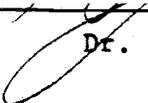


AN ABSTRACT OF THE THESIS OF

Gary L. Kiemnec for the degree of Doctor of Philosophy
in Soil Science presented on October 19, 1983.
Title: Diagnosis of Sulfur Deficiency and Sulfur Fertilization
Trifolium subterraneum L.

Redacted for privacy

Abstract approved: _____


Dr. Thomas L. Jackson

Sulfur (S) fertilization of Nangeela subclover (Trifolium subterraneum L.) was carried out on hill pastures in Western Oregon to determine the response to different particle sizes of elemental S and to evaluate indices of S status as they were affected by plant maturity. At the Roseburg, Oregon site elemental S and gypsum were applied annually during a four year period beginning in fall, 1974 at 0, 11, 22 and 44 kg S/ha. Single applications of elemental S having particle size diameters (mm) of <0.25, S(f), 0.5-0.59, S(mf), 0.70-0.83, S(mc) and 1.00-2.00, S(c) were applied at 88 and 176 kg S/ha were applied in September, 1974. All single applications were evaluated for residual S availability during the following four years. In 1975 dry matter yield responses to S fertilization were small or non-existent. All treatments increased yields in 1976 and 1977. Total four year yields for all single applications at 88 or 176 kg S/ha were not significantly different ($P=0.05$). Coarser particle sizes (S(mc), S(c)) of elemental S were more effective in increasing S accumulation in the last two years than in the first two years, while the reverse situation held true for S(f). Application of S(mf) resulted in the most constant plant accumulation of S (3.88 kg S/ha/yr) of any particle size. The S concentration of subclover leaves and petioles sampled at flowering was only

increased by higher application rates of gypsum and S(f) in 1975. Coarser materials (S(c), S(mf), S(mc)) were more effective in increasing S concentration of subclover in each succeeding year.

Chemical analysis of subclover leaves and petioles to determine indices of S deficiency were successful in identifying certain indices as being less dependent upon plant maturity than others. Total soluble S and sulfate-S/total S were least affected, sulfate-S, total nitrogen (N)/total S and non-protein organic N/non-protein organic S ratios moderately affected and total S most affected by plant maturity. The trichloroacetic acid (TCA) soluble N fraction was composed mainly of organic N. The TCA soluble S fraction was composed of organic S and sulfate-S with the percentage of organic S increasing with S fertilization.

Nitrogen:S ratios of the protein fraction, $(N:S)_p$, were affected by 1) the method of sample handling and extraction and 2) S fertilization. The trend towards a decrease in $(N:S)_p$ with increasing S fertilization was demonstrated for all methods of sample handling and extraction. It was observed that the $(N:S)_p$ of oven-dried plant material was greater than that of freeze-dried material when both were extracted with TCA. Ethanol extraction of fresh plant material resulted in lower $(N:S)_p$ ratios than either TCA extraction.

Oxidation of plant material in a combustion flask followed by a turbidimetric determination of sulfate-S resulted in higher S concentrations than dry ashing followed by either turbidity or methylene blue determination of sulfate-S.

Diagnosis of Sulfur Deficiency and
Sulfur Fertilization of Trifolium subterraneum L.

by

Gary L. Kiemnec

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed October 19, 1982

Commencement June 1984

APPROVED:

Redacted for privacy

Professor of Soil Science in charge of major

Redacted for privacy

Head of Department of Soil Science

Redacted for privacy

Dean of Graduate School

Date thesis is presented October 19, 1983

Typed by Gary L. Kiemnec for Gary L. Kiemnec

ACKNOWLEDGEMENTS

The author wishes to express his appreciation and gratitude to the following people:

Dr. T. L. Jackson, major professor, for constructive guidance and counsel on the research and writing of this thesis,
Dr. N. W. Christensen for thoughtful research suggestions and criticism of the written thesis,
Drs. D. Chilcote and D. Loomis for their constructive criticism of this thesis,
Dr. R. Peterson for advice on statistical questions,
Wayne Mosher, county extension agent, for field assistance and general enthusiasm,
Dianne Drlica, Steven Petrie and Ron Taylor for help in plant sampling and harvesting,
Dean Hanson for help with chemical procedures, and
David Tapley for laboratory assistance with N analyses.

Financial support for this research was provided by a grant from the Sulphur Institute.

TABLE OF CONTENTS

INTRODUCTION	1
LITERATURE REVIEW	4
Sulfur Availability in Soils	4
Metabolic Requirements of Sulfur	5
Sulfur Applications and Plant Responses	6
Sulfur Deficiency - Plant Analysis	8
Measures of Sulfur Deficiency in Subclover	12
MATERIALS AND METHODS	14
Field Experiment - Roseburg Site	14
Sulfur Methodology	17
Field Experiment - Corvallis Site	19
RESULTS	20
Field Experiments	20
Yield - Roseburg Site	20
Sulfur Concentration - Roseburg Site	22
Sulfur Accumulation - Roseburg Site	24
Yield - Corvallis Site	29
Sulfur Concentration - Corvallis Site	29
Chemical Analyses - N and S Determinations	31
Chemical Methods of S Determination	31
Soluble Fractions	31
Nitrogen Concentrations	35
Sulfur Concentrations	37
Critical Ranges	37
Nitrogen to Sulfur Ratios	39
Soil Reaction	43
DISCUSSION	44
Field Experiments	44
Roseburg Site	44
Corvallis Site	47
Sample Handling and Chemical Methods	47
Sulfur Determination	47
Critical Ranges	47
Nitrogen to Sulfur Ratios	51
Soil Reaction	54
CONCLUSION	57
BIBLIOGRAPHY	60
APPENDICES	68

LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Flow diagram for sample disposition in 1978.	18
2. Sulfur accumulation (cumulative minus check) of subclover/grass forage from 1975 to 1978 at the Roseburg site as influenced by source and particle size of sulfur applied at 88 kg S/ha in 1975.	27
3. Sulfur accumulation (cumulative minus check) of subclover/grass forage from 1975 to 1978 at the Roseburg site as influenced by source and particle size of sulfur applied at 176 kg S/ha in 1975.	28
4. Relationships between total N (N_t) and total S (S_t) as compared to the relationship between protein N (N^t) and protein S (S^t) for 3 sampling dates in 1978 at the Roseburg site. Points plotted are for total N and S; regression lines for N_t and S_t are those of Table 12 for oven-dried material. P	42

LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. Soil chemical properties of the Roseburg site.	15
2. Treatments applied to subclover plots at the Roseburg site.	15
3. Total dry matter forage yield over a four year period at the Roseburg site as influenced by rate, source and particle size of sulfur applied annually or as a single application.	21
4. Total sulfur concentration of subclover leaves and petioles at flowering over a four year period as affected by rate, source and particle size of sulfur applied annually or as a single application.	23
5. Sulfur accumulation of forage over a four year period as affected by rate, source and particle size of sulfur applied annually or as a single application.	25
6. Total forage dry matter yield and sulfur content of subclover leaves and petioles at three dates prior to flowering at the Corvallis site as influenced by rate and timing of gypsum applications in 1980.	30
7. Sulfur concentration of subclover leaves and petioles on April 27, 1978 as influenced by method of determination and S application as gypsum.	32
8. Comparison of total S, total TCA-soluble S and TCA-insoluble S at the March 30 sampling, each fraction independently analyzed.	33
9. TCA-soluble N and S fractions of subclover leaves and petioles in 1978 as influenced by sampling date and S fertilization as gypsum.	34
10. Nitrogen and S concentrations and N:S ratios of subclover leaves and petioles and subclover leaves and petiole fractions as influenced by S applications, sampling date, dry procedure and extractant.	36
11. Critical ranges of various S diagnostic indices of subclover leaves and petioles as influenced by sampling date in 1978.	38

12. Linear regression equations relating total N (N_t) to total S (S_t) for oven-dried subclover leaves and petioles, protein N (N_p) to protein S (S_p) for the TCA insoluble fraction^P of oven-dried and^P freeze-dried subclover leaves and petioles and N and S for the ethanol insoluble fraction of fresh^P subclover leaves and petioles at 3 dates in 1978. 40
13. The ratio of TCA-soluble non-protein organic N to TCA-soluble non-protein organic S in subclover leaves and petioles as influenced by sampling date and S fertilization. 43
14. Soil pH values in 1978 as influenced by S application in 1973 and depth of sampling. 43
15. Sulfur accumulation of forage for 1976 as affected by rate and source of sulfur applied annually. 46
16. Trichloroacetic acid soluble organic S fraction of subclover leaves and petioles as influenced by sampling date and S fertilization as gypsum. 50
17. Sulfur fractions of subclover leaves and petioles as a per cent of total S as influenced by S fertilization as gypsum and date of sampling. 55

DIAGNOSIS OF SULFUR DEFICIENCY AND SULFUR FERTILIZATION OF
TRIFOLIUM SUBTERRANEUM L.

INTRODUCTION

Subclover (Trifolium subterraneum L.) is a legume commonly grown on western Oregon hill pastures. Having originated in the Mediterranean area, it is well adapted to the hot, dry summers and cool, wet winters in western Oregon and provides high quality forage for cattle and sheep. Fertilization is critical to maintaining high levels of forage production. The amount of soil sulfur (S) is generally low in western Oregon hill pastures and responses to S fertilization are generally expected.

Before 1970 applications of ordinary superphosphate (0-20-0-10) generally provided sufficient S; the change to higher analysis phosphorus fertilizers has resulted in an increasing possibility for widespread S deficiencies, a search for other S sources, and a need for more precise definition of S requirements.

Sulfur deficiency symptoms in subclover occur as a light green to yellow coloration of the younger leaves and a general reduction in top growth; this is essentially a nitrogen (N) or protein deficiency since S deficiency limits metabolism of S-containing amino acids. Nitrogen fixation in subclover as measured by N or protein concentration is substantially reduced by S deficiency (Anderson and Spencer, 1950; Walker et al, 1956).

Subclover has shown yield responses to gypsum and elemental S both in Oregon (Jackson, 1972; Drlica and Jackson, 1979) and elsewhere (Metson, 1973; Jones and Ruckman, 1966). In Oregon the choice of elemental S is based on the economical advantage of elemental S over gypsum. Before plant uptake of S occurs elemental S must oxidize and the rate of oxidation is particle size dependent (Li and Caldwell, 1966). Larger particle sizes of elemental S oxidize slower, thus producing available S at a rate less than that produced by fine elemental S. The advantage of larger particle sizes may be

less frequent applications for adequate fertilization. The longevity of S availability of larger particle sizes must be evaluated to determine adequate but not excessive application rates. Knowledge of the S availability of different particle sizes would provide a basis for predicting response from 150 to 200 kg/ha applications of commercial elemental S material that contains a range of particle sizes. Jones and Ruckman (1969) in California have provided information on the duration of S availability of larger particle sizes of elemental S but did not evaluate smaller particle sizes.

Soil analysis (Ensminger and Freney, 1966; Reisenauer et al, 1973) has been used to determine the need for S applications but suffers the disadvantages of 1) low levels of sulfate sulfur ($\text{SO}_4\text{-S}$) in soils and the difficulty of accurate determination at these low levels and 2) the lack of complete knowledge of the contribution of the amounts and release rates of $\text{SO}_4\text{-S}$ from soil organic S fractions. An evaluation by Freney and Spencer (1967) concluded that soil testing was inferior to plant testing.

Determination of plant critical concentrations through plant tissue analysis can be used to diagnose nutrient conditions (Munson and Nelson, 1973), but factors influencing these concentrations need to be evaluated (Bates, 1971). Plant tissue analysis has been used to diagnose S deficient situations (Ensminger and Freney, 1966; Metson, 1973). Total S concentration, used as a diagnostic indicator by researchers (Jones, 1962; Seim et al, 1969; Spencer, 1978; Drlica and Jackson, 1979), often varies with plant maturity (Ensminger and Freney, 1966; Pumphrey and Moore, 1965b). This may account for the range of values reported in the total S critical level for subclover. To circumvent this problem other measures of S deficiency have been investigated. Sulfate S, $\text{SO}_4\text{-S}$:total S ratios and total N:total S ($(\text{N:S})_t$) ratios have been used as diagnostic indicators (Jones et al, 1980). Critical $(\text{N:S})_t$ ratios of legumes have been reported to average 17.5:1 (Dijkshoorn and Van Wijk, 1962) and for subclover to range from 16:1 to 19:1 (Spencer, 1980). However, $(\text{N:S})_t$ values of 25:1 for subclover at adequate

plant S levels have been observed under Oregon conditions (Drlica and Jackson, 1979). The use of $(N:S)_t$ assumes a constant protein N:protein S ($(N:S)_p$) of plants which is independent of plant maturity and S status.

Investigators have used a variety of chemical procedures to quantitatively determine S concentrations in plant samples and to extract S fractions from plant samples. The large number of different chemical procedures for both oxidation and determination of S is one indicator of the difficulty of obtaining accurate numbers in S analyses of plant tissue (Beaton et al, 1968). Additional complications arise when comparing S fractions which have been derived from different extraction procedures. Incomplete extraction of nonprotein S in the determination of $(N:S)_p$ has been discussed as a possible source for discrepancies in reported $(N:S)_p$ ratios (Freney et al, 1977). The small number of papers published in American Society of Agronomy publications since 1980 is probably a good indication of the frustration associated with trying to improve these technologies.

This study was undertaken to:

- 1) determine critical levels for different S fractions,
- 2) evaluate the effect of sample handling techniques on S fractions,
- 3) measure the response of subclover to S applications,
- 4) extend the work of Jones and Ruckman (1969) in evaluating the availability and longevity of different particle sizes of elemental S and
- 5) determine the effectiveness of S fractions in identifying S deficiency.

LITERATURE REVIEW

Sulfur Availability in Soils

Organic S comprises the largest reservoir of S in most soils (Whitehead, 1964). Organic S is oxidized to $\text{SO}_4\text{-S}$ before it becomes plant available. This is accomplished by the combined actions of a number of organisms: heterotrophic bacteria, actinomycetes and fungi (Gray and Williams, 1971). The release of $\text{SO}_4\text{-S}$ from organic matter is a complex phenomenon and release rates are not well defined (Freney and Stevenson, 1966; Freney and Swaby, 1975). Sulfur mineralization has some similarities to N mineralization, but the C:N:S ratio of the organic fraction of soils does not always indicate that the released nitrate to released sulfate ratio will correspond to the C:N:S ratio. If a soil is initially low in $\text{SO}_4\text{-S}$ and organic matter low in S content is added, $\text{SO}_4\text{-S}$ will be incorporated in humus production. This can occur under pasture conditions where organic matter is accumulating (Walker, 1955).

Inorganic S is the initial source of all S in the soil system. Inorganic S found as sulfides of iron, copper and nickel in igneous rocks can be oxidized through natural chemical weathering processes. Under appropriate environmental conditions, members of the genus Thiobacillus are a more important pathway of oxidation of sulfides. Sulfates are found in sedimentary rocks and availability is controlled, in part, by solubility. Potassium, magnesium and sodium sulfates are readily soluble, calcium sulfate is poorly soluble and barium sulfate is very insoluble.

Sulfate retention in soils is a function of kaolinitic clay mineral content, aluminum and iron oxide content, soil solution ionic strength and soil pH. Retention may be a result of 1) electrostatic attraction (ordinary anion exchange) due to positive charges on silicate minerals or 2) a ligand exchange of SO_4 for hydroxide (or H_2O) on aluminum or iron oxides or edges of silicate minerals (Gast, 1977). Organic matter may have some influence on the retention mechanism in soils (Chao et al., 1962). Lowering the soil pH

results in greater sulfate sorbtion (Ensminger, 1954; Harward and Reisenauer, 1966). Phosphate has greater retention in soils than $\text{SO}_4\text{-S}$ and is able to displace $\text{SO}_4\text{-S}$ from soil colloids (Mehlich, 1981).

Metabolic Requirements of Sulfur

Sulfur had been shown to be an essential element by Sachs and Knop in Germany in the middle of the 19th century, but its specific biochemical functions in cell metabolism as a constituent of organic compounds is a more recent discovery. After entering the plant as the sulfate ion, S is consecutively incorporated into amino acids and proteins (Kirkby and Mengel, 1978). Quantitatively, the main amino acids are cysteine and methionine; homocysteine and cystathionine are present in smaller amounts (Giovanelli et al, 1980; Thompson et al, 1970). The ferredoxin proteins are important compounds in the transfer of electrons in the light reactions of plants and as a reducing agent in the nitrogen fixation process. They contain S as cysteine and as part of a proposed iron-S linkage (Hughes, 1972). Another protein, coenzyme A, has a thiol group (-SH) which reacts with carboxylic acids to form thioethers which are critical reactive forms in processes such as amino acid synthesis and the tri-carboxylic acid cycle.

Other compounds are important S-containing metabolites. Glutathione, lipoic acid, biotin, sulpholipid and thiamine pyrophosphate perform essential functions in various cell reactions (Anderson, 1978).

