The unusual condition of sulfur deficiencies in many soils throughout the Pacific Northwest has long been recognized. Marked increases in yield and purity of stand of alfalfa in response to sulfur fertilization have been demonstrated. Analyses of such samples have frequently shown increased contents of crude protein and of total, inorganic and organic sulfur. These circumstances afford an unusual opportunity for a study of the effects of sulfur fertilization on the nutritive value of alfalfa protein with respect to its content of the sulfur-containing amino acids. It has been demonstrated that alfalfa consistently contains organic sulfur in amounts equivalent to 0.8-0.9% of its crude protein content. The organic sulfur in alfalfa is thought to exist in the form of proteins. It therefore appeared that all of the organic sulfur found in alfalfa should be accounted for as cystine and methionine.

An attempt was made to adapt microbiological assay procedures to the determination of cystine and methionine in alfalfa. The use of an all-purpose, synthetic medium, and the organism Leuconostoc mesenteroides P-60 resulted in uniformly reproducible standard curves.

Varied conditions of hydrochloric acid hydrolysis of alfalfa by autoclaving in sealed tubes resulted in inconsistent analyses and low recovery values. Adoption of a procedure for compensation for suspected toxic substances in the hydrolyzates permitted more uniform results, which however remained low. Increased sensitivity of the assay for cystine, by sterilization of glucose separately from the medium, permitted greater dilution of hydrolyzates and their inherent toxic effects. The basic defect of poor recovery of added amino acids was never overcome, intimating that destructive losses had occurred during hydrochloric acid hydrolysis.

Numerous enzyme digestions were attempted, the best of which did not permit successful assays for cystine and methionine. Subsequent acid hydrolysis of a digest filtrate resulted in assay values which indicated that enzyme action had successfully separated the protein fraction from the sample, but only accomplished partial proteolysis.
One of several combinations of hydrolysis of alfalfa by refluxing with sulfuric acid permitted consistent assay and recovery of methionine but not of cystine. Assay of several samples of known sulfur fertilization history showed increased methionine. This was accompanied by increased organic sulfur and crude protein contents. However, insufficient sulfur-containing amino acids were found to account for more than 50% of the organic sulfur.

Until liberation of cystine is more successfully accomplished, it does not appear likely that it will be possible to adequately characterize the proteins of such complex materials as alfalfa with respect to their sulfur-containing amino acids.
THE CYSTINE AND METHIONINE CONTENTS OF ALFALFA AS INFLUENCED BY SULFUR FERTILIZATION

by

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Typed by Miriam Schubert
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Thanks are also due to Dr. S. C. Fang, and Dr. L. F. Remmert for their help with microbiological assay techniques, and enzyme digestion procedures, respectively.

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To the above mentioned and others of the staff of the department, I am most appreciative for many helpful suggestions and for their interest in my progress.

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THE CYSTINE AND METHIONINE CONTENTS OF ALFALFA AS INFLUENCED BY SULFUR FERTILIZATION

INTRODUCTION

The presence of sulfur in plants and animals has been recognized since very early times. The mode of its incorporation and the role of its compounds in metabolism have been the objects of continued interest from the inception of the science of nutrition. Particular significance has been attached to the sulfur-containing amino acids, cystine and methionine.

The ultimate source of all mineral nutrients is the soil. The early recognition of mineral deficiencies in soils and their relation to food production has motivated numerous investigations. Oregon occupies a peculiarly interesting place in this picture, in that many of its soils are markedly deficient in sulfur. Since alfalfa frequently shows remarkable responses to sulfur fertilization, this plant was chosen as the object of investigations to be reported in this thesis. Special attention has been given to the cystine and methionine contents of the alfalfa plant.
HISTORICAL

General

Cystine was discovered by Wollaston in 1810 (71, p. 22). Until 1922, it was the only sulfur containing amino acid known, when however the work of Mueller (73, pp. 161-163) (74, pp. 157-169) showed the existence of methionine. From earliest nutritional studies, the amino acid components of protein have been recognized as criteria of quality. It was the work of Rose and co-workers (97, p. lxxxv) that established the indispensable nature of methionine, and not cystine as previously supposed. Later work by the same group (119, p. 410) suggested that cystine could replace a part of the methionine requirement.

This concept of sparing action has been widely accepted following the demonstration by Tarver and Schmidt (113, pp. 87-80) of a methionine to cystine conversion in the animal body. A mechanism of the reaction has been demonstrated by Binkley and duVigneaud (8, pp. 509-511), Stetten (107, pp. 503-506) and Horowitz (50, pp. 562-563). In mammalian nutrition the hypothesis now stands that a certain minimum amount of methionine is essential as such, since it is not synthesized in vivo. An additional amount of cystine or methionine is
necessary to meet requirements for aggregate sulfur containing amino acids.

The essential nature of these amino acids, and of other sulfur-containing metabolites, is of particular importance to Oregon and the Pacific Northwest. The unusual condition of local deficiencies of sulfur in many soils of the area cannot help but exert direct influence on the biological production of compounds of the element. Hence, this basic defect in the ultimate source of sulfur should be expected to affect all forms of life which require it for their metabolic processes.

In accounting for the total sulfur content of plant materials, an initial division may be made into inorganic and organic fractions. The latter is usually made up of a predominant percentage of protein, and the remainder largely consists of volatile compounds. Peterson (84, p.1297) sometime ago established that volatile sulfur compounds were negligible in alfalfa.

The long history of the response of alfalfa to sulfur fertilization forms the basis for present considerations. In 1916 Reimer (92, p.408) variously fertilized alfalfa grown on southern Oregon sulfur deficient plots. In all cases, the stands which had been supplied with sulfur showed marked increases in yield over the others. Later, Reimer and Tartar (91, pp.38-39), using sulfured and control plots, showed
yield increases of as much as 100%. In addition, the purity of the alfalfa stands was greatly improved. Chemical analyses of the resultant hays showed significant increases in their sulfur and nitrogen contents. These results have been repeatedly confirmed by such workers as Olson and St. John (79, pp.63-64) and Neller (77, pp.42-43), (76, p.73) in Washington, Neidig (75, p.135) in Idaho, and Evans and Greaves (28, pp.18-21) in Utah. The latter summed up many results and concluded that there is a high correlation between the organic sulfur and crude protein contents of alfalfa. A ratio was shown to be constant between 1 to 117 and 1 to 128, which was also expressed as organic sulfur being from 0.78 to 0.86% of the crude protein.

