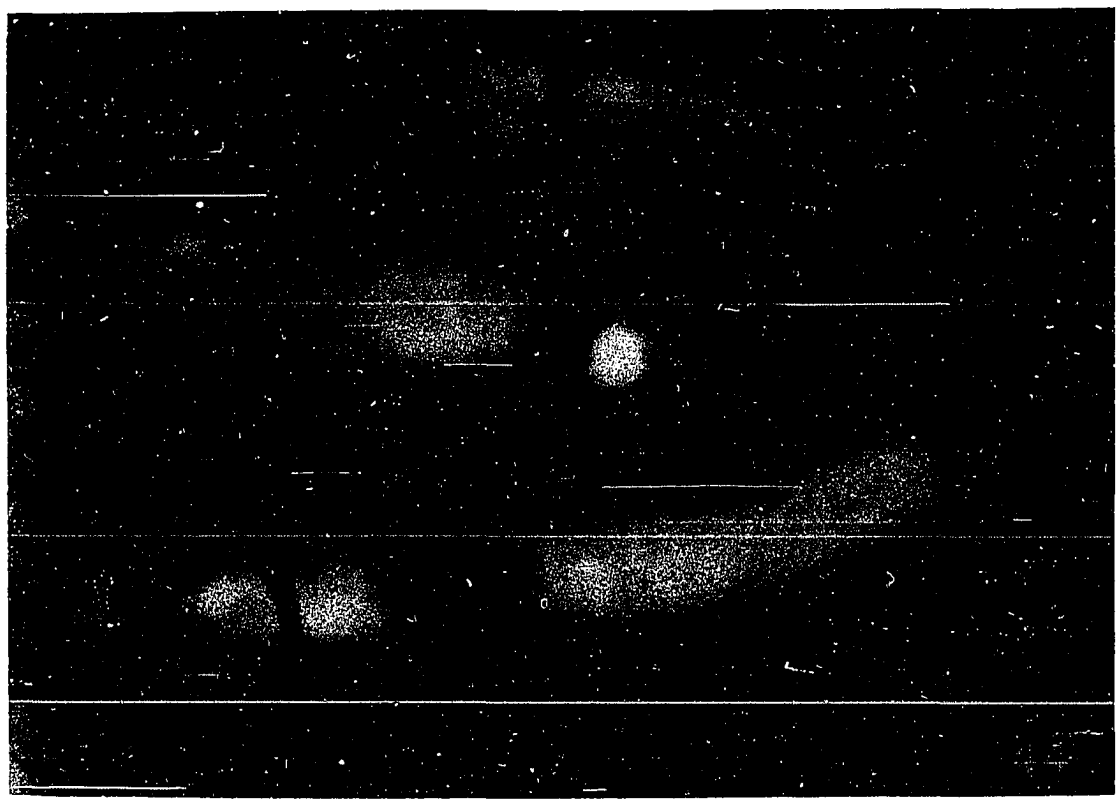


Figure 45. Chloroplast from high-bloat provoking alfalfa leaf collected at 10 AM.

Figure 46. Chloroplast from high-bloat provoking alfalfa leaf collected at 1 PM.