Protein synthesis requires N and S. Each plant protein has a genetically determined chemical structure and thus a specific $(\text{N:S})_p$ ratio. If there is no relative change among different plant proteins as the plant ages, a constant $(\text{N:S})_p$ for plant material should result. When available S is insufficient for plant needs, non-protein N, mainly in the form of amino acids for legume species, accumulates and the $(\text{N:S})_t$ ratio increases in comparison to $(\text{N:S})_p$. When the S supply is in excess, $\text{SO}_4\text{-S}$ and/or S-amino

acids accumulate and the $(N:S)_t$ ratio is lowered in comparison to $(N:S)_p$.

Sulfur Applications and Plant Responses

Acknowledgement that S had been overlooked as a necessary fertilizer was made over forty years ago by Alway (1940). Although S fertilizer usage has increased, the importance of S as a fertilizer is still not universally recognized (Amen and Dixon, 1979). However, as early as 1923 Powers (1923) noted response of clovers to S applications on Western Oregon hill pastures. The responses were associated with soils derived from basaltic parent material. More recent work (Pumphrey and Moore, 1965b; Dawson, 1969) has shown a variety of Western Oregon soils to be S deficient in a pasture situation. Basaltic soils in Australia have also been shown to be S deficient (Spencer, 1966).

Applications of S have become more critical as a result of several factors:

- 1) Combustion of low-S fuels in place of high-S fuels and increased pollution controls on low-S fuels.
- 2) Decline in the use of S as a fungicide.
- 3) Increased yields extracting more S from soils.
- 4) Increased use of high-analysis fertilizers and less use of fertilizers such as ordinary superphosphate (13% S) and ammonium sulfate (24% S).

Gypsum ($CaSO_4$) has been commonly applied as a source of S. Although low, the solubility of gypsum is adequate to provide available S for growing plants. The availability of sulfate from gypsum may be lost through incorporation into organic matter or via leaching. The rate at which sulfate is lost through leaching would be influenced by rainfall and the retention capacity of the particular soil.

Yield responses of legumes to applications of gypsum have been noted in various parts of the United States (Metson, 1973), in Ireland (Murphy, 1978; Hanley and Murphy, 1970) and responses in Australia and New Zealand have been summarized (McLachlan, 1974;

McLachlan, 1975). In Oregon, responses to gypsum have been observed on subclover (Jackson, 1972; Drlica and Jackson, 1979) and alfalfa (Pumphrey and Moore, 1965b). Annual application rates of gypsum to achieve maximum yields have been reported for grass/clover pastures and generally fall in the range of 22-45 kg S/ha (Spencer, 1963; Bentley and Green, 1954; Jones, 1964; McLachlan and DeMarco, 1968a; McLachlan and DeMarco, 1968b; McLachlan and DeMarco, 1971; McLachlan and DeMarco, 1973; McNaught and Chrisstoffels, 1961). Often, these or higher rates give variable residual responses resulting in increased yields from one to six years after application (Jones et al., 1970; Walker and Adams, 1958; McLachlan and DeMarco, 1968; Bentley and Green, 1954; Spencer, 1963; McLachlan and DeMarco, 1971).

Elemental S has been successfully used to supply S to S deficient legumes (McLachlan and DeMarco, 1968; Bentley and Green, 1954; Fox et al., 1964; Jones et al., 1970; Jones and Ruckman, 1966). Oxidation of elemental S is required before accumulation can occur. The rate of oxidation is dependent upon surface area and in turn, upon particle size (Attoe and Olson, 1966; Li and Caldwell, 1966; Burns, 1967; Fox et al., 1964). Larger particle sizes with smaller surface area/unit weight have slower oxidation. This effect of particle size has been translated into yield differences with application of coarser sizes (particle size diameter = 0.84 - 2.00 mm) in a field experiment resulting in small or no yield increases in the first one to three years (Jones and Ruckman, 1969). Elemental S having particle sizes of 0.25 - 0.61 mm (diam.) was not as effective as particle sizes of 0.15 mm or less in increasing the DM yield of white clover (Weir et al., 1963). The initial disadvantage of using larger particle sizes can be offset by a substantial residual value in later years (Jones and Ruckman, 1969).

Direct comparison of gypsum and elemental S have generally shown gypsum to be more effective as a S fertilizer than coarser particle sizes of elemental S in the first year of growth, but less effective in subsequent years (Jones and Ruckman, 1966; Jones and

Ruckman, 1969). Where more than 70% of the elemental S had a particle size diameter of 0.25 mm or more, applications of elemental S resulted in less lucerne production than gypsum applications (Ludecke, 1965). If applied early (approximately 6 months before planting or resumption of rapid pasture growth) elemental S in a flour form or fine enough to pass a 100 mesh screen is equivalent to gypsum in supplying available S (Jones and Ruckman, 1966).

Rainfall is a complicating factor in field studies comparing the effects of gypsum and elemental S. If moisture is too limited oxidation of elemental S could be restricted (Attoe and Olson, 1966). Under such conditions gypsum is superior to elemental S as a S fertilizer in the first year (Williams et al., 1964; McLachlan and DeMarco, 1968a). Under higher rainfall conditions gypsum and elemental S perform equally well in the first year (Jones, 1966); succeeding years often show less effect from gypsum due, at least in part, to leaching of the gypsum (Jones, 1966). Determination of what constitutes a leaching environment is difficult. Sinclair and Saunders (1982) assigned a value of zero to a rainfall leaching index if the rainfall was less than 500 mm. Studies where rainfall was greater than 760 mm experienced leaching of SO_4 from gypsum (Powrie, 1967; McKell and Williams, 1960). McLachlan and DeMarco established that 410-690 (1971) and 460-690 (1968) mm of rainfall were non-leaching situations. Western Oregon, with an average of 1020 mm or more of annual rainfall, would generally be considered a leaching environment.

In a grass-clover pasture the effect of added S is different for each component. Since grass has a competitive advantage for available S (Walker, 1957), situations of S deficiency result in grass-dominated pastures (Walker and Adams, 1958; McLachlan and DeMarco, 1973).

Sulfur Deficiency - Plant Analysis

Sulfur is present in the plant as sulfate, amino acids, and/or proteins. Any of these S fractions or total S may be analyzed to

diagnose S deficiency. And as the levels of these S fractions are subject to change with time and location in the plant (Spencer et al., 1977; Metson and Collie, 1972), sampling and interpretation of plant analysis results must recognize these variations.

Total S concentration in plants has been related to yield for a variety of crops and critical levels or ranges have been established (Ensminger and Freney, 1966; Eaton, 1966). Plant analysis for total S has the advantage of being a single analysis, but suffers from being dependent on plant maturity. There is apparently no consistent pattern to changes in S content over time; some species increase, some decrease and others remain stable in S concentration as they mature (Ensminger and Freney, 1966; Eaton, 1966).

Sulfate-S has been used as a diagnostic indicator of S deficiency (Ulrich et al., 1967; Jones and Martin, 1964; Spencer et al., 1977; Ensminger and Freney, 1966). Sulfate-S accumulates in the plant under conditions where S is more than adequate to meet plant growth requirements and is incorporated in amino acids and proteins under S deficient conditions. The yield of various forage crops has been related to $\text{SO}_4\text{-S}$ levels (Metson, 1973; Martin and Matocha, 1973). However, as with total S, the plant level of $\text{SO}_4\text{-S}$ varies over a growing season (Jones, 1963; Spencer et al., 1977; Metson, 1972). Over a shorter time period (0-32 days) $\text{SO}_4\text{-S}$ concentration is more constant (Jones, 1962; Smith and Dolby, 1977). The disadvantages of using $\text{SO}_4\text{-S}$ for determining plant S status are generally low concentrations of $\text{SO}_4\text{-S}$ for analysis, difficulty in determining whether all $\text{SO}_4\text{-S}$ has been extracted (Freney et al., 1977), or elaborate equipment (Johnson and Nishita, 1952). If the Johnson and Nishita procedure (1952) or a similar technique involving reduction via hydriodic acid is used for $\text{SO}_4\text{-S}$ detection, the S so reduced is often referred to as "reducible S."

Nitrogen to S ratios have shown promise in the diagnosis of S deficiency. Correlations of $(\text{N:S})_t$ with yield have been as good as or better than other measures of S deficiency (Smith and Dolby, 1977; Stewart and Whitfield, 1965). Total N to S ratios appear to

be less dependent on plant maturity than other measures of S deficiency (Spencer et al., 1977; Pumphrey and Moore, 1965a). By comparing $(N:S)_t$ to $(N:S)_p$ ratios an indication of plant S status may be obtained. An excess of S would lower the $(N:S)_t$ ratio below the $(N:S)_p$ ratio thereby indicating adequate S nutrition. A high N concentration in the plant would result in a higher $(N:S)_t$ ratio thus indicating a false S deficiency. Excess N would not accumulate in a legume where N is being provided mainly through N fixation, but could lead to erroneous conclusions for grass species (Smith and Dolby, 1977).

As discussed earlier, the theoretical basis of using $(N:S)_t$ is based on the concept of a constant $(N:S)_p$. Average $(N:S)_p$ ratios of 17.5 for legumes and 13.7 for grasses have been obtained from a survey of literature data (Dijkshoorn and Van Wijk, 1967). Other work has supported the concept of a constant $(N:S)_p$ ratio (Freney et al., 1977; Stewart and Porter, 1969; Kelly and Lambert, 1972; Stewart and Whitfield, 1965). Conversely, there is evidence that this ratio will decrease with time (Leggett et al., 1966) and increasing fertilization level (Metson and Collie, 1972; Jones et al., 1971; Gaines and Phatak, 1982). In addition to possible effects of S fertilization, it is conceivable that many of the discrepancies in reported $(N:S)_p$ ratios are a consequence of different extraction techniques. This has been a problem for researchers in determining whether the differences in reported $(N:S)_p$ ratios were real or due to incomplete extraction, protein degradation or incomplete precipitation of protein. Several different extraction methods are:

- 1) 0.5 gram oven-dried material boiled for 9 minutes with 33ml of 70% ethanol (ETOH), filtered and washed with ETOH until 100 ml of effluent is obtained (Westerman, 1975).
- 2) 0.3 gram oven-dried material extracted with 10 ml 0.5% acetic acid (80°C) for 20 minutes and centrifuged (5 min at 2500 rpm); tissue was re-extracted with the procedure (Gaines and Phatak, 1982).

- 3) oven-dried material extracted with 2% acetic acid to obtain $\text{SO}_4\text{-S}$; separate sample extracted with 5% TCA to obtain protein S. No mention of quantities, time or temperatures (Cairns and Carson, 1966).

Variations in extraction times have shown an effect on the amount of extractable S (Freney et al., 1977).

The effect of plant sampling and handling could influence the magnitude of S fractions through conversion of protein S to non-protein S. Work with phosphorus (P) has demonstrated changes in phosphate-P values from oven-drying as compared to freeze-drying (Mayland et al., 1978). Dry matter can be lost through oven-drying (Mayland, 1968) or air-drying (Handreck, 1972) and this would affect the value of any S fraction which is normally expressed on a dry weight basis. Using ratios eliminates effects of dry matter loss (assuming no volatilization of the element in question), but would indicate any conversion between fractions. Unpublished results quoted by Mayland et al. (1978) showed no effect of oven-drying on $\text{SO}_4\text{-S}/\text{total S}$ ratios.

Determination of S content of any fraction generally consists of two steps:

- 1) oxidation of organic-S to $\text{SO}_4\text{-S}$ and,
- 2) measurement of $\text{SO}_4\text{-S}$.

Step one is generally accomplished by either dry ashing, wet ashing or using a combustion flask (Beaton et al., 1968). Wet ashing with nitric and perchloric acids has resulted in underestimation of S content when compared to the combustion flask method of oxidation (Randall and Spencer, 1980). As with wet ashing, dry ashing may be criticized for being an open system, thus subject to volatilization losses, but problems have also occurred due to incomplete oxidation (Butters and Chenery, 1959).

Most determinations of $\text{SO}_4\text{-S}$ have employed one of four methods: gravimetry, turbidimetry, titrimetry or colorimetry (Beaton et al., 1968). The methylene blue colorimetric procedure of Johnson

and Nishita (1952) and various turbidimetric techniques (Beaton et al., 1968) have been commonly used in plant analysis.

Measures of Sulfur Deficiency in Subclover

Sulfur deficiency in subclover has been observed in Australia (Spencer, 1978), California (Jones, 1962; Jones, 1964) and Oregon (Jackson, 1972). To accurately diagnose the deficiency situation, plant analysis has been employed. Total S, $\text{SO}_4\text{-S}$, $\text{SO}_4\text{-S}/\text{total S}$ and $(\text{N:S})_t$ ratios have all been used to evaluate the S status of subclover (Jones et al., 1980; Spencer, 1978; Spencer et al., 1977).

Total S concentration has the advantage of a single step analysis and has been used as a diagnostic indicator by researchers (Bouma et al., 1969; McLachlan and DeMarco, 1968b; Spencer and Bouma, 1970; Spencer et al., 1977; Jones, 1962). The range of critical levels determined by these investigations is rather large, 0.07 - 0.25%. Comparisons are complicated by differences in environment (greenhouse versus field), plant part (old and new leaves, petioles, whole plants) and plant maturity (pre-flowering to harvestable). In field situations levels of total S may increase, decrease or both in the same season (Spencer, 1978). And this is apparently independent of S fertilization. Different plant parts contain different levels of S (Spencer et al., 1977), thus sampling procedures should be defined. In greenhouse studies whole plants are usually analyzed (Jones, 1962), while field studies generally use whole plants (Bouma et al., 1969; Spencer, 1978) or leaflets and petioles (Drlica and Jackson, 1979; Jones and Martin, 1964). Although field critical levels may have more practical applicability, variations in weather can be a problem in identifying critical levels. A difference of 0.04% in critical concentrations was noted for two consecutive years by Bouma et al. (1969).

With the advantages of a single analysis and elimination of oxidations problems, $\text{SO}_4\text{-S}$ has been investigated as a possible indicator of S status (Jones, 1962; Jones, 1963; Bouma et al., 1969).

Critical concentrations of 140 mg S/kg DM (Jones et al., 1980) and 170 mg S/kg DM (Jones, 1962) SO_4 -S have been reported for sub-clover at the flowering stage of growth. An early season value of 300 mg S/kg DM was given by Bouma et al. (1969). Sulfate-S concentrations generally decrease until flowering (Spencer et al., 1977; Jones and Martin, 1964), but appear to be more constant during flowering (Jones, 1962).

High levels of SO_4 -S in the plant might indicate a luxury consumption situation if the plant had a large amount of total S, but could be indicative of other nutritional problems if it represented a large proportion of the total S. Thus the ratio, SO_4 -S/total S, has been used as a diagnostic indicator for subclover (Jones et al., 1980). A critical range of 0.08 to 0.11 for this ratio was as constant or more constant over a 133 day growing period than other measures of S deficiency (Spencer et al., 1977).

The $(\text{N:S})_t$ ratio is another measure of S status for subclover (Jones et al., 1980; Spencer, 1978; Spencer et al., 1977). A greenhouse study demonstrated that the critical $(\text{N:S})_t$ ratio was dependent on the time of sampling and ranged from 18 to 23 (Jones et al., 1980). Clipping also affected the ratio in this study. A field study found the critical ratio to lie somewhere between 16 and 19 (Spencer, 1978). Under the assumption of a constant $(\text{N:S})_p$ ratio, a simple comparison of $(\text{N:S})_t$ to $(\text{N:S})_p$ would indicate S sufficiency/S deficiency. This assumption has held up in one greenhouse experiment (Freney et al., 1977). It was noted in this same study that variations in extraction techniques produced differences in amounts of extractable S and N compounds.

MATERIALS AND METHODS

Field Experiment - Roseburg Site

Sulfur was applied as either elemental S or gypsum to an established subclover-grass pasture near Roseburg, Oregon. This pasture was composed of Nangeela subclover (Trifolium subterraneum L.) and several grass species. This pasture had previously received single superphosphate (0-20-0-10) at 200 kg/ha in alternate years for approximately ten years. Under these conditions the pasture would not be expected to be severely S deficient, thus avoiding the incorporation of large amounts of S into organic matter production which occurs in severely S deficient pasture situations. The soil was a complex of the Nonpareil (member of the fine, loamy, mixed, mesic, shallow family of Dystric Xerochrepts) and Oakland (member of the fine, mixed, mesic family of Ultic Haploxeralfs) soil series, both developed from sedimentary parent material located on lower elevations in the Roseburg area. Soil depth varied from 40 to 60 cm. Soil chemical properties are summarized in Table 1. Rainfall for the experimental time period is presented in Appendix C-1. The 1.8 x 6 m individual plots were arranged in a randomized block design with 5 replications. Rates of application and particle sizes are listed in Table 2. The 11, 22 and 44 kg S/ha rates were applied annually beginning in fall, 1974. The 88 and 176 kg S/ha rates were applied in September, 1974 only. All plots received molybdenum at 1 kg/ha and boron at 2 kg/ha in the first year; phosphorus at 29 kg/ha was applied each year. Potassium (K) deficiency symptoms occurred in the first year and were confirmed by plant analysis. Therefore, K was applied annually at 100 kg K/ha. Annual harvests were taken for a four year period using a mechanical harvester cutting a 1 m swath from the center of each plot. Moisture samples were taken to determine dry matter content. Dry matter yield data was analyzed using a split plot in time design and also a randomized block design for each year.