Experimental feeding of alfalfa as a source of protein has shown decided disadvantages attributable to its sulfur fraction. Haag (34, p.369), using alfalfa as the sole source of protein, definitely established cystine as a limiting amino acid in its nutritive value for rats. Later Wright and Haag (121, p.266-267) used the same type of ration to study the effect on lactation of rats, and again found the inadequate cystine content of alfalfa to be a limiting factor. Morrison (72, p.100) states that the biological value of alfalfa proteins is 62% for rats. To partly explain this figure Mitchell and
Block (70, p. 602) report deficiencies of 33 and 44 percent, respectively, for cystine and methionine in alfalfa. They conclude this from comparison with the composition of whole egg protein, which they propose as a nearly perfect natural mixture of amino acids.

It has been conjectured by Cook (19, pp. 30-34) and Haag (33) that all the organic sulfur in alfalfa should represent protein, and be accounted for as cystine plus methionine. The basis for this lies in the previously stated findings, and their own analyses of alfalfa samples of known sulfur fertilization history. Some of the latter results are as follows:

1. Crude protein increases from 1 to 3%.
2. There is a corresponding increase in total sulfur content which largely represents accumulated sulfates.
3. The increase in total sulfur also includes an increase in organic sulfur which parallels the increase in crude protein.

This parallelism was exhibited by a calculated ratio of organic sulfur to total nitrogen for each sample, which gave strikingly constant average figures of 0.050 to 0.055. This may be taken to mean that the organic sulfur contents varied from 0.80 to 0.88% of crude protein. It was concluded therefore, that sulfur fertilization influences the amount of protein produced, but
does not alter the kind of protein in the mature plant.

In order to continue past work to its logical conclusion it seemed advisable to investigate the possibilities of hydrolyzing alfalfa proteins, in the intact hay, and to attempt to account for the organic sulfur fraction directly as cystine and methionine. This could then be followed by a more direct nutritional evaluation of the visible yield and purity benefits of sulfur fertilization.

Subsequent to the initiation of such a project, results of greenhouse pot culture studies have been reported (54, pp.25-29) and published (115, p.225) by Kingsley and co-workers. In the first of these, microbiological assays of several strains of alfalfa were carried out for their amino acid contents, including cystine and methionine. In determining total sulfur in the same samples they found that not over 27% of it was contributed by cystine plus methionine, which is interesting but inconclusive without inorganic sulfur determinations. In their latter paper, analyses of sand cultured plants, whose nutrient solutions supplied increasing amounts of sulfate ion, showed marked increases in cystine and methionine. An anomaly was presented however, in that the total nitrogen percentages decreased with added sulfate. This leads to striking increases of
cystine and methionine per gram of nitrogen with increasing levels of sulfur fertility, intimating an improvement of biological value of alfalfa protein.

A review of literature reports as to the absolute amounts of cystine and methionine in alfalfa shows highly variable values probably due to the variety of methods used. These figures are recalculated to a common basis and shown in Table I. Of these values it is interesting that only those of Block and Mitchell (10, p.248), and Lugg (63, p.409) suffice to be recalculated to give the same organic sulfur percents of crude protein found by Cook (19, p.v), and Evans and Greaves (28, p.20).

Chemical Analyses for Cystine and Methionine

The early quantitative determinations of cystine were performed by indirect chemical means, dependent on the sulfur content of a given protein. Among the first of these was the method of Van Slyke (117, pp.15-55) whereby groups of amino acids of similar chemical characteristics were separated from hydrolyzates. Cystine was precipitated along with basic amino acids through use of phosphotungstic acid, and a sulfur determination by the Benedict-Denis copper nitrate oxidation method served as the basis for calculation of the cystine content. Results from use of this method on alfalfa are given in Table I (21, p.440), (36, p.258). Vickery and White (118, pp.
**TABLE I**

**Literature Reports of the Cystine and Methionine Contents of Alfalfa**

(Recalculated in terms of gm. per 16 gm. N, therefore equivalent to percent of crude protein, N x 6.25).

<table>
<thead>
<tr>
<th>Ref. No.</th>
<th>Authors</th>
<th>Cystine</th>
<th>Methionine</th>
<th>Analytical Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10)</td>
<td>Block</td>
<td>1.6</td>
<td>2.3</td>
<td>Colorimetric</td>
</tr>
<tr>
<td>(21)</td>
<td>Dowell</td>
<td>0.35</td>
<td>-</td>
<td>Gravimetric</td>
</tr>
<tr>
<td>(36)</td>
<td>Hamilton</td>
<td>1.36</td>
<td>-</td>
<td>Gravimetric</td>
</tr>
<tr>
<td>(63)</td>
<td>Lugg</td>
<td>1.56</td>
<td>2.10</td>
<td>Gravimetric</td>
</tr>
<tr>
<td>(54)</td>
<td>Kingsley*</td>
<td>1.57</td>
<td>0.92</td>
<td>Microbiological</td>
</tr>
<tr>
<td>(115)</td>
<td>Tisdale*</td>
<td>1.36</td>
<td>1.11</td>
<td>Microbiological</td>
</tr>
<tr>
<td>(93)</td>
<td>Reisen</td>
<td>-</td>
<td>1.43</td>
<td>Microbiological</td>
</tr>
<tr>
<td>(109)</td>
<td>Stokes</td>
<td>-</td>
<td>0.83</td>
<td>Microbiological</td>
</tr>
<tr>
<td>(67)</td>
<td>Lyman</td>
<td>-</td>
<td>1.20</td>
<td>Microbiological</td>
</tr>
</tbody>
</table>

*Highest values.*
701-715) proposed a method of precipitation of cystine and cysteine as the cysteine cuprous mercaptide. Here again cystine content was calculated from the total sulfur content of the precipitate. It is recognized that there is no little chance for error on the basis of losses incurred through such isolation procedures. For the determination of both cystine and methionine in the same sample, Lugg (64, pp.2119) evolved a differential oxidation procedure, involving three separate sulfate determinations, following varying degrees of oxidative treatment.