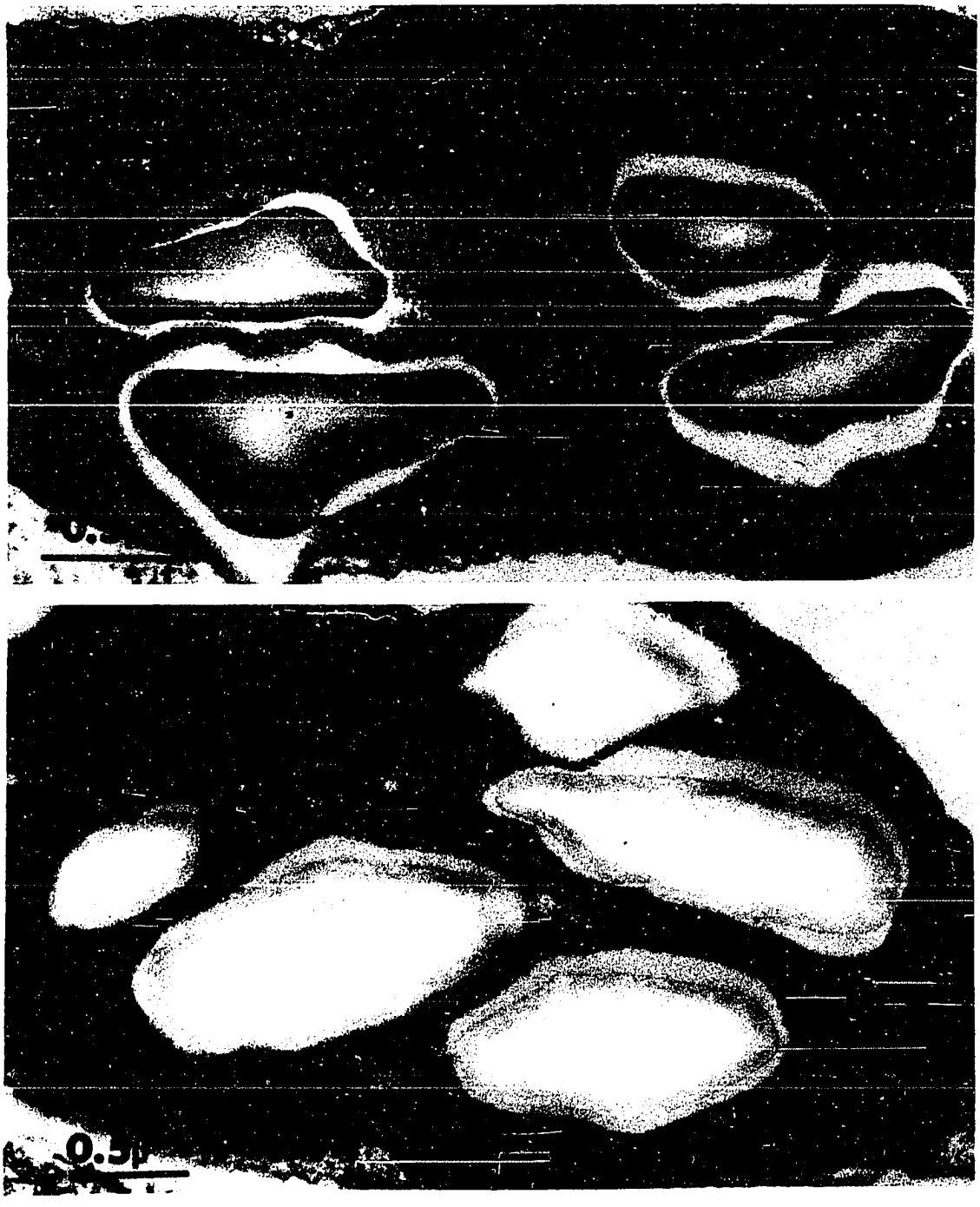


Figure 47. Chloroplast from high-bloat provoking alfalfa leaf collected at 4 PM.

Figure 48. Chloroplast from high-bloat provoking alfalfa leaf collected at 7 PM.