Table 1. Soil chemical properties of the Roseburg site.

Soil	Location	pH	SMP	CEC	Ca	Mg	K	P
		-----meq/100g-----					---ppm---	
Nonpareil- Oakland	SW 1/4, Sec 28, T 26 S, R 4 W	5.8	6.3	14.1	7.9	2.2	250	16

Table 2. Treatments applied to subclover plots at the Roseburg site.

S source	¹ Rates	² Particle diameter
	kg S/ha	mm
gypsum, S(gyp)	11, 22, 44, 88, 176	---
fine S, S(f)	11, 22, 44, 88, 176	less than 0.25
medium fine, S(mf)	88, 176	0.50 - 0.59
medium coarse, S(mc)	88, 176	0.70 - 0.83
coarse, S(c)	88, 176	1.00 - 2.00

1 - 11, 22, and 44 kg S/ha applied annually; 88 and 176 kg S/ha applied in fall 1974 only.

2 - S(f) passed a 60 mesh screen; S(mf) passed a 30 mesh screen but not a 35 mesh screen; S(mc) passed a 20 mesh screen but not a 25 mesh screen; S(c) passed a 10 mesh screen but not a 18 mesh screen.

Plant samples (approximately 25-50g fresh weight) were collected at random from plots at flowering (subclover leaves and petioles) and at harvest (total forage) for each of the four years. These samples were dried at 70°C for 48 hours analyzed for total S using a dry ash for oxidation and a turbidimetric technique for determination (Appendix A). Sulfur accumulation was calculated for the four year period and analyzed in the same manner as dry matter yield.

In 1978 in addition to those samples taken at flowering, samples were taken approximately 2 and 4 weeks prior to flowering. Separate samples from all 3 dates had the following disposition:

- 1) placed in paper bags at 25°C for 12 hours, then oven-dried at 70°C for 48 hours.
- 2) immediately placed on dry ice (-78°C) for 6-8 hours, then cooled in a dry ice-acetone mixture and freeze-dried.
- 3) five to 20 grams of fresh material was immediately placed in approximately 25 mls of 70% ethanol and boiled for 5 minutes; 48 hours later the plant material was captured on filter paper and washed with two 25 ml aliquots of 70% ethanol. Residue was oven-dried (70°C).

For oven-dried and freeze-dried material a sample was taken from each of five replications; for ethanol extraction, samples from four replications were taken and combined into two samples per treatment.

Oven-dried samples from all 3 sampling dates were ground in a Wiley mill to pass a 40 mesh screen and were analyzed for total N via a Kjeldahl technique (Jackson, 1958) and for total S via a combustion flask (Appendix A). A 0.3 gram sample was shaken for 30 minutes in 10 ml of a 5% (w/v) trichloroacetic acid (TCA) solution after which the solution was filtered and the residue washed with 10 ml of 5% TCA solution and 2 10 ml aliquots of distilled water. The residue (protein) was oven dried for 48 hours and ground to pass a 40 mesh screen and analyzed for S using oxidation flasks and turbidity and for N using a micro-Kjeldahl technique (Appendix A). The ex-

tract was brought to 50 ml in a volumetric flask. A 2 ml aliquot was analyzed for SO_4 -S using a methylene blue technique (Appendix A); a 5 ml aliquot was analyzed for NO_3 -N (Appendix A); a 5 ml aliquot was analyzed for total soluble N (non-protein organic N) using a micro-Kjeldahl procedure; a 35 ml aliquot was analyzed for total soluble S (non-protein organic S) by evaporating to near dryness before dry ashing. The freeze-dried material was treated similarly but only the residue was analyzed for N and S. Prior to each extraction for oven-dried and freeze-dried material, the filter paper was dried in a dessicator for a minimum of 7 days and weighed. Following the extraction the oven-dried paper plus residue was weighed to determine loss of dry matter. The residue from the alcohol extraction was analyzed for N and S. These procedures are summarized in a flow chart (Figure 1). Analyses of variance were computed for all fractions using a strip block in time design (Appendix B-6).

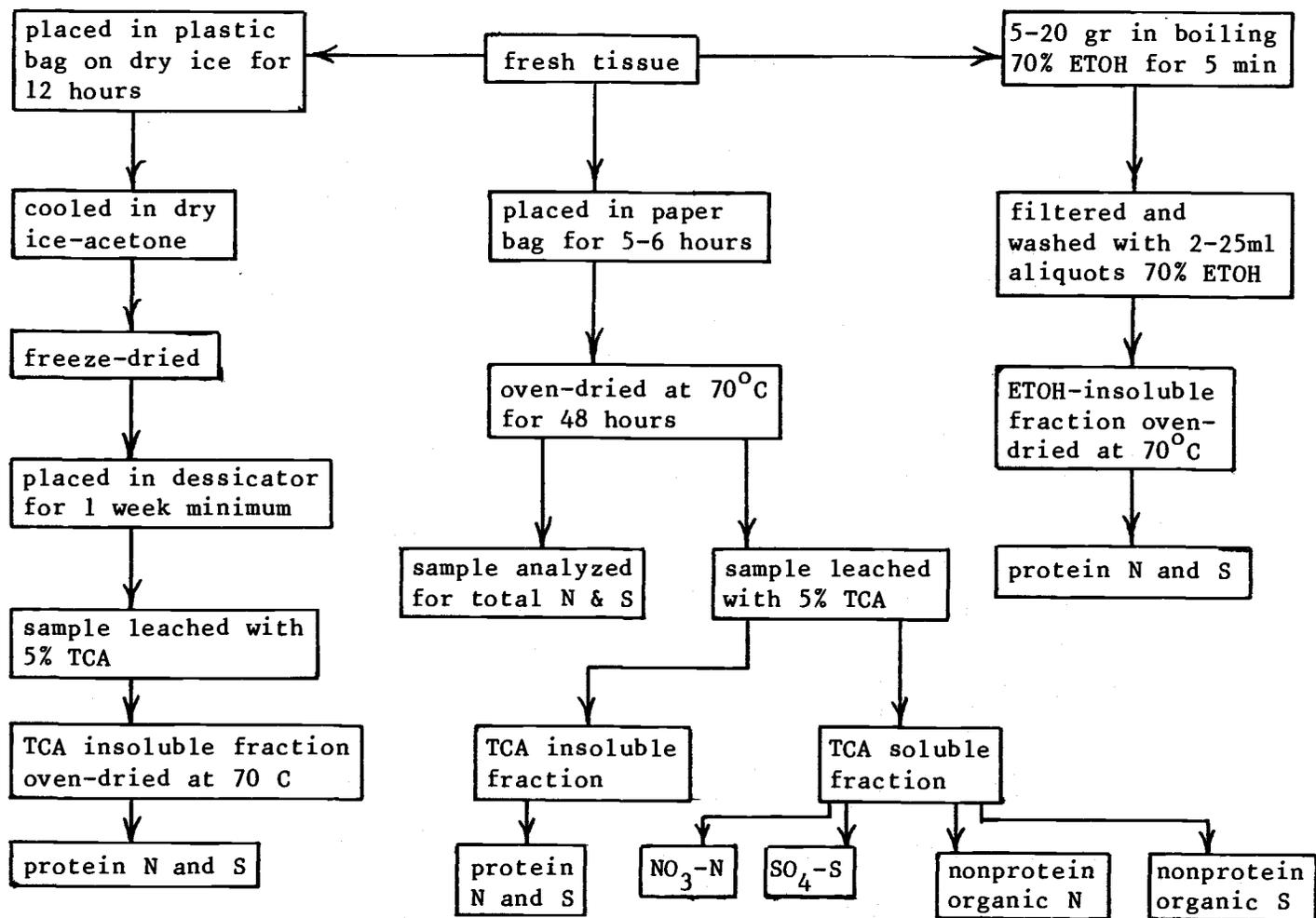
Sulfur Methodology

Plant samples from the April 27, 1978 sampling at the Roseburg site were analyzed for sulfur using four different techniques (Appendix A):

- 1) combustion of plant material in an oxidation flask and sulfate determination via turbidity,
- 2) combustion of plant material in an oxidation flask and sulfate determination via methylene blue,
- 3) dry ash combustion of plant material in an oxidation flask and sulfate determination via methylene blue and
- 4) dry ash combustion of plant material and sulfate determination via turbidity.

Results of comparisons among the four methods led to the selection of method (1) for all subsequent S analyses.

Figure 1. Flow diagram for sample disposition in 1978.



Field Experiment - Corvallis Site

Plots measuring 2.1 x 6.1 m were established near Corvallis, Oregon on an established subclover-grass pasture and had seasonal S treatments as gypsum applied for 2 years beginning in fall, 1978. The treatments are listed in Table 8. The treatments were replicated 4 times in a randomized block design.

Severe winter kill in 1978-1979 resulted in a grass dominated pasture with little or no clover. Plant samples and yield data were not taken in 1979. Subclover resumed growth in 1980 from seed naturally present in the soil and a good stand was achieved. On May 1, 1980 a 50 cm swath was harvested from the center of each plot and moisture samples taken to determine dry matter yield. Plant samples from 3 dates in 1980 were analyzed for total S using oxidation flasks and turbidity (Appendix A).

RESULTS

All S applications resulted in increases in four year cumulative dry matter (DM) yield and S accumulation. Within specific years the increases in DM yield were variable, whereas S accumulation data were more consistent. Therefore, S accumulation provides a better evaluation of the S availability of different particle sizes of elemental S. Subsequent sections deal with each topic individually - DM yield, S concentration and S accumulation - with a special focus on S accumulation in comparison of the effects of different particle sizes of elemental S on the availability of S. In these sections all references to 88 and 176 kg S/ha refer to single application in September, 1974 and are not the sum of annual treatments.

The plant analysis results for 1978 (Roseburg site) demonstrated an effect of S fertilization on all indices of S status. However, the effects of plant maturity were not the same on all S measurements. Protein N:S ratios were dependent upon method of sample handling and extraction technique, but regardless of method were influenced by plant maturity. All indices and extraction techniques are discussed in detail in later sections.

Field Experiments

Yield - Roseburg Site

In 1975 dry matter (DM) yield increases occurred with application of gypsum at 11, 44, 88 and 176 kg S/ha; application of S(f) resulted in increased DM yields at 44, 88 and 176 kg S/ha (Table 3). Coarser particle sizes of elemental S did not affect DM yield. In 1976 all treatments were effective in increasing DM yields. Annual applications of gypsum or S(f) at 44 kg S/ha resulted in the highest yields, 7.94 and 8.11 metric tons/ha, respectively. Again in 1977 all treatments were effective in increasing yields. Highest DM yields were achieved at 88 kg S/ha of gypsum and 176 kg S/ha of S(c).

Table 3. Total dry matter forage yield over a four year period at the Roseburg site as influenced by rate, source and particle size of sulfur applied annually or as a single application.

Source	¹ Rate	1975	1976	1977	1978	cumulative
	kg S/ha	-----metric tons/ha-----				
check	0	5.11	5.46	3.68	4.88	19.13
gypsum	11	5.80	7.52	4.99	6.77	25.08
	22	5.19	6.78	4.38	6.95	23.30
	44	5.87	7.94	5.05	7.11	25.97
	88	5.97	6.41	5.73	6.13	24.23
	176	5.66	7.14	4.99	6.28	24.07
S(f)	11	4.81	7.06	5.52	6.17	23.55
	22	5.30	6.88	4.15	6.33	22.67
	44	5.68	8.11	5.43	7.62	26.84
	88	5.52	7.35	5.21	5.27	23.35
	176	5.68	7.56	5.39	5.97	24.60
S(mf)	88	4.68	7.28	5.29	6.18	23.43
	176	5.09	7.29	4.66	6.89	23.92
S(mc)	88	5.28	6.79	4.98	5.95	23.00
	176	5.18	6.76	5.46	5.91	23.31
S(c)	88	5.45	7.37	4.97	5.22	23.01
	176	5.43	6.47	5.70	6.89	24.48
LSD(.05)		0.37	0.25	0.26	NS	2.99

¹ 11, 22 and 44 kg S/ha applied annually; 88 and 176 kg S/ha applied in Sept., 1974 only.

The overall yields in this year were low due to the extremely low rainfall during the winter period (Appendix C-1). The clover experienced moisture stress early in the following spring growing season. The F-test for treatment differences was not significant in 1978. Gopher infestation adversely affected yield during the last year this experiment was maintained; plant destruction and dirt mounds introduced variation in the yield data. Elemental S at 44 kg S/ha produced the greatest yield, 7.62 metric tons/ha.

Cumulative data show all single applications to be equal in their effect on DM yield. Annual applications of S(f) at 44 kg S/ha increased cumulative yield more than any other treatment. At the 5% probability level, there was equal response within the three annual rates of gypsum; for elemental S, the highest annual rate was superior to the two lower rates.

Sulfur Concentration - Roseburg Site

In the first year, sulfur concentration of subclover leaves and petioles was only increased significantly ($P = 0.05$) by application of S at 88 or 176 kg S/ha (Table 4). After 1975 all annual rates except for 11 kg S/ha in 1976 were effective in increasing the S concentration. The lack of response in 1975 may have been due to a moderate K deficiency or less than optimum S applications as evidenced by the increase in S concentration at 88 kg S/ha. Response from annual applications of S(f) was similar to response from annual applications of gypsum.

Single applications of gypsum increased S concentration of plant samples for the four year period; however, the annual applications were more effective during the third and fourth years. During the first year applications of S(f) produced the largest increases, 0.08 and 0.13 per cent for 88 and 176 kg S/ha respectively.

Coarser materials were not effective in increasing S concentration in 1975, but did result in increases in S concentration in 1976 and later. The largest gains in S concentration from coarser particle sizes ($>S(f)$) occurred in the last two years. In these years

Table 4. Total sulfur concentration of subclover leaves and petioles at flowering over a four year period as affected by rate, source and particle size of sulfur applied annually or as a single application.

Source	¹ Rate	1975	1976	1977	1978
	kg S/ha	-----S, %-----			
check	0	0.16	0.20	0.13	0.14
gypsum	11	0.17	0.20	0.15	0.18
	22	0.17	0.23	0.20	0.21
	44	0.18	0.23	0.25	0.21
	88	0.20	0.22	0.15	0.18
	176	0.20	0.22	0.16	0.19
S(f)	11	0.16	0.22	0.15	0.19
	22	0.16	0.23	0.18	0.19
	44	0.19	0.24	0.26	0.23
	88	0.24	0.24	0.17	0.19
	176	0.29	0.26	0.21	0.22
S(mf)	88	0.16	0.22	0.18	0.19
	176	0.16	0.24	0.23	0.25
S(mc)	88	0.16	0.21	0.17	0.22
	176	0.16	0.25	0.20	0.20
S(c)	88	0.17	0.22	0.15	0.17
	176	0.16	0.21	0.17	0.21
LSD(.05)		0.03	0.02	0.02	0.03

¹ 11, 22 and 44 kg S/ha applied annually; 88 and 176 kg S/ha applied in fall, 1974 only.

the S concentration from the S(mf) application was greater than or equal to any other particle size material. The S(mf), S(mc) and S(c) treatments were generally as effective or more effective in increasing S concentration in 1978 than annual treatments that had received the same amount of S over 4 years.

Sulfur Accumulation - Roseburg Site

Treatment effects on S accumulation are presented in Table 5. Cumulative data show that all treatments increased S accumulation, but distribution of this accumulation over the four year period was not the same for all treatments as is evidenced by a significant treatment by year interaction (Appendix B-2). In 1975 rates of S(f) or gypsum above 44 kg S/ha were statistically significant in increasing S accumulation. Except for S(mf) at 176 kg S/ha none of the coarser elemental S materials were effective (not statistically significant at $P = .05$) in increasing S accumulation. By 1976 and continuing through the two remaining harvest years, the coarser particle size materials resulted in increased yields and S removal.

In 1975 the response to application of gypsum the previous fall was a rate response; application of 176 kg S/ha gave the highest S accumulation. In subsequent years the 88 and 176 kg S/ha rates were less effective than annual rates of gypsum in increasing S accumulation.

Sulfur accumulation in 1975 with application of S(f) was also a rate response. Although uptake at 88 and 176 kg S/ha was the greatest of any treatment in 1975, this represents luxury accumulation. In each of the three remaining years annual application of 44 kg S/ha of S(f) resulted in the greater S accumulation than either single S(f) application. In terms of S accumulation, the single application of S(f) at 88 kg S/ha was statistically equal to the 22 kg S/ha rate in 1977 and to the 11 kg S/ha rate in 1978.

Of the single applications of elemental S at 176 kg S/ha, S(f) had the greatest S accumulation in 1975, but the lowest in 1978

Table 5. Sulfur accumulation of forage over a four year period as affected by rate, source and particle size of sulfur applied annually or as a single application.