One of the earliest of the direct chemical methods was that of Okuda (78, p.505). It involved redox titrimetry of a solution of cystine by KBrO₃, but not without inclusion of some histidine and tyrosine. A rather involved measurement of the redox system of cystine-cysteine interconversion was proposed by Baernstein (4, pp.125-234). By treating hydrolyzates with nascent hydrogen, any cystine present was reduced to cysteine, all of which was oxidized back to cystine with a known excess of iodine. The remaining iodine was titrated with hydrazine, and the nitrogen which was evolved was measured gasometrically. The method gives consistently high results which are attributed to reducing agents other than cysteine. The same author (3, pp.25-32)
devised a procedure for methionine which offers the opportunity to double check results. Hydrolysis of the sample was carried out with hydriodic acid, and the volatile methyl iodide evolved from demethylation of methionine was measured gasometrically. The homocysteine thiolactone remaining in the hydrolyzate was oxidized to homocystine, which in turn was converted to thiosulfate, and the latter titrated with potassium bi-iodate. High results were obtained due to non-specific sources of methyl iodide.

Among the chemical methods are those which involve specific reactions between the amino acid in question and color-developing reagents. An early beginning in this direction was made by Folin and co-workers (30, pp.421-434), (31, pp.103-108) utilizing phospho-18-tungstic acid and its combination with cysteine.

Perhaps the most eminently successful worker in devising colorimetric methods for both cystine and methionine has been M. X. Sullivan. He, with Hess (111, p. 221), reported that the reaction of cysteine with 1, 2 naphthoquinone-4-sulphonate is highly specific, with no interfering reaction with other disulfides, isocystine, homocystine, glutathione, or cysteine amine. In a more recent paper Sullivan, Hess, and Howard (112, pp.621-624) showed that by varying the means of reduction of cystine
to cysteine between use of sodium cyanide and sodium amalgam, a distinction can be made between cystine and cysteine originally present in the hydrolyzate. The popularity of the method may be best judged on the basis of the numerous reports of its use. Among these were reports by Prunty (88, pp.387-390), Pollard and Chibnall (86, pp.326-336), and Rossouw and Wilken-Jordan (98, pp.219-224).

The McCarthy and Sullivan method for methionine (68, pp.871-876), which depends on the color produced with sodium nitroprusside, was claimed to be the equal of its counterpart for cystine in specificity. Tests were negative for all other amino acids found in protein hydrolyzates. Those related compounds which gave no color included methionine sulfoxide, homocystine, cystine and cysteine. Technical difficulties have been corrected by Hess and Sullivan (41, pp.635-642) and Csonka and Denton (20, pp.329-338) have adapted the procedure to spectrophotometric measurement. Among others who have reported successful use of the method are Edwards, et al. (27, p.608) and Horn, Jones and Blum (48, pp.313-320).

The use of colorimetric methods necessitated decolorization of the varied degree of amber color resulting from sample hydrolysis. Sullivan and Hess (110, p.427) recommended charcoal decolorization even though
the hydrolyzate color was sometimes discharged by reducing action of reagent additions during the analysis procedure for cystine. In Prunty's modification (88, p.389) zinc reduction of cystine provided sufficient decolorization to do away with the necessity for use of charcoal. Decolorization was also noted prior to the McCarthy-Sullivan (68, p.374) methionine color development.

Microbiological Assays for Cystine and Methionine

General considerations. The use of microorganisms for the quantitative estimation of substances which they require for growth has resulted from the discovery of the extraordinary specificity of the lactic acid bacteria in their nutritional requirements. Original development of the methods centered around the water soluble vitamins as a group, individuals of which are ideally suited to determination by such means. The first report of the use of microbiological techniques for amino acid determination was that of Kuiken and co-workers in 1943 (56, p.266) which was patterned very closely after earlier vitamin assays.

The relative newness of microbiological methods has resulted in a wealth of recent applications in many fields, but outlines of underlying principles and basic techniques are less numerous. Of particular value in
initiating the present work have been the reviews of Dunn (22, pp.219-259), Schweigert and Snell (101, pp.497-510) and Snell (2, pp.85-118). According to Dunn et al. (23, pp.703-704) five prerequisites for obtaining satisfactory results by microbiological procedures must be met. The basal medium should contain the minimum concentration of each constituent that will produce a standard curve of maximum slope. This standard curve should also be reproducible and approximately linear, with a negligible blank titration or turbidity. In addition each assay should be applicable over at least a four-fold range of concentration, and at the same time only the lower portion of the standard curve should be used for comparison.

In order to establish the reliability of an assay procedure, Schweigert and Snell (101, p.505) proposed the following criteria.

1. Agreement with other methods.
2. Agreement of values calculated from various assay levels.
3. Agreement with repeated assay.
4. Agreement of recovery values.
5. Agreement by different organisms.

Media. The question of media for amino acid assay has been greatly simplified by an almost direct transfer
of knowledge gained during prior development of vitamin assays. The one component where this carry-over was very seldom feasible was the nitrogen source, where hydrolysed casein or peptone had been used to supply necessary amino acids. Instead, completely synthetic media containing purified amino acids are used.

Dunn, et al. (23, p.706) have worked out an amino acid mixture which has been widely used. The medium initially adopted for trial included amino acids in amounts intended to simulate hydrolyzed casein, plus those known to be deficient. The amounts of individual amino acids were then revised in order to provide the minimum amounts required for maximum acid production. The idea of a synthetic medium whose balance of amino acids is proportional to that of hydrolyzed casein, with supplements of cystine and tryptophane, has been used as such by Horn, Jones, and Blum (49, p.322).

Stokes and co-workers (109, pp.35-36) were the first to propose the idea of combining the necessary features of several methods and improvising a single medium suitable for organisms necessary for assay of the ten essential amino acids. Henderson and Snell (40, pp.15-29) have more recently extended and refined the idea of a uniform medium. They met the requirements of all of the most widely used organisms, and thereby simplified the
determination of 15 amino acids. The composition of the medium was determined by a compromise of literature reports which gave as good or better acid production than in previous use. The adequacy of the medium was checked by fractional to four-fold variations in concentration of single ingredients, or groups of related ones. Steele, et al. (106, pp.533-544) modified the medium for use with many assays that can be performed with *Leuconostoc mesenteroides* and *Leuconostoc citrovorum*.

The largest drawback in the use of synthetic media is the greater expense of pure isolated or synthesized amino acids over hydrolyzed natural products. It is of importance therefore to note the procedure of Toennies (116, pp.667-670), whereby a methionine and tryptophane free, and low cystine casein hydrolysate was produced by the oxidative action of hydrogen peroxide. The method has been utilized by Lyman, et al. (66, pp.427-431) who further succeeded in eliminating cystine and tyrosine from peptone, which was then used in an assay medium.