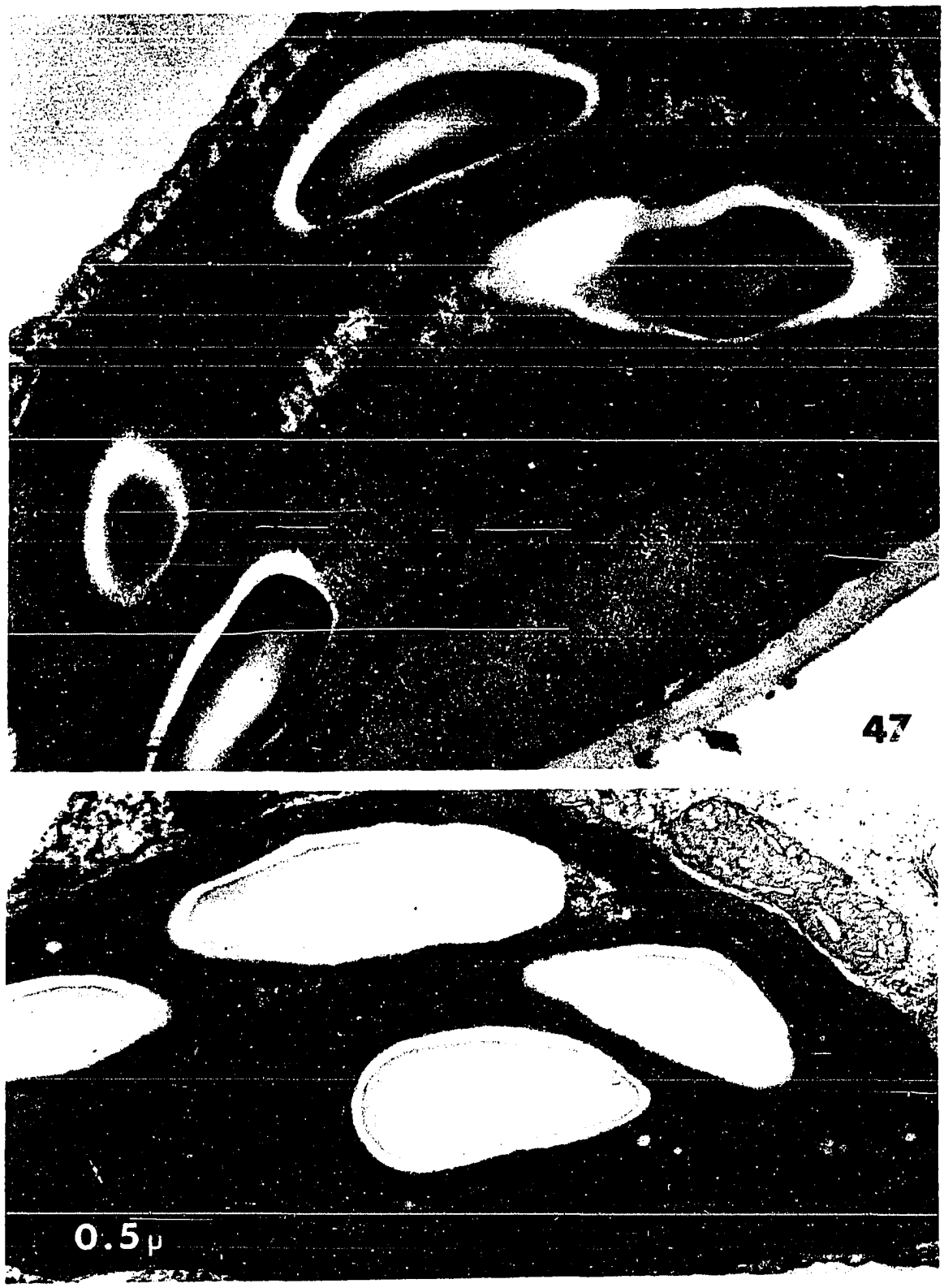

47
0.5 μ

Figure 49. Low-bloat bolus leaf following a 1-hour incubation in rumen fluid showing chloroplast limiting membranes (cm), starch grains (sg), stroma (s), grana (g) and osmiophilic granules (o).

Figure 50. High-bloat bolus leaf following a 1-hour incubation in rumen fluid showing the cell wall (cw), starch grains (sg) and osmiophilic granules (o).