Source	¹ Rate	1975	1976	1977	1978	Cumulative
	kg S/ha	-----kg S/ha-----				
check	0	5.25	2.56	2.74	5.27	15.81
gypsum	11	6.73	5.27	4.98	6.95	23.92
	22	6.66	6.12	7.17	6.75	26.70
	44	8.14	9.84	9.10	11.77	38.85
	88	10.85	5.31	4.95	7.56	28.68
	176	11.19	7.51	5.36	7.94	31.99
S(f)	11	4.91	6.08	5.29	7.24	23.52
	22	6.61	7.02	5.69	10.04	29.37
	44	7.82	12.69	11.43	15.11	47.06
	88	13.25	9.10	6.34	7.13	35.83
	176	17.26	9.93	10.47	9.42	47.08
S(mf)	88	5.85	6.30	6.37	9.55	28.07
	176	8.43	10.43	7.29	12.73	38.88
S(mc)	88	6.73	5.63	5.31	12.15	29.82
	176	6.66	5.58	7.38	11.39	31.01
S(c)	88	6.77	4.30	4.01	7.89	22.98
	176	5.96	4.24	5.38	15.00	30.58
LSD(.05)		1.55	1.66	1.61	2.28	3.96

¹ 11, 22 and 44 kg S/ha applied annually; 88 and 176 kg S/ha applied in fall, 1974 only.

while the reverse was true for S(c). The two coarser particle sizes had their greatest effect on increasing S accumulation in 1978.

Total four year data indicate equal S accumulation for annual applications of S(f) and gypsum at 11 kg S/ha, but S(f) providing for greater S accumulation at 22 (not statistically significant) and 44 kg S/ha. Sulfur accumulation from gypsum application at 176 kg S/ha was less than the 44 kg S/ha annual rate; however, at 88 kg S/ha S accumulation was statistically equal to the 22 kg S/ha rate. A single application of S(f) at 176 kg S/ha was equivalent to 4 years of an annual application of S(f) at 44 kg S/ha. This treatment showed the largest S accumulation of any single application of S. At 88 kg S/ha the 4 year total S accumulation was greater than the 22 kg S/ha annual rate. The two finer fractions of elemental S at 176 kg S/ha resulted in greater S accumulation than the two coarser fractions.

Statistical evaluation of the single application sources found there existed a significant source by year interaction (Appendix B-2). This evaluation of single S applications is more easily visualized by subtracting the check plots and preparing a cumulative graph (Figure 2 for 88 kg S/ha). At 88 kg S/ha application of S(f) resulted in less accumulation in each succeeding year. Sulfur as gypsum resulted in a more constant accumulation of 2.42 kg S/ha/yr averaged over the 4 year period. Sulfur(mf) also gave a nearly constant accumulation of 3.88 kg S/ha/yr, large enough that accumulation was almost equal to that from gypsum in 1978. Both S(mc) and S(c) produced linear S accumulation until the last year when response from both particle sizes increased, especially in the case of S(mc) which gave the largest response in the fourth year. The average accumulation for S(c) was 1.88 kg S/ha/yr. Excluding 1975 the general pattern of $S(f) > S(mf) = S(mc) > S(c)$ for cumulative S accumulation at 88 kg S/ha emerged.

At 176 kg S/ha (Figure 3) the pattern for S accumulation was similar to that at 88 kg S/ha with the greatest increase from coarse particle size during the third and fourth years. The similarity in

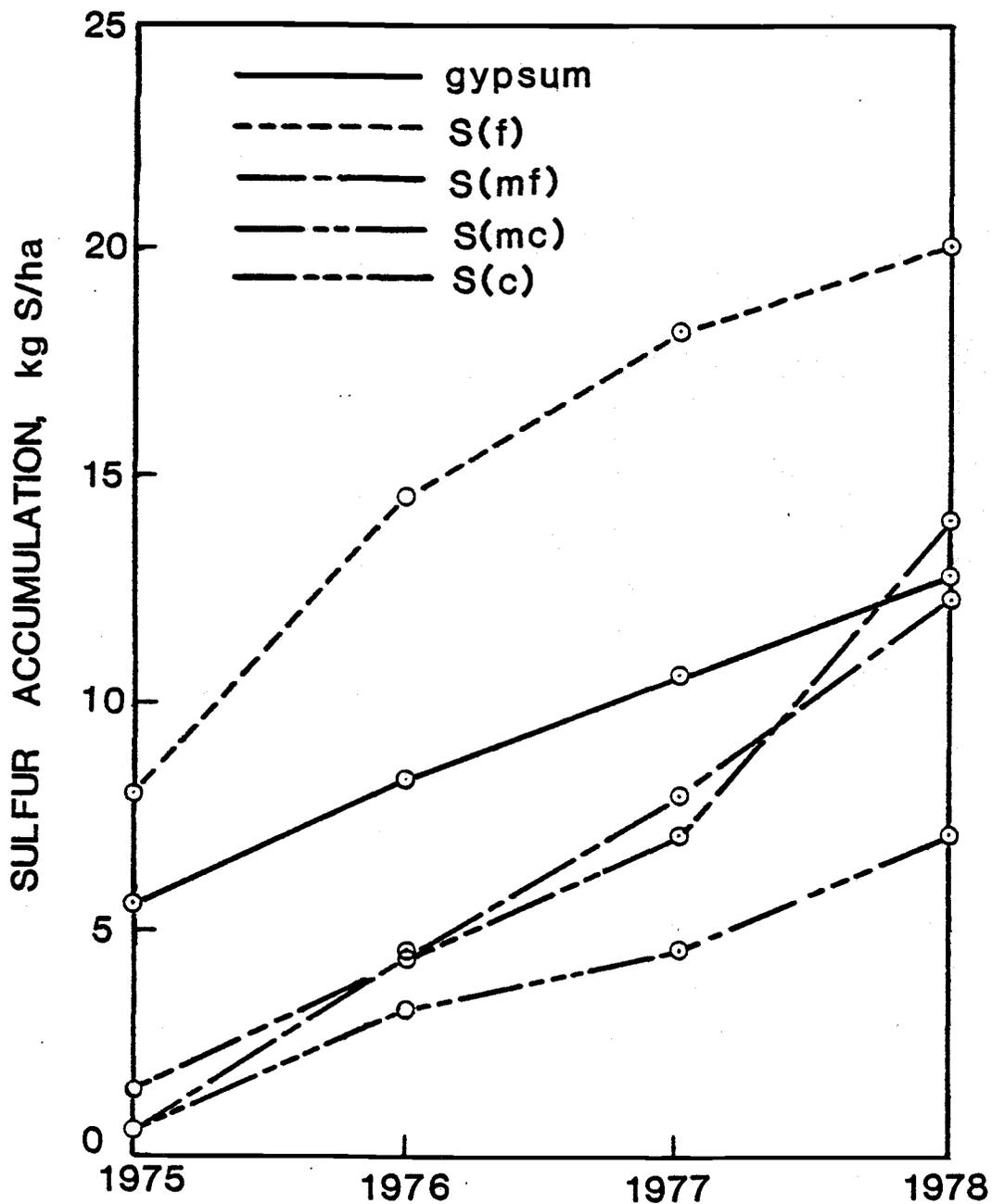


Figure 2. Sulfur accumulation (cumulative minus check) of subclover/grass forage from 1975 to 1978 at the Roseburg site as influenced by source and particle size of sulfur applied at 88 kg S/ha in 1975.

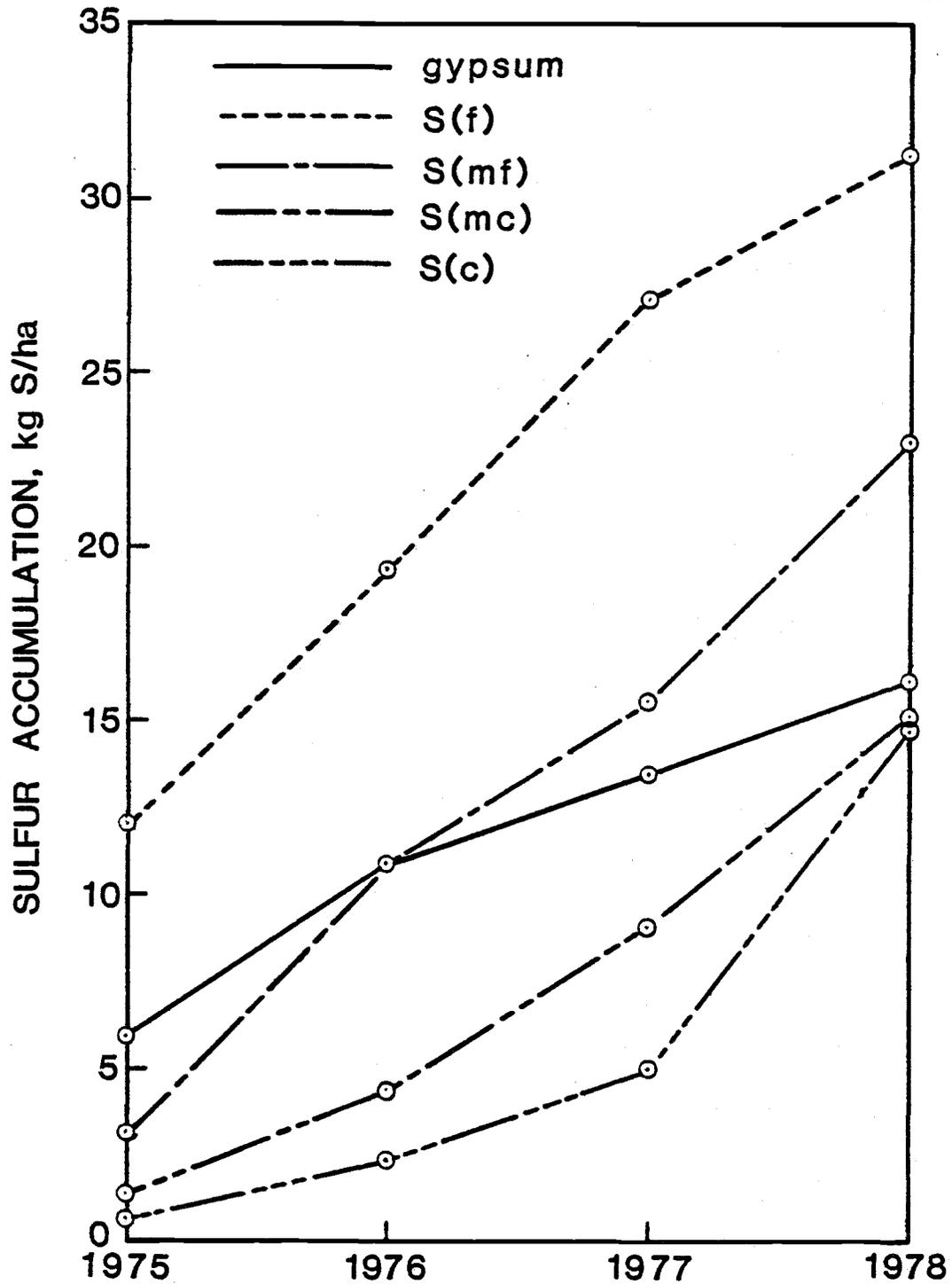


Figure 3. Sulfur accumulation (cumulative minus check) of subclover/grass forage from 1975 to 1978 at the Roseburg site as influenced by source and particle size of sulfur applied at 176 kg S/ha in 1975.

rate response is demonstrated by the absence of a significant rate by year interaction (Appendix B-2). The response from S(f) was linear until the last year when a decline in the increase over check occurred. The single application of gypsum had its greatest effect in the first two years. Elemental S(mc) and S(c) produced increasing S accumulation in each succeeding year with the last year resulting in the greatest increases over check. Excluding 1978 a comparison of cumulative S accumulation for elemental S demonstrated the pattern $S(f) > S(mf) > S(mc) > S(c)$.

At both 88 and 176 kg S/ha in 1978, the increase in S accumulation over check had decreased or remained constant for S(f) and S(gyp), while an increase had been noted for S(mf), S(mc) and S(c).

Yield - Corvallis Site

Yields were not statistically influenced by the treatments, although the highest mean yields corresponded to the largest amount of applied S (Table 6).

Sulfur Concentration - Corvallis Site

Total S concentrations for subclover leaves and petioles in 1980 are presented in Table 6. The highest rate of fall-applied S significantly increased percent S over the check plot at the March third sampling but not at the two later samplings. The spring applied S (44 kg S/ha) was broadcast on March 18 and this resulted in a large increase in S concentration at the later samplings. This probably was responsible for the significant treatment X sampling interaction. The S concentration after spring S application remained high for this treatment as compared to other treatments where the S concentration decreased or remained the same.

Table 6. Total forage dry matter yield and S content of subclover leaves and petioles at three dates prior to flowering at the Corvallis site as influenced by rate and timing of gypsum applications in 1980.

S level		Sampling			DM yield
Fall	Spring	March 7	April 15	April 30	
---kg S/ha---		-----S, %-----			---mt/ha---
0	0	0.243	0.267	0.248	4.87
22	0	0.278	0.263	0.262	4.87
44	0	0.283	0.268	0.276	5.21
22	22	-	-	-	5.15
22	44	0.260	0.349	0.365	5.63

LSD_{.05}(S level - same or different date): 0.035

LSD_{.05}(S level x date): sign. at 0.05 level

LSD_{.05}(yield): NS

Chemical Analyses - N and S Determinations

Chemical Methods of S Determination

Sulfur concentrations obtained from three different methods of analysis are presented in Table 7. Oxidation by flask or dry ashing followed by turbidity gave similar results at the two lower rates of applied S but the flask oxidation method resulted in better S recovery at the high rate of S. A dry ash oxidation followed by the methylene blue procedure gave noticeably lower S concentrations when compared to the other two methods. The oxidation flask procedure followed by methylene blue determination of $\text{SO}_4\text{-S}$ was attempted as a fourth method but results were inconsistent with approximately 75% of the samples registering abnormally low S values. It was suspected that $\text{NO}_3\text{-N}$ was present in these samples resulting in low S values (Johnson and Nishita, 1959). Since recommended procedures for eliminating $\text{NO}_3\text{-N}$ had been followed (Appendix A), this method was abandoned.

Preliminary work showed that the oxidation flask in combination with turbidity gave 98% recovery of methionine S; therefore this method was chosen for further S analyses whenever adequate S was present. For total soluble S where a low S content ($<0.07\%$ S) was encountered dry ashing was employed. The precision of these methods is shown in Table 8. The values presented are for individual samples and, in general, total S is equal to the sum of insoluble S plus total soluble S. Insoluble S was corrected for dry matter losses and is expressed on a per cent of dry matter content before extraction.

Soluble Fractions

The effects of sampling date and S fertilization on trichloroacetic acid soluble S and N fractions are summarized in Table 9. Soluble N concentration was not affected by S level but was affected by sampling date ($P=0.05$, Appendix B-6). Average soluble N concentration decreased from 0.72% at the March 30 sampling to 0.55% at

Table 7. Sulfur concentration of subclover leaves and petioles on April 27, 1978 as influenced by method of determination and S application as gypsum.

S level	Field Replication	Oxidation flask and turbidity	Dry ash and turbidity	Dry ash and methylene blue
kg S/ha		-----S, %-----		
0	1	0.156	0.124	0.125
0	2	0.134	0.160	0.117
0	3	0.165	0.145	0.126
0	4	0.188	0.190	0.134
0	5	0.155	0.164	0.135
	Average:	0.160	0.157	0.126
0	1	0.155	0.106	0.113
0 ¹	2	0.146	0.140	--
0 ¹	3	0.149	0.166	0.144
0	4	0.218	0.238	0.171
0	5	0.120	0.090	0.127
	Average:	0.158	0.148	0.139
22	1	0.217	0.162	0.168
22	2	0.204	0.208	0.168
22	3	0.243	0.226	0.187
22	4	0.202	0.206	0.180
22	5	0.213	0.214	0.200
	Average:	0.216	0.203	0.181
44	1	0.252	0.172	0.202
44	2	0.241	0.177	0.192
44	3	0.225	0.222	0.179
44	4	0.231	0.246	0.154
44	5	0.285	0.238	0.190
	Average:	0.247	0.211	0.183

¹ S application on this plot by error.

Table 8. Comparison of total S, total TCA-soluble S and TCA-insoluble S at the March 30 sampling, each fraction independently analyzed.

S level	Field replication	Total soluble S(1)	insoluble S(2)	(1)+(2)	Total S
kg S/ha		-----S, %-----			
0	1	0.025	0.202	0.227	0.231
	2	0.027	0.211	0.238	0.227
	3	0.036	0.196	0.232	0.230
	4	0.023	0.222	0.245	0.241
	5	0.026	0.214	0.240	0.210
22	1	0.061	0.225	0.286	0.283
	2	0.050	0.246	0.296	0.282
	3	0.067	0.232	0.299	0.300
	4	0.036	0.227	0.263	0.283
	5	0.059	0.248	0.307	0.304
44	1	0.070	0.226	0.296	0.306
	2	0.061	0.219	0.280	0.289
	3	0.064	0.220	0.284	0.290
	4	0.055	0.240	0.295	0.303
	5	0.073	0.217	0.290	0.304

Table 9. TCA-soluble N and S fractions of subclover in 1978 as influenced by sampling date and S fertilization as gypsum.