Due to its widespread biological occurrence, glucose has almost exclusively been used as the carbohydrate source in microbiological media. Camien, Dunn, and Salle (18, pp.36-38) have tested more than 20 of the most readily available sugars for their relative value in
acid yield by the action of 24 lactic acid bacteria. Glucose was shown to most consistently give highest production. The browning of media during heat sterilization has long been noted and some concern expressed as to possible detrimental effects. Hill and Patton (44, p. 481) attributed the color formation to the Maillard reaction between glucose as a reducing sugar and amino acids. They are of the opinion that the danger involved is due to formation of products not utilizeable by the bacteria, rather than formation of toxic products. They and Lankford, et al. (59, p. 368) simultaneously proposed separate sterilization of glucose, with later addition aseptically, in order to avoid destruction of essential nutrients. Depending on availability to individual organisms, Hill and Patton further suggested use of non-reducing sucrose.

Camien and Dunn (16, pp. 561-568) preferred the use of separate sterilization of glucose since sucrose is of limited availability to lactic acid bacteria. They reported that such a technique resulted in a ten-fold more sensitive method for cystine, with use of Leuc. mesenteroides. It is suggested that earlier assays are invalid due to unequal rates of reaction of the cystine in standards and samples. Lessened browning of their medium was also noted when its pH was decreased from the
generally used neutrality to 6.3. They continued use of
the lower value on the grounds that heat lability of
cystine could be expected to increase with pH.

Test organisms. In Tables II (a) and II (b) are
presented the cystine and methionine requirements of
several microorganisms very often used in microbiological
amino acid assays. On the basis of this incomplete sur­
vey, the bacterium most often named as having an absolute
requirement for both cystine and methionine was **Leuc. mesenteroides P-60**. There are other organisms listed as
often, for which one or the other of these two amino
acids is essential, but not both. So in the present
case of convenience of use of a single organism, **Leuc. mesenteroides** seemed best fitted. There were however a
number of conflicting reports as to the specificity of
this organism for the two amino acids.

Camien and Dunn (16, p.561) in an assay for cystine
have stated that the organism used was **Leuc. mesenteri­
oides P-60**, because in their experience it was most
specific in its requirement. Prior to this, Reisen, et
al. (94, pp.731-748) showed that only four of a number
of commonly used organisms required cystine, and of
these only **Leuc. mesenteroides P-60** did not show any re­
response to several related compounds. They also noted
that for **Leuc. mesenteroides P-60**, cystine and cysteine,
### TABLE II
The Cystine and Methionine Requirements of Various Microorganisms

(a) Methionine

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<thead>
<tr>
<th>Organism</th>
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<tr>
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<tr>
<td></td>
<td>Reisen (93)</td>
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<td></td>
<td>X</td>
</tr>
<tr>
<td></td>
<td>Dunn (25)</td>
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<td>Horn (49)</td>
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<td></td>
<td>Shankman (102)</td>
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<td>X</td>
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<tr>
<td></td>
<td>Hift (43)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Camien (17)</td>
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<td></td>
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<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td><em>fermenti</em></td>
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<td></td>
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<td></td>
</tr>
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<td><em>casei</em></td>
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<td></td>
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<td>X</td>
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<td>Stokes (108)</td>
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<td>Hift</td>
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<td><em>delbrueckii</em></td>
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<td>Hift (43)</td>
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## TABLE II (Cont.)

The Cystine and Methionine Requirements of Various Microorganisms (b) Cystine

<table>
<thead>
<tr>
<th>Organism</th>
<th>Author</th>
<th>Es-Ref.</th>
<th>Stim-Non</th>
<th>Utiliza-</th>
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<tr>
<td></td>
<td></td>
<td>sen-</td>
<td>tial</td>
<td>tion of</td>
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<tr>
<td></td>
<td></td>
<td>latory</td>
<td>tial</td>
<td>D form</td>
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<tr>
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<td>Kuiken (55,57)</td>
<td>X</td>
<td></td>
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<td>Barton-Wright (7)</td>
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<td>Reisen (94)</td>
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</tr>
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<td></td>
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<td><strong>Streptococcus Faecalis</strong></td>
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<td>X</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Speck (105)</td>
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<tr>
<td><strong>Leuconostoc Mesenteroides</strong></td>
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<tr>
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<td>Heller (39)</td>
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<tr>
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<td>Tisdale (115)</td>
<td>X</td>
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<td>X</td>
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<td>Speck (105)</td>
<td>X</td>
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<tr>
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<td><strong>Lactobacillus Fermenti</strong></td>
<td>Dunn (26)</td>
<td>X</td>
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<td></td>
<td>Reisen (94)</td>
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<td>Hutchings (51)</td>
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<td>Speck (105)</td>
<td>X</td>
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<tr>
<td></td>
<td>Hift (43)</td>
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<tr>
<td><strong>Lactobacillus delbrueckii</strong></td>
<td>Reisen (94)</td>
<td>X</td>
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<tr>
<td></td>
<td>Speck (105)</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hift (43)</td>
<td>X</td>
<td></td>
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</tr>
</tbody>
</table>
on an equivalent sulfur basis, were equivalent in activity. This latter point has been confirmed by Steele, et al. (106, pp.533-544) for Leuc. mesenteroides, and also Leuc. citrovorum. They further reported that Leuc. mesenteroides had a 10% response to D-methionine. However, Leuc. citrovorum showed no response to the D-isomer. Earlier, Lyman and co-workers (67, p.166) reported that Leuc. mesenteroides P-60 was unable to utilize D-methionine. Dunn, et al. (24, p.577) have also reported the D-form to be inactive for the organism.

The mutation of test organisms sometimes acts to invalidate assays. Hift and Wallace (43, pp.927-931) isolated from their stock culture a strain of Leuc. mesenteroides which required cystine, but had no need of methionine. Due to the frequently reported need of methionine by this organism, they considered this particular strain a mutation of the original bacterium. Naturally occurring mutations of Escherichia coli which affect its sulfur metabolism have been reported by Lampen, Roepke, and Jones (58, pp.55-66) and Simmonds (104, pp.717-722).