sg
49

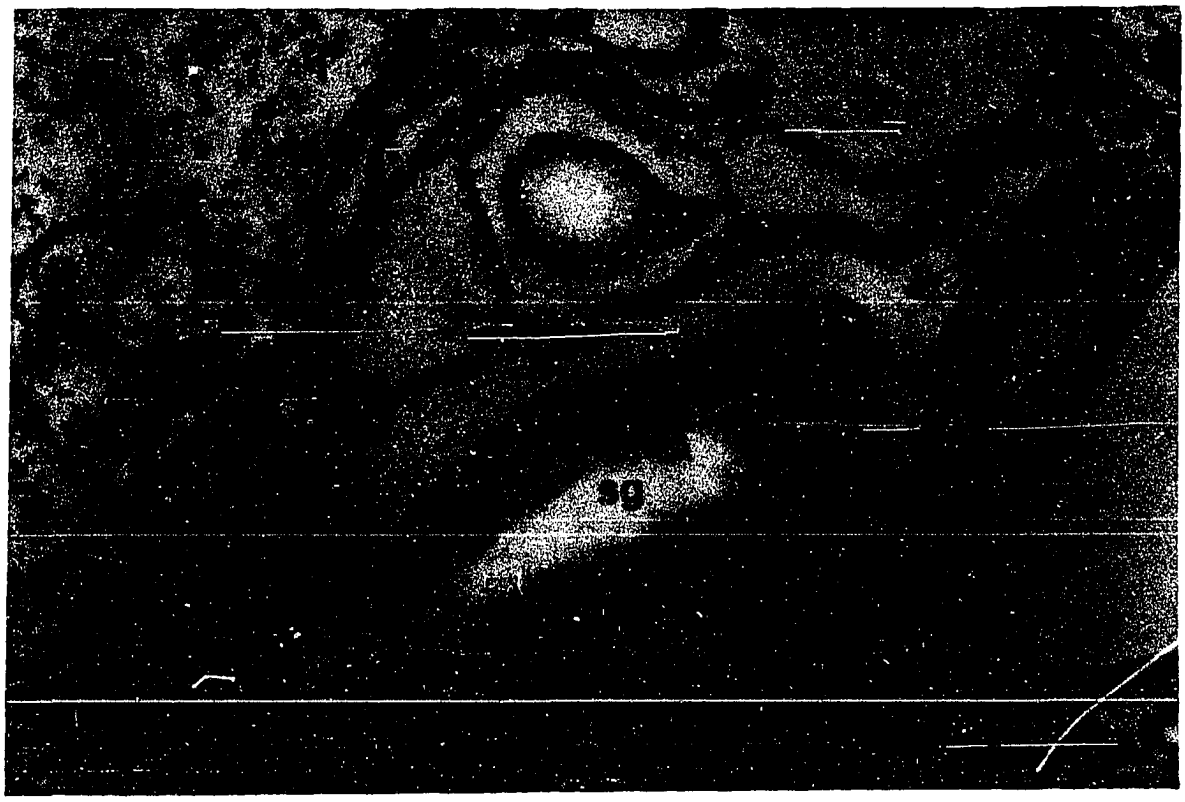

39

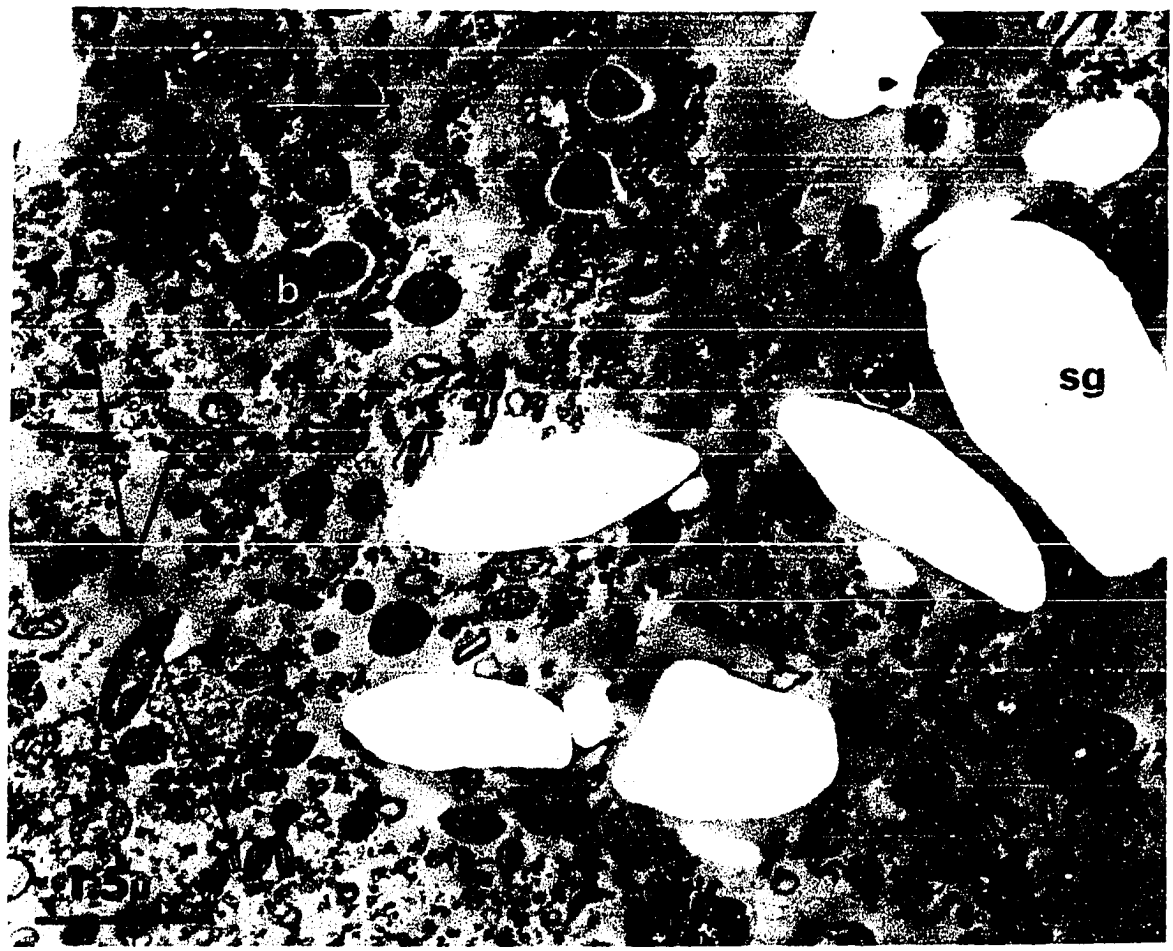
Figure 51. Non-bloat bolus leaf following a 2-hour incubation in rumen fluid showing grana (g) and osmiophilic granules (o).

Figure 52. Low-bloat bolus leaf following a 2-hour incubation in rumen fluid showing grana (g) and osmiophilic granules (o).


0.5μ

Figure 53. Low-magnification micrograph showing components present in bolus juice collected from a fistulated steer eating alfalfa soilage. Note the chloroplast fragments (cf), starch grains (sg) and bacteria (b).

Figure 54. High-magnification micrograph showing components present in bolus juice collected from a fistulated steer eating alfalfa soilage. Note the grana (g), osmiophilic granules (o) and starch grains (sg).


b
sg


sg

Figure 55. Low-magnification micrograph of rumen fluid collected from a fistulated steer 20 minutes after receiving alfalfa soilage. Note the chloroplast fragments (cf) and bacteria (b).

Figure 56. High-magnification micrograph of rumen fluid collected from a fistulated steer 20 minutes after receiving alfalfa soilage. Note the grana (g), osmiophilic granules (o) and bacteria (b).


cf
b
b
A
55
b
0.7μ

Figure 57. Low-magnification micrograph of a rumen protozoan containing starch grains (sg) in rumen fluid.


sg
1μ

Figure 58. High-magnification micrograph of a protozoan in rumen fluid showing ingested chloroplast membranes.

Figure 59. High-magnification micrograph of a rumen protozoan showing ingested starch grains.