S level	Sampling date		
	March 30	April 12	April 27
kg S/ha	-----soluble N, %-----		
0	0.70	0.51	0.49
22	0.72	0.55	0.56
44	0.73	0.58	0.51
Avg.	0.72	0.55	0.52
	-----soluble NO ₃ -N, %-----		
0	-		0.001
22	-		0.001
44	-		0.002
	-----total soluble S, mg S/kg DM-----		
0	270	260	280
22	550	480	510
44	650	650	610
	-----soluble SO ₄ -S, mg S/kg DM-----		
0	40	40	60
22	240	160	180
44	270	250	280

Soluble N:

LSD (S level): NS
 LSD_{.05} (date): 0.05
 LSD_{.05} (S level x date): NS

Total soluble S:

LSD (S level): 70
 LSD_{.05} (date): NS
 LSD_{.05} (S level x date): NS

Soluble sulfate-S:

LSD (S level): 40
 LSD_{.05} (date - at same S level): 35
 LSD_{.05} (S level x date): NS

the April 12 sampling. The average N concentration on April 27 was statistically equal to that on April 12.

Nitrate-N was analyzed on the April 27 sampling date and levels were below 0.003% N and could be disregarded as a significant contribution to the total N present in subclover.

Total soluble S increased with higher S applications. The soluble S concentration was not statistically influenced by sampling date (Appendix B-6).

Soluble SO_4 -S concentration increased with S application at each date. Sampling date had a significant effect on SO_4 -S concentration (Appendix B-6) but was significantly different only at the 22 kg S/ha rate. This was evidenced as a drop from 240 mg S/kg DM on March 30 to 160 mg S/kg DM on April 12. At 0 and 44 kg S/ha, the SO_4 -S concentration was virtually unchanged over the sampling period.

Nitrogen Concentrations

Total N concentrations generally increased with increasing rates of S and decreased as the plants matured (Table 10). From March 30 to April 12 the largest decrease, 1.23%, occurred at 22 kg S/ha. Nitrogen concentrations for all insoluble fractions also followed this trend. The N concentrations of insoluble fractions were greater than total N concentrations due to loss of soluble components. This loss reduced the dry matter by approximately one-third of the original sample in the case of the TCA-insoluble fractions. Total N and N_p concentrations for oven-dried and ethanol insoluble material were affected ($P=0.05$) by S rate and date of sampling (Appendix B-6). Protein N for freeze-dried material was significantly influenced by sampling date but not by S fertilization. It should be noted that protein N and S concentrations in Table 10 and associated analysis of variance tables in Appendix B-6 are based on the percentage in the dried insoluble material.

Table 10. Nitrogen and sulfur concentrations and N:S ratios of subclover and subclover fractions as influenced by sulfur applications, sampling date, drying procedure and extractant.

		TCA insoluble											
		Total			oven-dried			freeze-dried			ETOH insoluble-fresh		
S level		N	S	N:S	N	S	N:S	N	S	N:S	N	S	N:S
kg S/ha		-----%-----			-----%-----			-----%-----			-----%-----		
							<u>March 30</u>						
0		4.32	0.217	20.0	5.99	0.304	19.8	5.64	0.285	20.0	5.30	0.263	20.2
22		5.12	0.290	17.6	6.54	0.336	19.5	5.95	0.319	18.7	5.73	0.308	18.6
44		4.93	0.298	16.5	6.56	0.339	19.3	6.06	0.318	19.1	5.90	0.349	17.0
							<u>April 12</u>						
0		3.72	0.183	20.4	5.55	0.273	19.9	5.08	0.266	19.0	4.44	0.249	17.8
22		4.01	0.226	17.7	5.99	0.308	19.5	5.14	0.285	18.0	4.90	0.288	17.0
44		4.23	0.263	17.4	6.03	0.323	18.6	5.49	0.312	17.6	5.14	0.304	17.0
							<u>April 27</u>						
0		3.41	0.151	22.8	4.82	0.232	20.8	4.69	0.230	20.4	4.30	0.218	19.8
22		3.82	0.209	18.2	5.31	0.281	18.9	4.95	0.251	19.8	4.80	0.268	17.6
44		4.12	0.247	16.8	5.80	0.305	19.1	5.33	0.299	17.8	5.03	0.303	16.6
LSD(.05)		0.22	0.020		0.35	0.024		0.40	0.026		0.14	0.018	

Sulfur Concentrations

Total S concentrations increased with higher S applications and decreased with time (Table 10). This was a significant effect (Appendix B-6).

Insoluble fractions had S concentrations which followed the same pattern as total S. Protein S for all extractions were highly significant for date of sampling and S fertilization (Appendix B-6).

For TCA-insoluble fractions, S concentrations for oven-dried material were greater than that for freeze-dried. Sulfur concentrations for ethanol insoluble material were less than those for freeze-dried at 0 kg S/ha for all three dates, but varied in the same manner as the other fractions at the other two S levels for the three dates.

Critical Ranges

The variation in 1978 yield data made it difficult to relate yield increases from application of S to a specific critical level; therefore, critical ranges were related to yield increases. These ranges were obtained by using the value of the particular index at 0 kg S/ha as the low end of the scale and the value at 44 kg S/ha as the high end of the scale. The plants at 0 kg S/ha were definitely deficient (Table 5), thus values near the low end of the range would be considered S deficient while values at 22 kg S/ha would be considered marginally adequate and values the high end of the range would be considered S sufficient. These critical ranges are summarized in Table 11. Total S and $\text{SO}_4\text{-S}$ were significantly affected by sampling date; total soluble S was not significantly affected by sampling date.

For total S the largest drop, 0.08%, occurred at 22 kg S/ha from March 30 to April 30. On April 30 the range from low to high, 0.10%, was larger than the range on the other two dates.

Sulfate-S remained relatively constant over time for 0 and 44 kg S/ha; however, a decrease occurred from March 30 to April 12 at 22 kg S/ha. The range from low to high S status was similar for

Table 11. Critical ranges of various sulfur diagnostic indices of subclover leaves and petioles as influenced by sampling date in 1978.

Diagnostic index	Sampling date	Critical range (low to high)
		-----S, %-----
Total S	March 30	0.22 - 0.29 - 0.30
	April 12	0.18 - 0.24 - 0.26
	April 27	0.15 - 0.21 - 0.25
		--mg S/kg DM--
Sulfate-S	March 30	40 - 240 - 270
	April 12	40 - 160 - 250
	April 27	60 - 180 - 280
Total Soluble S	dates NS	270 - 510 - 640
		-----ratios-----
SO ₄ -S/total S	March 30	0.02 - 0.08 - 0.09
	April 12	0.02 - 0.08 - 0.09
	April 27	0.03 - 0.09 - 0.11
(N:S) _t	March 30	20.0 - 17.6 - 16.5
	April 12	20.4 - 17.7 - 17.4
	April 27	22.8 - 18.2 - 16.8

each date.

The ranges of the ratio, SO_4-S /total S, was not drastically altered by date of sampling and showed S deficiency when SO_4-S was 2-3% of total S and definite S sufficiency when the percentage reached 9-11%.

The $(N:S)_t$ ratios increased with time for plants at 0 and 22 kg S/ha, but showed no consistent trend at 44 kg S/ha. The range from low to high was wider on April 27 than on the previous two dates.

Total soluble S at 270 mg S/kg DM represented the low end of the critical range and 510 mg S/kg DM the high end.

Nitrogen to Sulfur Ratios

Total N:S ratios (Table 10) decreased with increasing rates of applied S. At each date the difference between the check and either rate of sulfur was larger than the difference between the rates of sulfur. A highly significant relationship existed between N_t and S_t at all three dates (Table 12).

Protein N:S ratios from oven-dried, TCA-insoluble material, in general, showed a small decline with added S (Table 10). The magnitude of this decline increased with plant maturity. On March 30 the difference between $(N:S)_p$ for 0 and 44 kg S/ha was 0.5, while on April 27 this difference had increased to 1.7. For freeze-dried material there was a general decline in $(N:S)_p$ with increasing S levels. As with the oven-dried fraction, the degree of decline increased with time. Ethanol insoluble $(N:S)_p$ varied inversely with increasing S levels for all dates except 44 kg S/ha on April 12 and had the largest decreases in $(N:S)_p$ as compared to freeze-dried or oven-dried material. For the most part the $(N:S)_p$ ratio showed a decline in the order: TCA-oven-dried > TCA-freeze-dried > ethanol insoluble-fresh for all 3 rates and dates except 0 kg S/ha on March 30. The relationship between N_p and S_p was highly significant for all extractions except TCA on freeze-dried material on the April 30 sampling (Table 12).

Table 12. Linear regression equations relating total N (N_t) to total S (S_t) for oven-dried subclover leaves and petioles, protein N (N_p) to protein S (S_p) for the TCA insoluble fraction of oven-dried and freeze-dried subclover leaves and petioles and N_p and S_p for the ethanol insoluble fraction of fresh subclover leaves and petioles at three dates in 1978.

Treatment	equation	coefficient of determination
<u>March 30</u>		
oven-dried	$N_t = 2.54 + 8.40S_t$	0.74**
oven-dried	$N_p = 1.14 + 14.22S_p$	0.70**
freeze-dried	$N_p = 1.52 + 11.78S_p$	0.69**
ethanol	$N_p = 3.58 + 6.73S_p$	0.84**
<u>April 12</u>		
oven-dried	$N_t = 2.35 + 7.50S_t$	0.71**
oven-dried	$N_p = 1.04 + 13.60S_p$	0.76**
freeze-dried	$N_p = 0.78 + 14.10S_p$	0.74**
ethanol	$N_p = 1.48 + 11.92S_p$	0.72**
<u>April 27</u>		
oven-dried	$N_t = 2.42 + 6.72S_t$	0.74**
oven-dried	$N_p = 1.14 + 12.76S_p$	0.87**
freeze-dried	$N_p = 1.70 + 9.46S_p$	0.48*
ethanol	$N_p = 2.36 + 8.90S_p$	0.86**

** - significant at the 0.01 level

* - significant at the 0.05 level

Protein N and S concentrations for oven-dried and freeze-dried material have been corrected for dry matter loss, thus N_p and S_p relate to concentrations in the original dried tissue before extraction; N_p and S_p for ethanol extracted material relate to concentration in the ethanol insoluble fraction. See Appendix B-3 for analyses of variance of regression lines.

Total N was plotted against total S for each separate date (Figure 4). There are two lines on each graph for each date, one representing the best fit regression line (points plotted) relating N_t and S_t for oven-dried material and one representing the best fit regression line for $(N:S)_p$ for oven-dried material (points not plotted).

It can be seen that there is a shift in points from a more grouped arrangement on March 30 with little separation between the two S levels to a more scattered arrangement on April 12 and April 30. The intersection of the $(N:S)_t$ line with the $(N:S)_p$ line would be the situation of $(N:S)_t = (N:S)_p$, i.e. the critical level. The $(N:S)_t$ ratios at these intersections are 19.0, 18.4 and 18.1 for March 30, April 12 and April 27, respectively.

The ratio of TCA-soluble non-protein organic N to non-protein organic S was calculated and the effect of sampling date and S fertilization on this ratio is presented in Table 13. Nitrate-N concentration was extremely low and therefore non-protein organic N was considered to be equal to total soluble N (Table 13). Non-protein organic S was calculated as total soluble S minus SO_4-S . A large drop in the ratio occurred from March 30 to April 12 for all rates of applied S; the decrease was greatest for 0 kg S/ha and the smallest for 44 kg S/ha. There was little change in the ratio from April 12 to April 27. As the level of S fertilization increased, the ratio decreased at all dates. The difference between 0 and 22 kg S/ha was greater than that between 22 and 44 kg S/ha for all sampling dates.

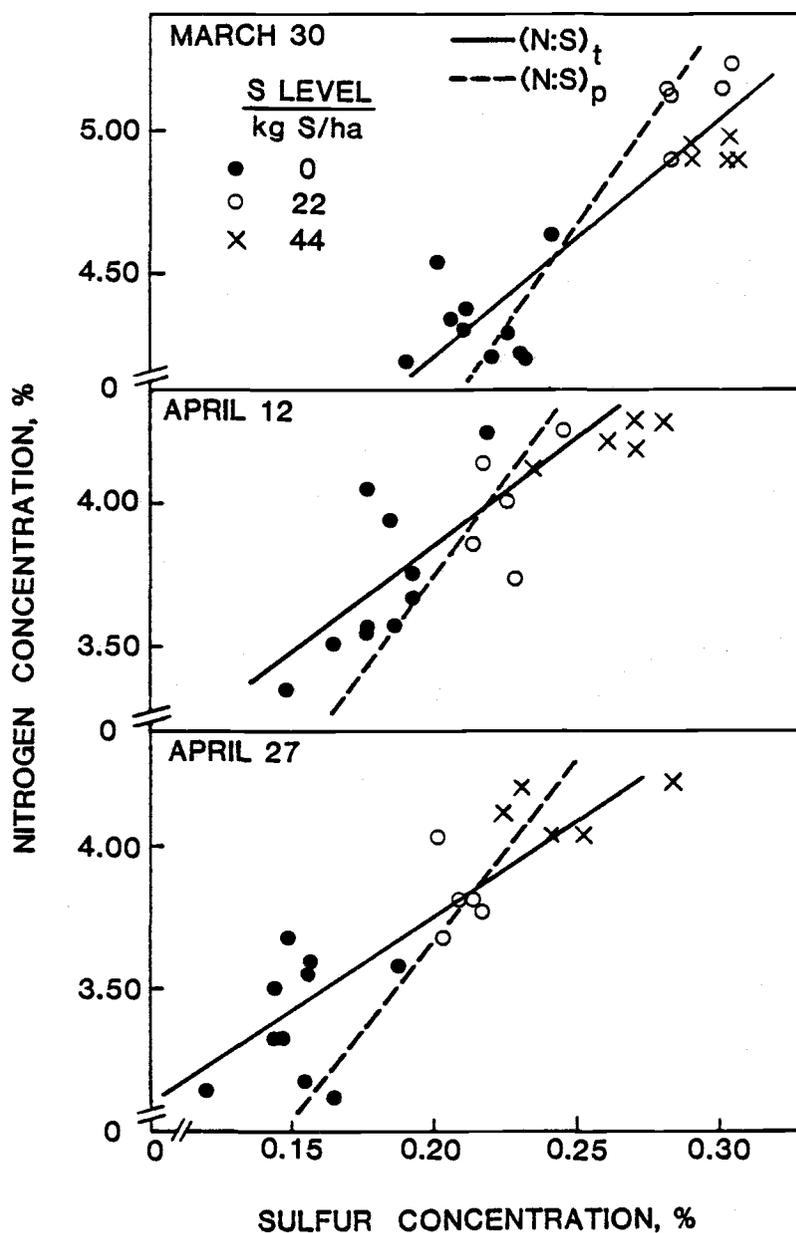


Figure 4. Relationships between total N (N_t) and total S (S_t) as compared to the relationship between protein N (N_p) and protein S (S_p) for 3 sampling dates in 1978 from the Roseburg site. Points plotted are for total N and S; regression lines for N and S are those of Table 12 for oven-dried material.

Soil Reaction

The soil pH of selected treatments was determined in March, 1978. These results are presented in Table 14. The greatest reduction in soil pH in the top 5 cm occurred with S(c) at 176 kg S/ha (0.3 pH unit). Smaller reductions occurred with S(f) at 88 and 176 kg S/ha. Deeper samples showed a diminished effect of S additions on soil pH.

Table 13. The ratio of TCA-soluble non-protein organic N to TCA-soluble non-protein organic S in subclover leaves and petioles as influenced by sampling date and S fertilization.

S level kg S/ha	Sampling date		
	March 30	April 12	April 27
0	30	23	22
22	23	17	17
44	19	15	15

Table 14. Soil pH values in 1978 as influenced by S application in 1973 and depth of sampling.

Sulfur		Depth (cm)	
Source	Rate	0-5.1	5.1-15.2
kg S/ha			
check	0	5.7	5.8
S(f)	88	5.7	5.8
S(f)	176	5.6	5.8
S(c)	176	5.5	5.7

DISCUSSION

Field ExperimentsRoseburg Site

The yield responses in 1975, although statistically significant, were small. Potassium deficiency and/or a moderate S deficiency may have been responsible for the small response. Except for the 88 and 176 kg S/ha rates of S(f) and gypsum, S concentrations (Table 5) also showed little effect from S treatments in 1975. For the remaining three years annual applications of S(f) and gypsum produced increases in yield with no discernible advantage between sources. The F-test for annual sources was non-significant (Appendix B-1). While some research (Jones and Ruckman, 1966 and 1977; Jones, 1970; Lobb, 1962; Bentley and Green, 1954) has shown these sources to be equally effective in increasing grass-legume or alfalfa yields in the first year after application, other research (Ludecke, 1965; Weir et al, 1963; Fox et al, 1964; McLachlan and Marco, 1968) has demonstrated gypsum to be superior. Work by Fox (1964) has shown a different response between field and greenhouse experiments with gypsum increasing yields over elemental S in field trials, but showing no advantage in a greenhouse study. Comparison between studies is made difficult by differences in particle sizes of elemental S and distribution and amounts of rainfall. Since it is known that particle size is critical for oxidation (Burns, 1967; Attoe and Olson, 1966) it is not surprising to find variable results with different particle size applications. In fact, gypsum particle size may also be an important factor (McLachlan and DeMarco, 1968). Where rainfall is limited oxidation of elemental S would be restricted but the solubility of larger particle sizes of gypsum would also be reduced. Where limited rainfall came in the cooler months gypsum was superior to elemental S in the first year (Conrad, 1950).