The nomenclature of organisms as used by microbiological analysts sometimes has appeared confused. Camien, et al. (18, p.37) reported that Leuc. mesenteroides P-60 did not utilize sucrose. This fact is
entirely at variance with the Bergey classification of Breed, et al. (13, pp.346-348) of the type species *Leuc. mesenteroides*. To reconcile this disagreement Carnien suggests that *Leuc. mesenteroides*, strain P-60, should probably be classified as *Leuc. citrovorum*.

**Specific assays.** A source of inconvenience has been the relative lack of development of assays for both cystine and methionine, utilizing the same medium and the same microorganism for both amino acids. A second disadvantage is the application of most assays to purified proteins only, and not complex food materials.

Dunn, et al. (23, p.713) while investigating the amino acid requirements of *Leuc. mesenteroides*, suggested that their combination of medium and organism would be useful for assay for cystine and methionine. Barton-Wright (6, pp.267-278) has done so, but used *L. arabinosus* for cystine assay. Heller and Kirch (39, pp.345-349) have used the cystine assay only. In both cases, however, the method was applied solely to purified proteins. Dunn and co-workers have maintained their pre-eminence in the field of amino acid assay for many years, and other cystine and methionine methods that they report are (16, pp.561-568) and (24, pp.577-586).

The method of Lyman, et al. (67, pp.161-171), using their hydrogen peroxide oxidized peptone hydrolysate
medium, ia. well developed for assay for methionine. They have applied the procedure to both purified proteins and complex materials, including alfalfa, using *Leuc. mesenteroides* P-60. It is unfortunate however, that in spite of the absence of cystine in their medium, they were unable to obtain reproducible and smooth standard curves for assay. Horn, Jones, and Blum (49, p.325) have presented results of methionine assays of 30 proteins and foods, using *L. arabinosus* with their previously mentioned medium.

The assay developed by Reisen, et al. (93, pp.355-348) for methionine is adapted to use for both proteins and foods, including alfalfa. They have pointed out dangers involved in contamination of individual amino acids with others, in this case having had trouble with large quantities of methionine in naturally isolated L-leucine. This is confirmation of the earlier report of Baernstein (3, p.32), and for that reason, both authors suggest careful selection of a synthetic product.

Of the all-purpose media, that of Stokes, et al. (109, pp.35-49) was devised for assay of the ten essential amino acids. It therefore included methionine, which was determined in alfalfa. Kingsley (54, pp.17-18) and Tisdale, et al. (115, pp.221-222), with minor changes, have used the method for the determination of
cystine and methionine in alfalfa. The similar method of Henderson and Snell (40, pp.15-29) is often used due to its adaptability to assay of 15 amino acids, but cystine was not originally among them. Kemmerer and Acosta (52, pp.527-534) have applied the method to assay of the essential amino acid content of several vegetables.

Unusual assays. A method has recently been reported by Boyd and co-workers (11, pp.1027-1035) which varies somewhat from the ordinary in that the organism used is not one of those producing lactic acid. It is of interest since in investigating the growth requirements of the organism Clostridium perfringens (Welchii) BP6K, the authors (12, p.1017) found both cystine and methionine to be essential. An advantage lies in the fact that rigid aseptic techniques need not be used, due to rapid growth of the organism. Sodium azide was also used because of its bacteriostatic action against many other organisms, as reported by Lichstein and Soule (61, pp.221-230).

Block and Mitchell (10, p.251) have pointed out that one of the chief sources of error in protein analysis is hydrolysis, which induces alternative losses of amino acids. The goal of protein analysts therefore should be development of methods eliminating the need for preliminary hydrolysis of samples. One of the few
of these reported followed the discovery by Regeney (90, p.159) of the "leucineless" Neurospora mutant. The observation was made that the organism could be used for the determination of leucine in intact casein, due to the elaboration by the Neurospora of the necessary proteolytic enzymes. The investigations of Kidder and Dewey (53, pp.425-432) into the nutritional requirements of the protozoon *Tetrahymena geleii*, resulted in the development of a synthetic medium for the organism, which included methionine as essential. Rockland and Dunn (96, pp.541-543) adapted this information to a microbiological assay for tryptophane, utilizing proteolysis of the samples by the organism.

Microbiological assays are subject to one disadvantage not encountered with chemical methods, namely, susceptibility of the microorganisms to toxic materials, which result in decreasing values with sample increments. Removal or dilution of toxic elements are too often impractical. After reviewing these difficulties, Thompson and Kirby (114, pp.127-130) have presented a method which provides compensation for toxicity. In essence, the principle is to determine the standard growth response to the substance being assayed in the presence of the same concentration of toxic substances as are in the extract being assayed. Valid results are obtained only
if the growth response of the organism is a linear function of the assayable substance. Also required are blank tubes which show no growth.

**Hydrolytic Liberation of Cystine and Methionine**

Irrespective of reliability of analytical methods, the quantitative determination of amino acids remains a problem. The crux of the situation lies in the consideration of the magnitude of hydrolytic losses in preparing an intact proteinaceous sample for determination.

Attempts to evaluate this problem have followed two general approaches. The first involves submitting a mixture of a purified protein, and one of various other substances, particularly carbohydrates, to hydrolysis. Recovery of amino acids is judged by comparative assays of this hydrolyzate and that of the protein alone. By this method, cystine and methionine have been shown to be especially readily destroyed by hot acid treatments in the presence of carbohydrates, according to Block and Bolling (9, pp.142-144). Lyman, et al. (67, p.168) showed that methionine losses were at least 10%. That heat of itself is damaging is indicated by nutritional impairment of casein when autoclaved with glucose, as was reported by Patton and co-workers (80, pp.68-69), (81, pp.623-624),(82, pp.659-660) and McInroy, et al. (69, pp.256-260). A review (1, p.218) attributes these
losses to the Maillard reaction.

The second general method used to assess hydrolytic losses is to add a pure amino acid to the material to be hydrolyzed. The combination is assayed comparatively with an hydrolyzate of the complex material alone. This constitutes the common recovery experiments. Martin and Synge (2, p.11) have questioned the validity of the procedure since it has not been established that the stability of the amino acid alone and in peptide linkage are the same. Nevertheless, methionine recoveries from acid hydrolyzates appear to be fairly successful. Most of the reports of microbiological assays previously mentioned herein, give values of 100 ±10 percent.