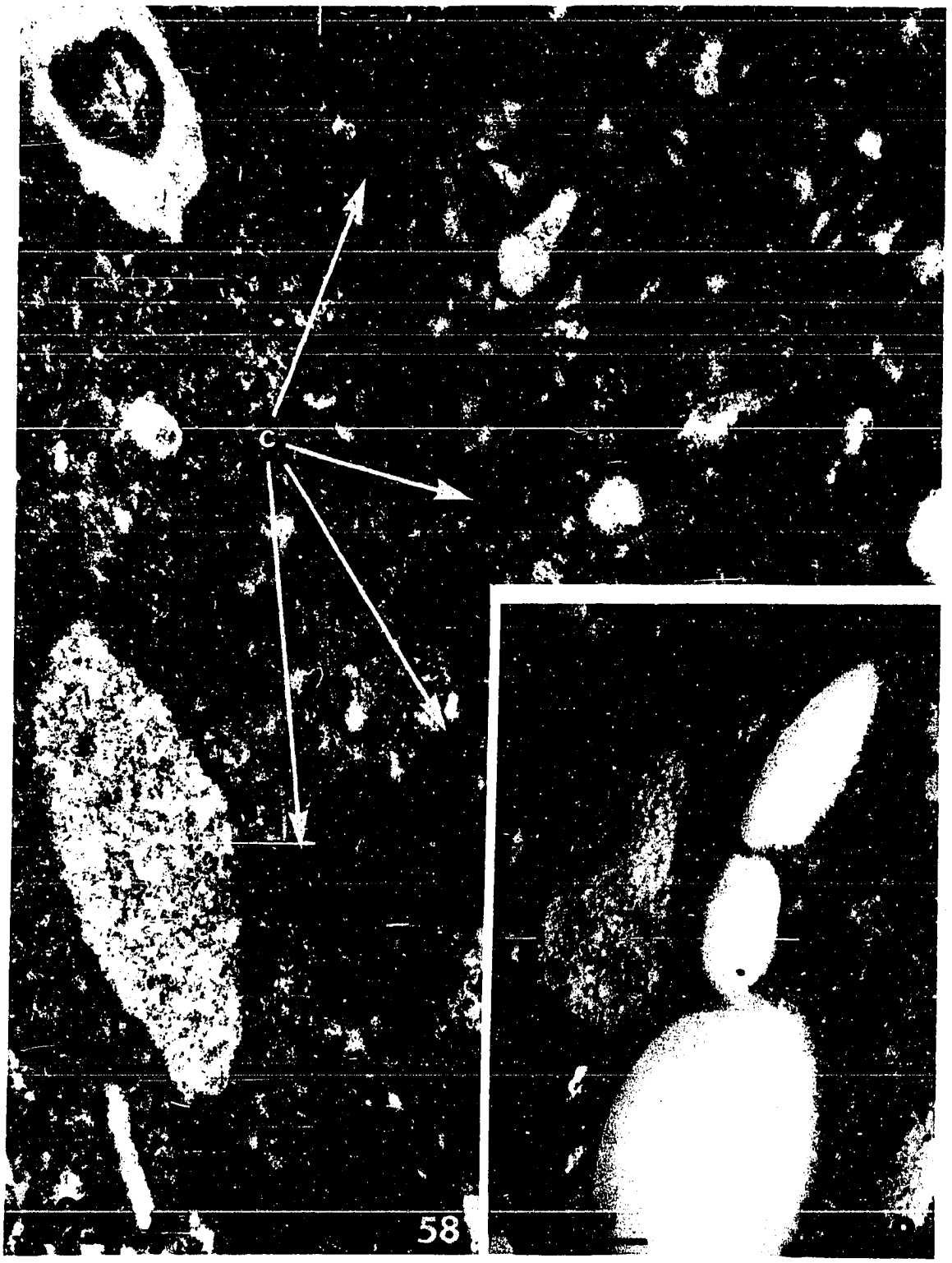
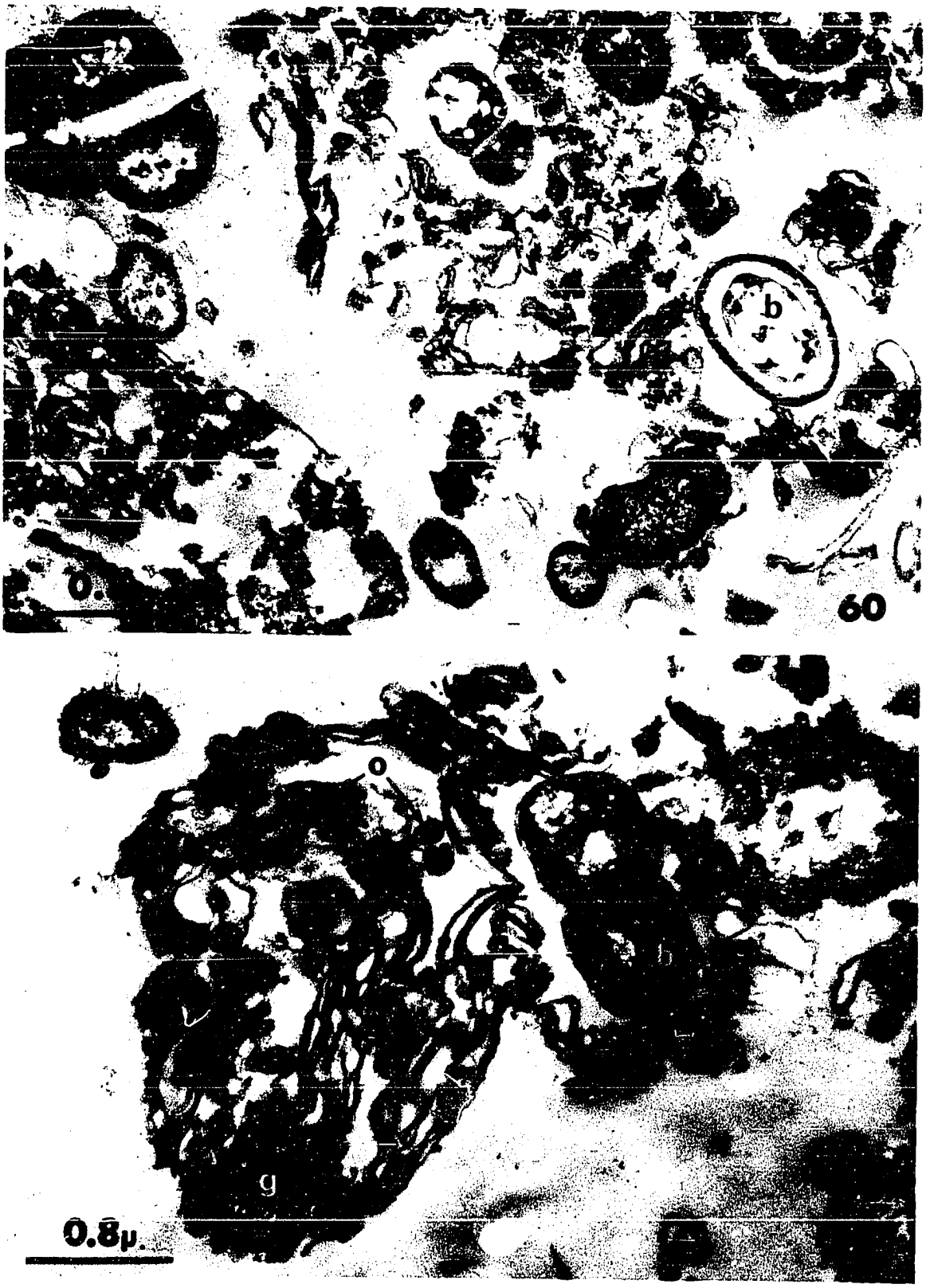

C
58

Figure 60. Low-magnification micrograph of ruminal foam collected from a fistulated steer 20 minutes after initially receiving alfalfa soilage. Note several varieties of bacteria (b) containing gas vacuoles.

Figure 61. High-magnification micrograph of ruminal foam collected from a fistulated steer 20 minutes after initially receiving alfalfa soilage. Note the grana (g), osmiophilic granules (O) and bacteria (b).


b
60
o
b
g
0.8μ.