Yield results from single applications were erratic and no consistent pattern emerged. All F-tests for sources, rate and

interactions were non-significant for single applications (Appendix B-1). It is interesting to note that gypsum at 88 or 176 kg S/ha was still effective in 1978. Both yield and S accumulation were greater than the check. This indicates a residual value for gypsum four years after application. It was noted that grass had become more prevalent in the plot area in 1978. Since grass is more competitive than clover under S stress conditions and is able to make better use of residual S, the increase in the grass component may have contributed to forage yield in 1978. Walker and Adams (1958) reported increased grass and decreased clover content with an overall increase in forage yield four years after gypsum applications. Other investigations (Walker and Adams, 1964; McLachlan and DeMarco, 1971) have also shown a residual effect from applied gypsum. Even if a grass influence was present it should be noted that the S concentration of subclover was still significantly increased by gypsum at 88 or 176 kg S/ha in 1978.

Fine elemental S provided more available S than other single applications over the four year period. This is supported by the S concentrations data where a more consistent increase was evidenced by S(f) and by the S accumulation data where S(f) was as good as or better than other applications in three out of four years. Only in 1978 did S(f) fall below other particle sizes in supplying S. This may represent the time limit of S(f) to supply adequate S for plant needs. The cumulative S accumulation was also the highest for S(f) at 88 or 176 kg S/ha. The average accumulation for S(c) was 1.88 kg S/ha/yr which was between the 0.5 kg S/ha/yr for 44.8 kg applied S/ha and 5.0 kg S/ha/yr for 448 kg applied S/ha for a similar particle size range reported by Jones and Ruckman (1969) for a subclover-grass pasture in California.

Visual observations in 1980 verified that all single applications showed residual value. Both S(c) and S(mc) were expected to have residual value beyond the four year study period. Maximum uniform S availability over a four year period from a single application would be achieved with a mixture of S(f) and S(mc).

An analysis of variance for S accumulation resulted in several interactions for annual sources being significant (Appendix B-2). The source x rate interaction was significant and both annual rate x year and source x year interactions were highly significant. Due to the limited response in 1975 the only significant differences were within rates for S(f). Data from 1976 is presented in Table 15.

Table 15. Sulfur accumulation of forage for 1976 as affected by rate and source of sulfur applied annually.

Source	Rate		
	11	22	44
	-----kg S/ha-----		
S(f)	6.08	7.02	12.69
gypsum	5.27	6.12	9.84

LSD (0.05) = 2.00

From this table it can be seen that the rate x source interaction is a result of a significant difference at 44 kg S/ha only. Data from 1977 is similar while data from 1978 showed significant differences at both higher S rates but not at the lowest S rate. The fact that significant differences between sources occurred at different rates for different years underscores the annual rate x year and source x year interactions.

Corvallis Site

Yield were not significant but there is an indication that spring rates are more effective than fall applications. This can be seen at 44 kg S/ha in the increased S concentration from 0.260% before S application to 0.349% after S application.

Samples from check plots had high S concentrations, substantially beyond reported critical levels under Oregon conditions (Drlica and Jackson, 1979). At these S levels a yield response would not be expected. Yields were not as high as those from the Roseburg site (Table 5 vs Table 8), but the Corvallis site represents a cooler environment and maximum yield potential would be less than that of the Roseburg site.

Sample Handling and Chemical Methods

Sulfur Determination

Comparisons of results derived from different S analyses can be complicated when considering the reliability of various chemical procedures. Volatile losses associated with dry or wet ashing techniques are minimized or eliminated with the use of oxidation flasks. In S deficient plant material the use of oxidation flask plus turbidity have approximately equal values with that of dry ash plus turbidity (Table 3). At 44 kg S/ha there was incomplete recovery with dry ash as compared to the oxidation flask. The dry ash oxidation in combination with methylene blue determination gave consistently lower results than those for the other two methods. The reason for this is unknown.

Critical Ranges

Critical ranges for total S of subclover encompass values for critical levels at similar growth stages found by other authors (Spencer and Bouma, 1970; Drlica and Jackson, 1979). To accurately assess S status, total S would seem to be a less desirable measure

due to variation with time and difficulty in interpretation of growth stage.

The level of $\text{SO}_4\text{-S}$, although affected by stage of maturity, had its only significant drop at 22 kg S/ha from March 30 to April 12 (Table 9). This may have been the result of an luxuriant S supply at an early growth stage but as temperatures warmed and plant growth increased the S supply at this rate was not able to keep pace with plant growth. For the same time period the $\text{SO}_4\text{-S}$ level did not show as dramatic a decrease at 44 kg S/ha. Sulfur accumulation was significantly higher at 44 kg S/ha when compared with 22 kg S/ha. If $\text{SO}_4\text{-S}$ is to be used as a measure of S status, an early sampling may not give an adequate assessment for the entire growing period. Jones (1963,1964) found sampling subclover at the flowering stage to be a better indicator of S status than earlier samplings. In a greenhouse study (Jones, 1962) a critical level for subclover was reported to be 170 ppm $\text{SO}_4\text{-S}$. Another greenhouse study (Spencer et al., 1977) demonstrated that 61-day-old plants (pre-flowering) needed 30 ppm $\text{SO}_4\text{-S}$ in young leaves and 500 ppm $\text{SO}_4\text{-S}$ in young petioles to provide for 90% of maximum yield at 133 days. In this same study Spencer suggests field critical levels may be higher than those obtained in the greenhouse. Comparison of critical values between field and greenhouse studies must be done with the understanding of the large and sometimes sudden variations in the field environment as opposed to the more constant conditions of the greenhouse. The environmental effect could be directly on the plant or indirectly through the N fixation process. Under field conditions grazing can also be a complicating factor.

Total soluble S was not significantly affected by sampling date, thus being a good choice for an indicator of sulfur status. This S fraction was of similar magnitude to values found for alfalfa and alsike clover by Walker and Bentley (1961). They indicated that this fraction was as good as $\text{SO}_4\text{-S}$ in determining the need for S fertilization. The practical disadvantage is the additional step of extraction before oxidation.

The TCA-soluble organic S fraction calculated as total soluble S minus soluble $\text{SO}_4\text{-S}$ is presented in Table 15. Comparing these numbers with those of $\text{SO}_4\text{-S}$ it can be seen that under conditions of S deficiency (0 kg S/ha) $\text{SO}_4\text{-S}$ drops to one sixth to one third of its level at 22 kg S/ha depending on sampling date while the organic S fraction decreased only to two thirds of its value for a similar comparison. Other work (Spencer, 1959) has also shown $\text{SO}_4\text{-S}$ to be more sensitive to S deficient conditions than the organic S fraction. The relative insensitivity of the organic S fraction to S status suggests a pool of organic S which must be satisfied before a similar size $\text{SO}_4\text{-S}$ pool can exist. This pool is likely to consist of cysteine and methionine, two S-containing amino acids which have been shown to decrease under S deficiency (Bardsley and Jordan, 1957). The organic S fraction was not affected by sampling date. The lack of change with time of the organic S fraction and its size relative to $\text{SO}_4\text{-S}$ resulted in total soluble S being independent of plant maturity for the time period in this study.

The critical range of $\text{SO}_4\text{-S}/\text{total S}$ was fairly constant as the plant matured. Jones (1980) found the $\text{SO}_4\text{-S}/\text{total S}$ ratio for subclover to be a relatively stable indicator of S status over a 133 day growing period. In this study 90% of maximum growth was obtained when $\text{SO}_4\text{-S}$ represented 8 to 11% of total S. This is very close to the range obtained for the 22 to 44 kg S/ha rates in the present experiment. The insensitivity of this ratio to plant maturity would make it a good candidate for a diagnostic indicator.

The ratio of organic non-protein N to organic non-protein S, $(\text{NPN}/\text{NPS})_{\text{org}}$, has been suggested as a possible indicator of S status (Metson and Collie, 1972). If $(\text{N}:\text{S})_{\text{p}}$ is relatively constant, changes in $(\text{N}:\text{S})_{\text{t}}$ which consists mainly of protein N and protein S would not be as sensitive as $(\text{NPN}/\text{NPS})_{\text{org}}$ to fluctuations in S supply. Comparing 0 kg S/ha to 22 kg S/ha it can be seen that a greater sensitivity to S deficiency existed with $(\text{NPN}/\text{NPS})_{\text{org}}$ (Table 13) than with $(\text{N}:\text{S})_{\text{t}}$ (Table 10). Since

Table 16. Trichloroacetic acid soluble organic S fraction of subclover leaves and petioles as influenced by sampling date and S fertilization as gypsum.

S level	Sampling date		
	March 30	April 12	April 27
kg S/ha			
0	230	220	220
22	310	320	330
44	380	400	330

organic NPS was relatively constant, the drop in $(\text{NPN}/\text{NPS})_{\text{org}}$ from March 30 to April 12 was mainly due to the drop in organic NPN. The organic NPN was insensitive to S fertilization. Since NPN would be expected to increase under severe S deficiency, subclover in the check plots experienced only moderate S deficiency. The fact that organic NPN decreased from March 30 to April 12 while organic NPS did not may be indicative of a lag period of nodulation and N fixation in supplying fixed N during a period of increased growth while the S supply was increasing at a rate similar to growth.

Nitrogen to Sulfur Ratios

The critical ranges for $(\text{N}:\text{S})_t$ obtained in this experiment were similar to values in the literature. Subclover $(\text{N}:\text{S})_t$ values of 16:1 to 19:1 under field conditions were given by Spencer (1978) and 16:1 to 23:1 under greenhouse conditions by Jones et al (1980). A critical range for $(\text{N}:\text{S})_t$ of 17:1 to 19:1 for white clover was reported by McNaught (1970) under field conditions. A value of 19.3:1 for $(\text{N}:\text{S})_p$ of subclover was obtained by Freney et al (1977). For legumes in general, 17.5:1 was the critical value arrived at by Dijkshoorn and Van Wijk (1962). In a greenhouse study Spencer (1978) found the critical $(\text{N}:\text{S})_t$ to decrease during the vegetative phase of growth. This is in agreement with the decrease, 19.0 to 18.1, obtained for the $(\text{N}:\text{S})_t$ critical level in the present study. The decrease in the $(\text{N}:\text{S})_t$ critical level with time as determined by $(\text{N}:\text{S})_t = (\text{N}:\text{S})_p$ does not follow the increasing pattern with time of $(\text{N}:\text{S})_t$ for 0 kg S/ha. Where no S was applied S concentration decreased faster than N concentration, thus raising the $(\text{N}:\text{S})_t$ ratio. At 22 kg S/ha the declines in N and S concentrations were not as widely different resulting in a slight increase in $(\text{N}:\text{S})_t$. A comparison of $(\text{N}:\text{S})_t$ with the critical $(\text{N}:\text{S})_t$ - 19.0, 18.4, and 18.1 for March 30, April 12, and April 27, respectively - demonstrated that only plants at 0 kg S/ha were deficient on March 30 and April 12, but that plants at 0 and 22 kg S/ha were deficient on April 27. Although there is this slight

discrepancy between these two methods, both methods do indicate that S fertilization at 22 kg S/ha is becoming marginal at the latest sampling date.

The constancy of $(N:S)_p$ is a necessity if it is to be used as a reference point for comparison with $(N:S)_t$. Although there was a highly significant relationship between N_p and S_p (Table 12) at each date for all three procedures except freeze-dried on April 30, the relationship was not constant. The F-test for determination of a difference between regression lines for freeze-dried and oven-dried material was highly significant for all dates (Appendix B-5). Since the sampling procedure was different for ethanol extracted material a comparison for this material was not attempted. For the $(N:S)_p$ ratios obtained via different extraction methods to be equal and unaffected by S fertilization, all equations would be required to have equal slopes and pass through the origin. This is not the case for the equations obtained here (Table 12). All methods of determining $(N:S)_p$ resulted in $(N:S)_p$ demonstrating an inverse relationship to S fertilization. This is in contrast to some work (Stewart and Porter, 1966; Leggett et al., 1966; Stewart and Whitfield, 1965), but in agreement with others (Jones et al., 1971; Westerman, 1975; Metson and Collie, 1972). The decrease in $(N:S)_p$ could be a result of changing proportions of different plant proteins. Work by Mertz and Matsumoto (1956) showed that relative proportions of cytoplasmic proteins were affected by S deficiency. Byers (1971) demonstrated that changes in chloroplastic protein content could occur with age while cytoplasmic protein content remained relatively constant.

Freeze-dried plant material is more closely related to fresh tissue than oven-dried material (Smith, 1973), thus a comparison between ETOH and TCA as extractants for $(N:S)_p$ determination would be appropriate and informative. Protein N:S ratios from the freeze-dried TCA insoluble fraction were generally greater than the $(N:S)_p$ ratios from the ETOH insoluble fraction. Mathematically this was a result of a decrease in N_p while S_p decreased little

or none at all when comparing the TCA insoluble fraction with the ETOH insoluble fraction. Averaged over all treatments and dates this decrease equaled 5.9% for N_p and 0.7% for S_p . Two possible reasons for this discrepancy in N_p values are 1) incomplete precipitation of all protein by ETOH and 2) incomplete extraction of NPN by TCA. Plant proteins have been shown to be hot, ETOH soluble (Gaines, 1976) while TCA has been considered to be a better protein precipitant than methyl alcohol (Greenwald, 1915). Slow heat coagulation has also been disadvantageous to protein precipitation recovery (Singh, 1960). In contrast, Byers (1976) reported $(N:S)_p$ ratios of 15:1 for lucerne and 16:1 for lupin when extracted with 5% TCA - results similar to those obtained using 80% ethanol as an extractant. If the $(N:S)_p$ ratios obtained by ethanol extraction were, in fact, the true $(N:S)_p$ values a comparison with $(N:S)_t$ should indicate S deficiency and coincide with yield data. It can be seen (Table 10) that on April 12 and 27 S deficiency would be predicted for 22 and 44 kg S/ha, i.e., $(N:S)_t > (N:S)_p$. This result appears incongruous and leads to uncertainty in $(N:S)_p$ ratios obtained from ethanol extraction of fresh tissue.

Oven-dried plant material and freeze-dried material were handled similarly and were amenable to statistical analysis. The result of this analysis showed a significant effect of drying procedure ($P=0.05$ level) on N_p and S_p (Appendix B-7). The increase in N_p and S_p with oven-dried material as compared to freeze-dried material was, for the most part, probably due to losses in dry matter content of the TCA-insoluble fraction. Incomplete extraction of NPN and NPS in the oven-dried material could also be responsible for the increase. Since the percentage increases (averaged over all treatments and dates) in N_p (8.8%) and S_p (5.3%) were not equal, loss of dry matter cannot be solely responsible for this increase. If the 5.3% increase in S_p represents dry matter loss, then oven-drying affected the plant material in such a way as to impair NPN extraction with TCA or directly alter the NPN fraction. Danley

and Vetter (1971) found freeze-ground samples to have more water soluble NPN than oven-dried samples. Much of the research involved in extraction of NPN has been done on fresh tissue (Bisset, 1954; Lewis and Pate, 1973; McKee et al., 1955). A comparison of fresh and dried subclover tissue showed equal amounts of extractable NPS (Freney, 1977); however, the amounts of extractable NPN and NPS have been shown to be dependent on extraction techniques (Freney, 1977; Metson and Collie, 1972). From the significant N_p interactions of drying procedure with S rate and sampling date as compared to no interactions for S_p , it would appear that the protein N fraction is more susceptible to changes in extraction techniques (Appendix B-7). The lack of significance for S rates of protein N for freeze-dried material as compared to that for oven-dried also supports this view (Appendix B-6). If dry matter loss occurred to the extent of causing an 8.8% increase in N_p , there may have been hydrolysis and loss of S_p to the TCA-soluble fraction. Although proteolysis and amino acid decomposition has been suggested (Handreck, 1972) as a possible source of confusion for SO_4-S determination, definitive data is yet forthcoming on protein hydrolysis resulting in a transfer of organic S from protein to an amide fraction under drying conditions normally encountered in plant analysis.

To observe the relative changes in plant S fractions, the levels of SO_4-S , organic S, and protein S were calculated as percentages of total S (Table 16). Total S was the sum of total soluble S and protein S, and S_{org} equaled the difference between total soluble S and SO_4-S . For each rate SO_4-S percentages did not change over time. At 0 and 22 kg S/ha the organic S percentage increased while protein S decreased with plant maturity. At 44 kg S/ha organic S showed no definite pattern over the three dates. Apparently there is a need for an organic S fraction which will be fulfilled, even at the expense of protein S.