Several studies have been reported using the recovery method, which emphasize serious cystine losses. Halwer and Nutting (35, pp.521-530) showed by chemical analyses that cysteine added to purified protein was only 50% recovered whether hydrolyzed by reflux or by autoclaving in sealed tubes. Losses were equal whether or not the samples were predigested, and they were intensified by additions of glucose. Reisen, et al. (94, pp. 737-738) showed further 50% losses of cystine when autoclave sterilized in a microbiological medium. Their proposed alternative was separate filtration sterilization of cystine standards and samples, and subsequent aseptic
addition to autoclaved media. That these recent experiences represent a continuation of a longstanding problem is indicated by similar findings of earlier chemical analysts such as Lugg (65, p.1028) and Bailey (5, p.1404).

Pigman and Goepp (85, p.375) have proposed a general form that the Maillard reaction between reducing sugars and amino acids may take during acid and heat treatments of proteins. They point out that it should be similar to the manner in which the most common nitrogenous derivatives of carbohydrates are formed. This latter is a dehydration reaction between aldehyde groups of sugars with amino groups of proteins by splitting out water. The work of Schubert (99, p.343), (100, pp.601-603) showed that cysteine is a special case yielding a ring compound, since the water lost arises partly from sulfhydryl groups.

Racemization will manifest itself as destruction in microbiological assays, due to specificity of most test organisms for the naturally occurring L-isomers. According to Martin and Synge (2, p.9) alkali causes extensive racemization of amino acids. Incomplete knowledge indicates that varying and unpredictable degrees of racemization of different amino acids occur in acid hydrolyzates of different proteins. It is interesting to note that Hollander and de Vigneaud (46, pp.244-245)
used an extended acid reflux of L-cystine to produce the inactive mixture in 75% yield from which D-cystine was first isolated. Dunn, et al. (24, pp. 577-585) have reported that \textit{L. fermenti} fully utilizes DL-methionine. They cited their higher than usual results as evidence of racemization of methionine by acid hydrolysis.

In spite of the afore mentioned difficulties, the most widely used method of liberating amino acids from their protein linkages has been acid hydrolysis. As may be seen from Table III there has been a wide variation in the procedures and conditions used. These differences may have been dependent on the type of sample, the type of analytical method used, and the amino acid determined. This is particularly evidenced by the development of chemical methods by Sullivan and co-workers (110, pp. 423-423) (42, pp. 353-355) (68, p. 374) (41, p. 638). The hydrolysis described in the latter paper consisted of sulfuric acid reflux in an atmosphere of nitrogen. It was preferred in preparation of a single sample for both cystine and methionine analysis.

The work of Stokes, et al. (109, pp. 37, 44-46) is representative of the wide use of hydrochloric acid for preparation of samples for microbiological assay. They used 10% HCl (ca. 3.25N) in an autoclave, where pressure and higher temperature contributed to lessened time.
TABLE III
Various Procedures Reported for Hydrolytic Liberation of Amino Acids from Proteins and Complex Materials with Acid (a) For Chemical Analysis

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Sample Size (gm.)</th>
<th>Acid Used</th>
<th>Acid Strength</th>
<th>Acid Vol (ml)</th>
<th>Method of Heating</th>
<th>Hours Heated</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified proteins</td>
<td>-</td>
<td>HI</td>
<td>57%</td>
<td>-</td>
<td>Reflux</td>
<td>6</td>
<td>Baernstein</td>
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<td>Casein</td>
<td>1</td>
<td>HCl</td>
<td>20%</td>
<td>5</td>
<td>*</td>
<td>2</td>
<td>Sullivan</td>
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<tr>
<td>Casein</td>
<td>1</td>
<td>HCl</td>
<td>20%</td>
<td>5</td>
<td>*</td>
<td>7</td>
<td>Sullivan</td>
</tr>
<tr>
<td>Casein</td>
<td>0.5</td>
<td>HCl</td>
<td>20%</td>
<td>2</td>
<td>*</td>
<td>10</td>
<td>McCarthy</td>
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<td>0.25</td>
<td>H2SO4</td>
<td>6N</td>
<td>6</td>
<td>Reflux</td>
<td>12</td>
<td>Hess</td>
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<td>&quot;</td>
<td>HI</td>
<td>57%</td>
<td>6</td>
<td>Reflux</td>
<td>18</td>
<td>Hess</td>
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<tr>
<td>Tobacco mosaic virus protein</td>
<td>&quot;</td>
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<td>57%</td>
<td>3</td>
<td>&quot;</td>
<td>24</td>
<td>Hess</td>
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<td>Purified proteins</td>
<td>-</td>
<td>HCl</td>
<td>20%</td>
<td>-</td>
<td>Reflux</td>
<td>6</td>
<td>Hess</td>
</tr>
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<td>Purified proteins</td>
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<td>HCl</td>
<td>20%</td>
<td>-</td>
<td>&quot;</td>
<td>6</td>
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<td>Wool</td>
<td>-</td>
<td>H2SO4</td>
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<td>-</td>
<td>&quot;</td>
<td>24</td>
<td>Rossouw</td>
</tr>
<tr>
<td>Proteins &amp; foods</td>
<td>-</td>
<td>HCl</td>
<td>20%</td>
<td>-</td>
<td>Reflux</td>
<td>24</td>
<td>Csonka</td>
</tr>
<tr>
<td>Corn germ &amp; beef</td>
<td>1</td>
<td>HCl</td>
<td>20%</td>
<td>25</td>
<td>Reflux</td>
<td>18</td>
<td>Horn</td>
</tr>
<tr>
<td>Corn germ &amp; beef</td>
<td>1</td>
<td>HCl</td>
<td>20%</td>
<td>30</td>
<td>Reflux</td>
<td>24</td>
<td>Edwards</td>
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</table>

* 125°C. oil bath reflux.
TABLE III (Cont.)