Soil Reaction

Soil application and oxidation of elemental S results in a

Table 17. Sulfur fractions of subclover leaves and petioles as a per cent of total S as influenced by S fertilization as gypsum and date of sampling.

S level	SO ₄ -S	TCA-soluble S (organic S)	TCA insoluble S (protein S)
kg S/ha	-----%-----		
<u>March 30</u>			
0	2	11	87
22	9	10	81
44	10	13	77
<u>April 12</u>			
0	2	12	86
22	7	14	79
44	9	15	76
<u>April 27</u>			
0	3	13	84
22	8	15	77
44	11	13	76

decrease in soil pH. A large reduction in soil pH would have a negative effect on plant growth. The lowering of soil pH with additions of elemental S were not of sufficient magnitude to adversely affect plant growth. Over four years soil pH decreased only slightly at the highest rate of S(f), 176 kg S/ha. In 1978 the depression of soil pH at 176 kg S/ha was greater with S(c) than with S(f). Plant S accumulation with S(c) indicates that oxidation of this particle size occurred to the greatest extent in 1978 which corresponds to the decrease in soil pH associated with this treatment.

CONCLUSION

Applications of S as gypsum or different particle sizes of elemental S were responsible for increasing DM yields and S accumulation of a subclover/grass pasture during a four year period (1975 - 1978) at Roseburg, Oregon. Sulfur concentration in subclover leaves and petioles were also increased during this period. An annual application of S(f) at 44 kg S/ha resulted in the greatest amount of total DM yield for the four year period. Single applications of different particle sizes of elemental S at 88 and 176 kg S/ha in 1974 showed no advantage of any particle size for the four year total of DM yield. Particle size did, however, have a large influence on S concentration of subclover leaves and petioles and S accumulation of forage over time. Sulfur accumulation at 88 kg applied S/ha occurred most rapidly in the first two years with S(f), but with S(mc) in the last year; S(mf) resulted in a constant S accumulation (3.88 kg S/ha/yr). At 176 kg applied S/ha, S accumulation occurred most rapidly with S(f) and S(mf) in the first two years, but with S(mc) and S(c) in the last year. The S concentration of subclover leaves and petioles only responded to high S applications rates (88 and 176 kg S/ha) of S(f) and gypsum in 1975. The greatest increases in S concentration with coarser materials came in 1977 and 1978. The overall effect of larger particle sizes having less S availability than smaller sizes in the first year or two years after application was observed. This relationship diminished, and in most cases, inverted with time. All 88 and 176 kg S/ha rates were still effective in increasing yields in 1978. To avoid yearly applications in supplying adequate S for a four year period, a single application of a mixture of S(f) and S(mc) at 88 kg S/ha would be recommended.

Chemical analysis of subclover leaves and petioles to determine indices of S deficiency were successful in identifying certain indices as being less dependent upon plant maturity than others. Critical ranges were established for all indices. Total soluble S

and the $\text{SO}_4\text{-S}/\text{total S}$ ratio were least affected, $\text{SO}_4\text{-S}$, $(\text{N:S})_t$ and $(\text{NPN:NPS})_{\text{org}}$ moderately affected and total S most affected by plant maturity. Total soluble S was shown to contain an organic fraction which constituted >50% of this soluble fraction and was not influenced by plant maturity. Total soluble N consisted primarily of organic N with virtually no $\text{NO}_3\text{-N}$ present. This N fraction was not influenced by S fertilization, but did decrease in concentration at the second sampling date.

Protein N:protein S ratios of subclover leaves and petioled were influenced by S fertilization. This effect was demonstrated for three different methods of sampling handling and extraction. Ethanol extraction of fresh tissue was the method which resulted in the greatest decrease in $(\text{N:S})_p$ with increasing S fertilization. In general the magnitude of $(\text{N:S})_p$ ratios followed the extraction relationship of: TCA oven-dried > TCA freeze-dried > ethanol-fresh. The difference between TCA oven-dried and TCA freeze-dried $(\text{N:S})_p$ ratios was a consequence of a differential increase in N_p versus S_p for oven-dried as compared to freeze-dried residue. A comparison of N_p and S_p values for TCA freeze-dried and ethanol-fresh insoluble material showed the major difference to be in the N_p concentrations, thus identifying a difference in NPN extraction or protein precipitation between the two extractants. Since $(\text{N:S})_p$ was affected by S fertilization and plant maturity the concept of a constant $(\text{N:S})_p$ ratio does not apply under the conditions of this experiment. There was a statistically significant ($P=0.05$) relationship between N_p and S_p for all extractions and the relationship was not constant between sampling dates. For oven-dried material a regression equation relating N_p and S_p was used to obtain $(\text{N:S})_t$ critical levels of 19.0, 18.4 and 18.1 for March 30, April 12 and April 27, respectively.

Three different chemical methods of determining S concentration in plant tissue were evaluated with the results demonstrating oxidation in a combustion flask followed by $\text{SO}_4\text{-S}$ determination via a turbidity procedure to give the highest S concentration values. Dry

ashing followed by a reduction technique for SO_4 -S resulted in the lowest reading for S concentration.

BIBLIOGRAPHY

- Alway, F. J. 1940. A nutrient slighted in agricultural research. *J. Amer. Soc. Agron.* 32:913-921.
- Amen, W. J., and R. C. Dixon. 1979. Sulfur: a new look at a poor stepchild. *Agric. Age.* Nov.-Dec. p. 52-53.
- Anderson, A. J., and D. Spencer. 1950. Sulfur in nitrogen metabolism of legumes and non-legumes. *Austr. J. Sci. Res.* 3:431-449.
- Anderson, J. W. 1978. Sulphur in biology. University Park Press. Baltimore.
- Attoe, O. J., and R. A. Olson. 1966. Factors affecting rate of oxidation in soils of elemental sulfur and that added in rock phosphate-sulfur fusions. *Soil Sci.* 101:317-324.
- Bates, T. E. 1971. Factors affecting critical nutrient concentrations in plants and their evaluation: a review. *Soil Sci.* 112:116-130.
- Beaton, J. D., G. R. Burns, and J. Platou. 1968. Determination of sulphur in soils and plant material. *The Sulfur Institute Tech. Bull. No. 14.*
- Bentley, J. R., and L. R. Green. 1954. Stimulation of native annual clovers through application of sulfur on California foothill range. *J. Range Mgmt.* 7:25-30.
- Berg, M. G., and E. H. Gardner. 1978. Methods of soil analysis used in the soil testing laboratory at Oregon State University. *Oreg. Agric. Exp. Stn. Spec. Rep. 321.* p. 29-32.
- Bouma, D., K. Spencer, and E. J. Dowling. 1969. Assessment of the phosphorus and sulphur status of subterranean clover pastures. 3. Plant tests. *Austr. J. Exp. Agr. Animal Husb.* 9:329-340.
- Burns, G. R. 1967. Oxidation of sulphur in soils. *The Sulphur Institute Tech. Bull. No. 13.*
- Butters, B., and E. M. Chenery. 1959. A rapid method for the determination of total sulphur in soils and plants. *Analyst.* 84:239-245.
- Cairns, R. R., and R. B. Carson. 1961. Effect of sulphur treatments on yield and nitrogen and sulphur content of alfalfa grown on sulphur-deficient and sulphur-sufficient grey wooded soils. *Can. J. Plant Sci.* 41:709-715.

- Chao, T. T., M. E. Harward, and S. C. Fang. 1962. Soil constituents and properties in the adsorption of sulfate ions. *Soil Sci.* 94:276-283.
- Dawson, M. D. 1969. Sulphur on pasture legumes in Oregon. *Sulphur Inst. J.* 5:16-18.
- Dijkshoorn, W., and A. L. Van Wijk. 1967. The sulphur requirements of plants as evidenced by the sulphur-nitrogen ratio in the organic matter. A review of published data. *Plant and Soil.* 26:129-157.
- Drlica, D. M., and T. L. Jackson. 1979. Effects of stage of maturity on P and S critical levels in subterranean clover. *Agron. J.* 71:824-828.
- Eaton, F. M. 1966. Sulfur-diagnostic criteria for plants and soils. Univ. of Calif. Press.
- Ensminger, L. E. 1954. Some factors affecting the adsorption of sulfate by Alabama soils. *Soil Sci. Soc. Am. Proc.* 18:259-264.
- _____, and J. R. Freney. 1966. Diagnostic techniques for determining sulfur deficiencies in crops and soils. *Soil Sci.* 101:283-290.
- Fox, R. L., H. M. Atesalp, D. H. Kampbell, and H. F. Rhoades. 1964. Factors influencing the availability of sulfur fertilizers to alfalfa and corn. *Soil Sci. Soc. Am. Proc.* 28:406-408.
- _____, R. L., A. D. Flowerday, F. W. Hosterman, H. F. Rhoades, and R. A. Olson. 1964. Sulfur fertilizers for alfalfa production in Nebraska. *Nebr. Agr. Exp. Stn. Res. Bull.* 214.
- Freney, J. R., and K. Spencer. 1967. Diagnosis of sulphur deficiency in plants by soil and plant analysis. *J. Austr. Inst. Agric. Sci.* 33:284-288.
- _____, _____, and M. B. Jones. 1977. On the constancy of the ratio of nitrogen to sulphur in the protein of subterranean clover tops. *Commun. Soil Sci. Plant Anal.* 8:241-249.
- _____, and F. J. Stevenson. 1966. Organic sulfur transformations in soils. *Soil Sci.* 101:307-316.
- _____, and R. J. Swaby. 1975. Sulphur transformations in soils. p. 31-39. *In Sulphur in Australasian agriculture.* Sydney University Press. Sydney.
- Gaines, T. P. 1976. Determination of protein nitrogen in plants. *J. Assoc. Off. Anal. Chem.* 60:590-593.

- Gaines, T. P., and S. C. Phatak. 1982. Sulfur fertilization effects on the constancy of the protein N:S ratio in low and high sulfur accumulating crops. *Agron. J.* 74:415-418.
- Gast, R. G. 1977. Surface and colloid chemistry. p. 27-73. In *Minerals in soil environments*. Soil Sci. Soc. Am., Inc. Madison, WI.
- Giovanelli, J., S. H. Mudd, and A. H. Datko. 1980. Homocysteine biosynthesis in plants. p.81-92. D. Cavallini, G. E. Gaull, and V. Zappia (eds.). In *Natural sulfur compounds*. Plenum Press. New York.
- Gray, T. R. G., and S. T. Williams. 1971. *Soil micro-organisms*. Longman. London.
- Handreck, K. A. 1972. Sulphur status of plants by sulphate-sulphur: sample handling as a source of misinterpretation. *Plant soil* 37:203-207.
- Hanley, P. K., and M. D. Murphy. 1970. Crop responses to sulphur in Ireland. p. 68-79. In *Sulphur in agriculture*. An Foras Taluntais. Dublin, Ireland.
- Harward, M. E., and H. M. Reisenauer. 1966. Reactions and movement of inorganic soil sulfur. *Soil Sci.* 101:326-335.
- Hughes, M. N. 1972. *The inorganic chemistry of biological processes*. John Wiley and Sons, Inc. New York.
- Iismaa, O. 1959. Micro determination of sulphur in plant material. *J. Austr. Inst. Agric. Sci.* 25:136-138.
- Jackson, T. L. 1972. Effects of fertilizers and lime on the establishment of subterranean clover. *Oregon Agr. Exp. Stn. Circ. Info.* 634.
- Johnson, C. M., and H. Nishita. 1952. Microestimation of sulfur in plant materials, soils and irrigation waters. *Anal. Chem.* 24:736-742.
- Jones, M. B. 1962. Total sulfur and sulfate sulfur content in subterranean clover as related to sulfur responses. *Soil Sci. Soc. Am. Proc.* 26:482-484.
- _____. 1963. Effect of sulfur applied and date of harvest on yield, sulfate sulfur concentration, and total sulfur uptake of five annual grassland species. *Agron. J.* 55:251-254.
- _____. 1964. Effect of applied sulfur on yield and sulfur uptake of various California dryland pasture species. *Agron. J.* 56:235-237.

- Jones M. B., and W. E. Martin. 1964. Sulfate-sulfur concentration as an indicator of sulfur status in various California dryland pasture species. *Soil Sci. Soc. Am. Proc.* 28:539-541.
- _____, _____, and J. E. Ruckman. 1970. Effectiveness of various sulphur sources applied to annual-type grasslands of California. *Proc. XI Int. Grassld. Congr.* p. 373-376.
- _____, and J. E. Ruckman. 1966. Gypsum and elemental sulfur as fertilizers on annual grassland. *Agron. J.* 58:409-412.
- _____, and _____. 1969. Effect of particle size on long-term availability of sulfur on annual-type grasslands. *Agron. J.* 61:936-939.
- _____, _____, W. A. Williams, and R. L. Koenigs. 1980. Sulfur diagnostic criteria as affected by age and defoliation of subclover. *Agron. J.* 72:1043-1046.
- Jones, R. K., P. J. Robinson, K. P. Haydock, and R. G. Megarrity. 1971. Sulphur-nitrogen relationships in the tropical legume *Stylosanthes humulis*. *Austr. J. Agric. Res.* 22:885-894.
- Kelly, J., and M. J. Lambert. 1972. The relationship between sulphur and nitrogen in the foliage of *Pinus radiata*. *Plant Soil* 37:395-407.
- Legget, G. E., B. A. Stewart, and D. W. Jones. 1966. Sulfur deficiency of sugar beets. *Proc. 17th Ann. Fert. Conf. Pacific Northwest*, July 12-14. p. 157-164.
- Li, P., and A. C. Caldwell. 1966. The oxidation of elemental sulfur in soil. *Soil Sci. Soc. Am. Proc.* 32:535-540.
- Ludecke, T. E. 1965. Further aspects of sulphur nutrition of legumes. *N. Z. Grassld. Assoc. Proc.* 27:129-138.
- McKell, C. M., and W. A. Williams. 1960. A lysimeter study of sulfur fertilization of an annual-range soil. *J. Range Mgmt.* 13:113-117.
- McLachlan, K. D. 1974. Handbook on sulphur in Australian agriculture. CSIRO Melbourne, Australia.
- _____. 1975. Sulphur in Australasian agriculture. Sydney Univ. Press Sydney, Australia.
- _____, and D. G. DeMarco. 1968a. The influence of time of application of gypsum and elemental sulphur on the pasture response to sulphur. *Austr. J. Exp. Agr. Animal Husb.* 8:725-730.

- McLachan, K. D., and D. G. DeMarco. 1968b. The influence of gypsum particle size on pasture response on a sulphur deficient soil. *Austr. J. Exp. Agr. Animal Husb.* 8:203-209.
- _____, and _____. 1971. Current and residual effects of calcium sulphate applications on a sulfur deficient basaltic soil. *Austr. J. Exp. Agr. Animal Husb.* 11:64-70.
- _____, and _____. 1973. A comparison of fertilizer programmes for the development and maintenance of sown pasture on a sulphur deficient basaltic soil. *Austr. J. Exp. Agr. Animal Husb.* 13:75-80.
- McNaught, K. J. 1970. Diagnosis of mineral deficiencies in grass-legume pastures by plant analysis. *Proc. XI Int. Grassld. Congr.* p. 334-338.
- _____, and P. J. E. Chrisstoffels. 1961. Effect of sulphur deficiency on sulphur and nitrogen levels in pastures and lucerne. *N. Z. J. Agric. Res.* 4:177-196.
- Martin, W. E., and J. E. Matocha. 1973. Plant analysis as an aid in the fertilization of forage crops. p. 393-426. *In* Soil testing and plant analysis. *Soil Sci. Soc. Am. Inc.* Madison, WI.
- Mayland, H. F. 1968. Effect of drying methods on losses of carbon, nitrogen and dry matter from alfalfa. *Agron. J.* 60:658-659.
- _____, D. T. Westermann, and A. R. Florence. 1978. Drying method effects on extractable phosphorus levels in plant tissue. *Commun. Soil Sci. Plant Anal.* 9:551-557.
- Mehlich, A. 1981. Charge properties in relation to sorption and desorption of selected cations and anions. p. 47-75. *In* Chemistry in the soil environment. *Am. Soc. Agron., Soil Sci. Soc. Am.* Madison, WI.
- Mengel, K., and E. A. Kirkby. 1978. Principles of plant nutrition. *DerBund.* Bern, Switzerland.
- Metson, A. J. 1972. Iron pyrites as sulphur fertilizers. II. Forms of sulphur fertiliser in relation to growth of, and total sulphur, sulphate sulphur, and nitrogen/sulphur ratio in, a grass-clover pasture. *N. Z. J. Agric. Res.* 15:565-584.
- _____. 1973. Sulphur in forage crops. *Technical Bull. No. 20.* The Sulphur Institute.