Various Procedures Reported for Hydrolytic Liberation of Amino Acids from Proteins and Complex Materials with Acid (b) For Microbiological Analysis

<table>
<thead>
<tr>
<th>Type of Sample</th>
<th>Sample Size (gm.)</th>
<th>Acid Used</th>
<th>Acid Strength</th>
<th>Acid Vol. (ml)</th>
<th>Method of Heating</th>
<th>Hours Heated</th>
<th>Authors</th>
<th>Ref. No.</th>
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<td>Purified proteins</td>
<td>-</td>
<td>HCl</td>
<td>8N</td>
<td>80</td>
<td>Reflux</td>
<td>8</td>
<td>Heller</td>
<td>(39)</td>
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<tr>
<td>Casein &amp; Soy Globulin</td>
<td>-</td>
<td>HCl</td>
<td>6N</td>
<td>-</td>
<td>Reflux</td>
<td>30</td>
<td>Patton</td>
<td>(91,82)</td>
</tr>
<tr>
<td>Purified proteins</td>
<td>1</td>
<td>HCl</td>
<td>3.5N</td>
<td>10</td>
<td>*</td>
<td>30</td>
<td>Patton</td>
<td>(91,82)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>HCl</td>
<td>3N</td>
<td>20</td>
<td>*</td>
<td>10</td>
<td>Boyd</td>
<td>(11)</td>
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<td>0.2</td>
<td>HCl</td>
<td>4N</td>
<td>10</td>
<td>*</td>
<td>5</td>
<td>Henderson</td>
<td>(106)</td>
</tr>
<tr>
<td>Proteins &amp; Foods</td>
<td>1</td>
<td>HCl</td>
<td>10%</td>
<td>20</td>
<td>*</td>
<td>10</td>
<td>Stokes</td>
<td>(109)</td>
</tr>
<tr>
<td></td>
<td>0.5-2</td>
<td>HCl</td>
<td>2N</td>
<td>-</td>
<td>*</td>
<td>5-10</td>
<td>Reisen</td>
<td>(93)</td>
</tr>
<tr>
<td>Vegetables</td>
<td>-</td>
<td>HCl</td>
<td>6N</td>
<td>100</td>
<td>Reflux</td>
<td>24</td>
<td>Lyman</td>
<td>(67)</td>
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<tr>
<td>Alfalfa</td>
<td>-</td>
<td>HCl</td>
<td>20%</td>
<td>-</td>
<td>Reflux</td>
<td>24</td>
<td>Horn</td>
<td>(49)</td>
</tr>
</tbody>
</table>

* Autoclaved sealed tubes.
The sample and acid were enclosed in a sealed tube to prevent loss of HCl gas. Using casein as a representative protein, they varied the time of hydrolysis from five to 30 hours, and obtained unchanged values for all ten essential amino acids. In spite of not having run recovery values, they stated that these results unequivocally emphasized the stability of amino acids to acid hydrolysis. Reisen, et al. (93, p.358) and Henderson and Snell (40, pp.24-25) reported that use of 2 to 3 N HCl for five hours was sufficient to hydrolyze purified proteins, with 10 hours necessary for complex materials. When longer periods of hydrolysis did not significantly alter results, release of amino acids was assumed complete, without destruction.

For hydrolysis of alfalfa, Tisdale et al. (115, p.221) reported that both cystine and methionine recoveries plotted against HCl concentrations gave maxima at 4% strength (ca. 1.3 N), which indicated its use. Heating was specified for 10 hours by autoclaving in sealed tubes. The prior report of Lyman, et al. (67, pp.165-166) disagrees. They found that sealed tube autoclave treatment of alfalfa with 1 N HCl always gave low methionine results, regardless of extension of time. Refluxing of alfalfa for 24 hours with 6 N HCl was recommended.
A problem in the choice of an acid, and its volume and strength for hydrolytic use, is presented by the salts produced by neutralization before analysis. Hydrochloric acid has been favored due to the relatively innocuous nature of sodium chloride for most microorganisms. Camien, Dunn and Salle (18, p.39) have surveyed the lactic acid bacteria most used for microbiological assay purposes, and found that the salt tolerance of Leuc. mesenteroides was among the highest. They reported that only above a 1% level of NaCl, was impairment of growth noticeable, to which point Henderson and Snell (40, p. 20) and Reisen, et al. (93, p.357) have agreed. It is not surprising that the salt tolerance of Leuc. mesenteroides was shown to be high, for it is the organism commonly associated with sauerkraut fermentation. According to Prescott and Dunn (87, p.285), NaCl concentration in such vats is at a 2.5% level.

Consideration of the possibility of use of proteolytic enzymes seemed to offer the prospect of resolving the problems of racemization and destruction of amino acids during sample hydrolysis. In the extensive review of Martin and Synge (2, p.9), they stated they were not aware of any such case, providing the reaction medium itself did not effect the unfavorable changes. It has been pointed out by Schweigert and Snell (101, p.503)
that enzyme liberation of tryptophane has been used with some success. (As by Wooley and Sebrell (120, pp.148-150) and Horn and Jones (47, p.158).) They caution however, that the procedure should be extended with care to other amino acids, and microbiological assays for them. Thorough investigations are required as to the availability to microorganisms of the amino acids contained in various peptides which remain after enzyme digestion. Block and Mitchell (10, p.252) have criticized the use of enzyme hydrolysis on the basis that breakdown is very seldom complete, and that many days of digestion are required. Adequate blanks are also required, since the enzymes themselves are largely protein and contribute amino acids by autolysis.

The bases for dependable selection of an enzyme for most complete digestion of a particular substrate are two-fold. The first of these is a comparison between an initial Kjeldahl total nitrogen determination (29, p.27), and Van Slyke amino nitrogen analyses (29, p.429), at intervals throughout the course of the digestion. The ideal final finding is an equivalence of the two. An example of the use of this procedure is the work of Bubl (14, p.35), who showed that the digestion of casein with Takamine protease was 95% complete in 72 hours. It was also by this means that Lowry and Thiessen
determined that certain enzymes could release amino acids from the nutritionally impaired product resulting from autoclaving casein and glucose.

The second procedure is based on the work of Hiller and Van Slyke (45, pp.258, 264), who reported that Witte's peptone was entirely soluble in 5% trichloroacetic acid, which latter is a noted protein precipitant. On the basis of amino and peptide nitrogen determinations this peptone was characterized as a mixture of amino acids and low molecular weight intermediate degradation products of protein. Remmert (95) has therefore suggested a procedure which utilizes so-called acid soluble nitrogen determinations. After an initial total nitrogen analysis, aliquots of a digest are removed at intervals and added to equal volumes of 10% trichloroacetic acid. Total nitrogen determinations of the filtrates of the latter give approximations of the proportion of the original total nitrogen which is liberated as peptones or lesser molecules as proteolysis progresses.