- Metson, A. J., and T. W. Collie. 1972. Iron pyrites as sulphur fertilisers. III. Nitrogen-sulphur relationships in grass and clover separates of pasture herbage in a field trial at Golden Bay, Nelson. *N. Z. J. Agric. Res.* 15:585-604.
- Munson, R. D., and W. L. Nelson. 1973. Principles and practices in plant analysis. p. 223-248. *In* Soil testing and plant analysis. Soil Sci. Soc. Am. Inc. Madison, WI.
- Murphy, M. D. 1978. Responses to sulphur in Irish grassland. p. 95-107. *In* Sulphur in forages. An Foras Taluntais. Dublin.
- Neter, J., and W. Wasserman. 1974. Applied linear statistical models. Irwin, Inc. Homewood, Illinois. p.160-165.
- Powers, W. L. 1923. Sulfur in relation to soil fertility. Oregon Agric. Coll. Exp. Sta. Bull. 199.
- Powrie, J. K. 1967. Fragmented rock gypsum as a sulphur fertilizer. *J. Austr. Inst. Agric. Sci.* 33:127-129.
- Pumphrey, F. V., and D. P. Moore. 1965a. Diagnosing sulfur deficiency of alfalfa (Medicago sativa L.) from plant analysis. *Agron. J.* 57:364-366.
- _____. and _____. 1965b. Sulfur and nitrogen content of alfalfa herbage during growth. *Agron. J.* 57:237-239.
- Randall, P. J., and K. Spencer. 1980. Sulfur content of plant material: A comparison of methods of oxidation prior to determination. *Commun. Soil Sci. Plant Anal.* 11:257-266.
- Reisenauer, H. M., L. M. Walsh, and R. G. Hoelt. Testing soils for sulphur, boron, molybdenum, and chlorine. p. 173-200. *In* Soil testing and plant analysis. Soil Sci. Soc. Am. Inc. Madison, WI.
- Seim, E. C., A. C. Caldwell, and G. W. Rehm. 1969. Sulfur response by alfalfa (Medicago sativa L.) on a sulfur-deficient soil. *Agron. J.* 61:368-371.
- Sinclair, A. G., and W. M. H. Saunders. 1982. Sulphur fertilizers in New Zealand. Quantities and forms required for pasture production. *Sulphur in agric.* 6:15-22.
- Smith, F. W., and G. R. Dolby. 1977. Derivation of diagnostic indices for assessing the sulphur status of Panicum maximum Var. Trichoglume. *Commun. Soil Sci. Plant Anal.* 8:221-240.
- Spencer, K. 1963. The residual value of a sulphatic fertilizer applied to a basaltic soil. *Austr. J. Exp. Agr. Animal Husb.* 3:180-183.

- Spencer, K. 1978. Sulphur nutrition of clover: effect of plant age on the composition-yield relationship. *Commun. Soil Sci. Plant Anal.* 9:883-895.
- _____, and D. Bouma. 1970. Sulphur content of clover as an indicator of pasture response. *Proc. XI Int. Grassld. Congr.* p. 341-344.
- _____, K., M. B. Jones, and J. R. Freney. 1977. Diagnostic indices for sulphur status of subterranean clover. *Austr. J. Agric. Res.* 28:401-412.
- Stewart, B. A., and L. K. Porter. 1969. Nitrogen-sulfur relationships in wheat (*Triticum aestivum* L.), corn (*Zea mays*), and beans (*Phaseolus vulgaris*). *Agron. J.* 61:267-271.
- _____, and C. J. Whitfield. 1965. Effects of crop residue, soil temperature, and sulfur on the growth of winter wheat. *Soil Sci. Soc. Am. Proc.* 29:752-755.
- Technicon Industrial Method No. 100-70W. Sept., 1973. Technicon Industrial Systems. Tarrytown, New York.
- Technicon Industrial Method No. 334-74A/A. Jan., 1976. Technicon Industrial Systems. Tarrytown, New York.
- Thompson, J. F., I. K. Smith, and D. P. Moore. 1970. Sulfur requirement and metabolism in plants. p. 80-96. In Symposium: sulfur in nutrition. AVI Publishing Co., Inc. Westport, CN.
- Ulrich, A., M. A. Tabatabai, K. Ohki, and C. M. Johnson. 1967. Sulfur content of alfalfa in relation to growth in filtered and unfiltered air. *Plant Soil* 26:235-252.
- Whitehead, D. C. 1964. Soil and plant-nutrition aspects of the sulphur cycle. *Soil fertilizers* 27:1-9.
- Walker, T. W. 1955. Sulphur responses on pastures in Australia and New Zealand. *Soil fertilizers* 18:185-187.
- _____. 1957. The sulphur cycle in grassland soils. *J. British Grassld. Soc.* 12:10-18.
- Walker, T. W., A. F. R. Adams, and H. D. Orchiston. 1956. The effects of levels of calcium sulphate on the yield and composition of a grass and clover pasture. *Plant soil.* 8:290-300.
- _____, and _____. 1958. Residual effects of calcium sulphate on the yield and composition of a grass-clover association. *Plant soil* 10:176-182.

- Weir, R. G., B. Barkus, and W. T. Atkinson. 1963. The effect of particle size on the availability of brimstone sulphur to white clover. *Austr. J. Exp. Agr. Animal Husb.* 3:314-318.
- Westerman, D. T. 1975. Indexes of sulfur deficiency in alfalfa II. Plant analyses. *Agron. J.* 67:265-268.
- Williams, W. A., C. M. McKell, and J. N. Reppert. 1964. Sulfur fertilization of an annual-range soil during years of below normal rainfall. *J. Range Mgmt.* 17:1-4.

APPENDICES

APPENDIX A

Dry ash oxidation

A 0.5 gram plant sample was placed in a 50 ml beaker to which 2 ml of 70% ETOH and 3 ml of saturated magnesium nitrate solution were added. Ethanol was driven off with low heat. Only 2 ml of magnesium nitrate solution were added with aqueous samples. The samples were then ashed in a muffle furnace at 550°C for four hours. After cooling, 10 ml of 3N HCL was added to dissolve the ash. This solution was filtered into 50 ml volumetric flasks and brought to volume with distilled water.

Oxidation flask

This method is based on that of Iismaa (1959) with certain changes as noted below. The sample size was 50-100 mg. The trapping solution consisted of 17 ml of 0.4N KOH and 1.0 ml of 50% H₂O₂ and the flask was shaken prior to ignition to coat all inside surfaces. After standing one hour after ignition, 2 ml of 7.5N HCL was added and the flask was shaken to coat all inside surfaces. An aliquot of this solution was taken for further analyses.

Turbidimetric procedure for sulfate-S determination

From a dry ash combustion a 10 ml sample aliquot was added to 1.0 ml of a 50% conc. HCL, 50% conc. acetic acid solution and allowed to stand one hour. To this was added 1.0 ml of turbidity solution (0.6 gram gelatin dissolved in 200 ml distilled water to which was added 4 grams of BaCl₂), mixed for 15-20 seconds and, after 30 minutes, was swirled for 5 seconds and read for absorption at 500 nm.

From combustion in an oxidation flask, a 2.5 ml sample was taken and spiked with 2.5 ml of an aqueous K₂SO₄ solution (5 mg S/L). For blanks, 2.5 ml of blank solution was diluted with 2.5 ml of distilled water. For standards, 2.5 ml of blank solution was

added to 2.5 ml of standard solution. The same procedure as for dry ash was then followed except for using 0.5 ml of the HCL:acetic acid mixture and 0.5 ml of the turbidity solution.

Methylene blue procedure for sulfate-S determination

The method used was based on the technique of Johnson and Nishita (1952) as modified by the Soil Testing Laboratory at Oregon State University (1978). When this procedure was used in conjunction with dry ashing, aliquots were taken directly from the ashed solution. When this procedure was used in conjunction with the oxidation flask combustion, one ml of 1 N HCL was added to a 2.0 ml sample aliquot in the reduction flask and heated to dryness to drive off any $\text{NO}_3\text{-N}$ present. Two ml of distilled water was then added to the reduction flask.

Nitrate-N determination

The procedure paralleled that of Technicon (1973) with the following modifications: 1) the NH_4Cl reagent was not made with alkaline water, but 0.1 gram of Na_2EDTA /liter was added to the NH_4Cl reagent and 2) the column was conditioned with 100 ppm $\text{NO}_3\text{-N}$ solution for five minutes.

Micro-Kjeldahl technique

Five ml or 100 mg of sample was combined with 1/8 tsp digestion mix (1000 g Na_2SO_4 , 25 g CuSO_4 , and 10 g Se powder), 1/8 tsp thiosulfate and 5 ml conc. sulfuric acid and pre-digested on low heat for 15-20 minutes. Then the digestion mixture was refluxed at high heat (approx. 450 C) for 3 hours or until clear. After cooling the solution was brought to volume. Ammonium-N was determined using a Technicon (1976) procedure.

APPENDIX B

Table B-1. ANOVA for dry matter yield of forage for Roseburg site for four years.

Source	df	Mean square	F-test
Total	339		
Block	4	1.1889	
Year	3	71.3965	133.47**
Block X Year	12	0.5349	
Treatment	16	3.8171	4.26**
annual rate (ar)	2	11.4282	12.75**
source (s)	1	0.8254	0.92 ns
ar X s	2	0.5549	0.62 ns
single rate (sr)	1	1.2478	1.39 ns
source (s)	4	0.3501	0.39 ns
sr X s	4	0.2793	0.31 ns
Treatment X Year	48	1.2439	
ar X year	6	1.3430	1.50 ns
s X year	3	0.7319	0.82 ns
ar X s X year	6	0.4247	0.47 ns
sr X year	3	1.2757	1.42 ns
s X year	12	1.0967	1.22 ns
sr X s X year	12	0.9763	1.09 ns
Error	256	0.8963	

** significant at the 1% level

ns not significant at the 5% level

Table B-2. ANOVA for S accumulation of forage at the Roseburg site for four years.

Source	df	Mean square	F-test
Total	339		
Block	4	7.4961	
Year	3	174.313	238.80**
Year X Block	12	0.7299	
Treatment	16	85.906	33.75**
annual rate (ar)	2	254.029	99.77**
source (s)	1	22.700	8.92**
ar X s	2	11.931	4.68**
single rate (sr)	1	145.672	57.22**
source (s)	4	76.668	30.11**
s X sr	4	12.501	4.91**
Treatment X Year	48	23.206	9.11**
ar X year	6	10.373	4.07**
s X year	3	13.200	5.18**
ar X s X year	6	3.472	1.36 ns
sr X year	3	3.646	1.43 ns
s X year	12	57.178	22.46**
sr X s X year	12	11.235	4.41**
Error	256	2.546	

** significant at the 1% level

ns not significant at the 5% level

Table B-3. ANOVA for regression of total N on total S and protein N on protein S for different drying procedures for 3 sampling dates.

Comparison-treatment	Source	df	Mean square
<u>March 30</u>			
N _t vs S _t (oven-dried)	total	19	0.15096
	regression	1	2.24431
	residual	18	0.03467
N _P vs S _P (oven-dried)	total	19	0.12032
	regression	1	1.76015
	residual	18	0.02921
N _P vs S _P (freeze-dried)	total	19	0.09540
	regression	1	1.30956
	residual	18	0.02795
<u>April 12</u>			
N _t vs S _t (oven-dried)	total	19	0.11497
	regression	1	1.58880
	residual	18	0.03291
N _P vs S _P (oven-dried)	total	19	0.13552
	regression	1	1.95929
	residual	18	0.03421
N _P vs S _P (freeze-dried)	total	19	0.10071
	regression	1	1.42401
	residual	18	0.02720
<u>April 27</u>			
N _t vs S _t (oven-dried)	total	19	0.23535
	regression	1	3.38212
	residual	18	0.06053
N _P vs S _P (oven-dried)	total	19	0.13486
	regression	1	2.23420
	residual	18	0.01823
N _P vs S _P (freeze-dried)	total	19	0.09969
	regression	1	0.90803
	residual	18	0.05479

Table B-4. ANOVA for regression of protein N on protein S of the combination of oven-dried and freeze-dried data and the regression of N concentration on S concentration of the combination of oven-dried and TCA insoluble material data for 3 sampling dates.

Combination	Source	df	Mean square
<u>March 30</u>			
N vs S (Freeze ^P + oven- dried)	total	19	0.13659
	regression	1	4.01436
	residual	18	0.03455
N vs S (total + TCA insoluble)	total	19	0.17380
	regression	1	5.30981
	residual	18	0.03865
<u>April 12</u>			
N vs S (Freeze ^P + oven- dried)	total	19	0.13902
	regression	1	3.98543
	residual	18	0.03780
N vs S (total + TCA insoluble)	total	19	0.16328
	regression	1	4.71856
	residual	18	0.04341
<u>April 27</u>			
N vs S (Freeze ^P + oven- dried)	total	19	0.12199
	regression	1	3.37674
	residual	18	0.01364
N vs S (total + TCA insoluble)	total	19	0.28294
	regression	1	8.85944
	residual	18	0.05724

Table B-5. Values of the F-test for comparison of N vs S regression lines for 3 sampling dates. Mean square values were calculated according to Neter and Wasserman (1974).

Comparison	March 30	April 12	April 27
freeze-dry (protein) vs oven-dry (protein)	14.23**	13.85**	17.13**
oven-dry (total) vs oven-dry (protein)	14.09**	13.18**	11.73**

** significant at the 1% level

Table B-6. ANOVA for N and S fractions of subclover.

Source	df	Total N conc.		Soluble N conc.		Soluble S conc.		Total S conc.	
		Mean square	F-test	Mean square	F-test	Mean square	F-test	Mean square	F-test
block (B)	4	5.796		27.775		1.0602		2423.33	
S rate (S)	3	175.593	25.90**	35.261	0.33ns	52.5151	59.32**	185698.	65.85**
S X B	12	6.780		106.664		0.8852		2820.00	
date (D)	2	504.415	310.58**	2470.400	59.22**	0.0447	0.10ns	5180.00	5.87**
D X B	8	1.624		41.712		0.4480		882.08	
S X D	6	8.832	3.71**	11.978	0.41ns	0.6351	1.27ns	1871.11	2.01ns
S X D X B	24	2.371		29.568		0.5007		928.75	
		<u>Total S conc.</u>		<u>Protein N (fr-dry)</u>		<u>Protein N (oven-dry)</u>		<u>Protein S (fr-dry)</u>	
block (B)	4	0.02443		0.20705		0.39068		0.0006572	
S rate (S)	3	2.69470	50.02**	0.80702	2.52ns	1.78362	11.27**	0.0086195	10.25**
S X B	12	0.05387		0.32027		0.15828		0.0008413	
date (D)	2	2.17132	360.68**	4.33661	89.91**	5.88212	33.63**	0.0123662	96.24**
D X B	8	0.06020		0.04823		0.17492		0.0001285	
S X D	6	0.04414	2.40ns	0.08895	1.73ns	0.18403	2.93*	0.0006722	1.86ns
S X D X B	24	0.01840		0.05151		0.06298		0.0003610	
		<u>Protein S (oven-dry)</u>		<u>Protein N (ETOH)</u>		<u>Protein S (ETOH)</u>			
block (B)	4	0.0008976		0.32805		0.000018			
S rate (S)	3	0.0111443	16.83**	0.72105	24.99**	0.008675	53.32**		
S X B	12	0.0006621		0.02885		0.000163			
date (D)	2	0.0167740	158.81**	1.56465	42.27**	0.002722	128.60**		
D X B	8	0.0001056		0.03702		0.000021			
S X D	6	0.0005097	1.54ns	0.00256	0.33ns	0.000176	0.74ns		
S X D X B	24	0.0003316		0.00777		0.000240			

ns - not significant at the 5% level

* - significant at the 5% level

** - significant at the 1% level

Table B-7. ANOVA for protein N and S for the combined data for oven- and freeze-dried material.

Source	df	Protein S		Protein N	
		Mean square	F-test	Mean square	F-test
block (B)	4	0.0012606		0.52057	
S rate (S)	3	0.0194141	15.59**	2.44231	5.46*
S X B	12	0.0012449		0.44704	
date (D)	2	0.0289322	171.86**	9.97812	66.99**
D X B	8	0.0001684		0.14894	
S X D	6	0.0010113	2.04ns	0.24890	3.42*
S X D X B	24	0.0004962		0.07286	
drying proc.(DP)	1	0.0054675	27.57**	5.63333	118.60**
DP X S	3	0.0000035	0.02ns	0.14828	3.12**
DP X D	2	0.0002077	1.05ns	0.24059	5.07**
DP X S X D	6	0.0000171	1.42ns	0.02410	0.51ns
error	48	0.0001983		0.04752	

ns - not significant at the 5% level

* - significant at the 5% level

** - significant at the 1% level

Table C-1. Rainfall distribution for the Roseburg site for four years.

Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
-----cm-----												
1974									0.00	4.65	8.79	16.28
1975	13.23	13.46	14.15	5.99	2.31	0.38	1.88	2.84	0.33	10.57	11.40	15.47
1976	14.73	6.91	9.68	3.86	1.73	0.66	1.27	8.38	2.26	0.76	2.77	2.13
1977	4.01	6.17	8.41	2.24	6.78	1.30	0.00	4.39	8.00	3.71	12.19	14.25
1978	9.07	9.32	3.61	6.88	1.93							