An early use of enzymes for digestion of proteins for amino acid analytical purposes was that of Pollard and Chibnall (86, pp.331-332). They found that a sequence of peptic, tryptic, and ereptic digestions of isolated grass proteins gave much higher chemically determined values for cystine than when they used acid
hydrolysis. Horn, Jones and Blum (48, pp. 317-319) used papain digestion of purified proteins with only fair success to analyze for methionine by their modified McCarthy and Sullivan reaction.

There are few reports of the cystine or methionine activity of identified peptides for either chemical analytical reactions, or microorganisms used for microbiological assay. McCarthy and Sullivan (68, p. 872) have found that glycylmethionine is fully as positive as methionine in its reaction with sodium nitroprusside. Reisen, et al. (94, p. 748) reported that cystine-containing glutathione is inactive for Leuc. mesenteroides. On the other hand, the mutant strain reported Rift and Wallace (43, p. 929) was found to use glutathione just as well as cystine.
THE PROBLEM

The frequent occurrence of soil sulfur deficiency in the Northwest, the marked improvement in quantity and quality of alfalfa in response to sulfur fertilization, and the limiting amounts of cystine and methionine in alfalfa protein form the basis for this problem.

The first objective was to provide a simple, accurate method for the microbiological assay of cystine and methionine in alfalfa, one which if possible might be extended to routine determinations in plant materials in general. Necessary considerations included selection of a suitable microorganism and basal medium, together with an appropriate assay procedure, and establishing a hydrolytic preparation of samples compatible with most consistent results. The intent was to adapt existing methods with only as many modifications as were necessary to suit the material under consideration.

The ultimate aim was to apply such a procedure to assay of alfalfa samples of known and varied sulfur fertilization histories for their cystine and methionine contents. It was hoped that a relationship between chemically determined total nitrogen, and total and inorganic sulfur contents could be discovered. Due to the constancy of organic sulfur, total nitrogen ratios, and
the lack of demonstrated organic sulfur other than that of a protein nature, a working hypothesis was proposed that the organic sulfur of alfalfa should be accounted for as its combined cystine and methionine contents.
Assay Method

Of the analytical methods for cystine and methionine the chemical procedures are of most long-standing usefulness. In spite of this they have continuously been subject to revisions to overcome shortcomings as to specificity, difficulty in wide application especially to complex materials, and time consuming performance. The newer microbiological methods, although not in all respects perfected nor completely investigated, seem in a large measure to overcome the afore mentioned difficulties of chemical analysis. At any rate their relatively recent origin leaves room for numerous untried applications of which the present problem seemed worthy of trial.

Basal medium. The basal medium selected for use was unchanged from that described by Henderson and Snell (40, p.17). Its use was indicated primarily in consideration of its recent well demonstrated applicability to the determination of methionine in purified proteins. In addition, this medium was shown to be adequate for the assay of at least 14 other amino acids. The advantage of such all-purpose medium, which not only could be applied to the work at hand, but be further extended to
many routine analyses, is self-evident. The medium is entirely synthetic, each constituent being chemically characterized. This disregards the relatively greater expense of the individual compounds necessary, but eliminates the variable and unknown composition factors incurred with use of natural products to supply basal ingredients.

The relatively standardized procedures for microbiological assay as related in the above paper were followed at the outset, with only two major exceptions. Conditions were later varied further as occasions demanded. The initial variances with the prototype were the attempt to adapt it to cystine assay, which was not proposed by the authors, and the present choice of microorganism for methionine assay. On the basis of the earlier outline (Table I), the convenience of use of a single microorganism for both cystine and methionine assay seemed best fulfilled in choosing *Leuconostoc mesenteroides* P-601.

In order to gain a working familiarity with the analytical tool to be used, it seemed advisable that first attempts should be limited to practice standard curves for cystine and methionine. But even such a simple beginning was not without its difficulties.

1Supplied through the courtesy of Dr. Vernon H. Cheldelin, Department of Chemistry, Oregon State College.
which ultimately took considerable time to resolve. Acid production by the organism, in the usual 72 hour incubation time, sufficient to require at least a 10 ml. titration with approximately 0.1 N NaOH, was found to occur within ranges of 0 to 100 ml of standard DL-methionine (equivalent to 0 to 50 ml L-methionine), and 0 to 60 ml L-cystine standard. Curves were therefore prepared with five increments of one ml. of standard cystine and methionine solutions containing 20 and 12 per ml., respectively. These, and two subsequent repetitions, presented the immediate problem of blank tubes for both amino acids which titrated at least 5 ml. in each case, or over half the useful ranges of acid production. After the possibility of contamination of the assay organism had been eliminated by plating the culture, several other possible reasons for the high blanks were investigated. Identification of contaminated ingredients was first attempted, using a procedure suggested by Lewis (60). Series of blank tubes were prepared, to duplicates of which in turn were added individual amino acids in dry form equivalent to the amounts already present. By this procedure contamination of a basal amino acid should theoretically show up as a titration double that of the true blank. As it was experienced here, the doubled-blank actually occurred only where one amino acid was contaminated. It
was also considerably less than doubled when gross contamination already placed the true blank near the midpoint of the total response curve.

Addition of dry amino acids for this test was necessitated by the procedure in the original method of combining all those to be included in the basal medium, in a single solution. In cases of contamination, where at most only a few of them were apt to be at fault, it required discarding the entire solution to be rid of each offending ingredient as it was identified. For this reason, each individual basal amino acid was thereafter prepared in a solution by itself. This also made possible an exact doubling of amino acids from separate stocks in further contamination studies, and subsequently yielded more conclusive results than first obtained.

Tracing the resolution of difficulties with methionine contamination first, the initial studies definitely showed that L-leucine was grossly contributory, and so was replaced from a different source. In addition DL-aspartic acid and L-glutamic acid (a student preparation) were suspected, while all other solutions than amino acids were exonerated. A subsequent standard curve was much improved with blank tubes dropping to 2 ml. titrations, which however were still too high to permit accurate assays. With the idea that the L-amino acids isolated
from natural sources were more apt to be naturally con-
taminated than the synthetic DL-forms, those of the
former which had been used, were tested further, with
DL-aspartic acid also retested. Off these the latter was
again implicated slightly, as were L-tyrosine and L-
tryptophane, which latter two were replaced. At the
same time, L-cystine gave an added response which was
thought to be due to a possible cross-contamination with
methionine by blowing when dried in a vacuum oven prior
to preparation of standard solutions of these two.
Thereafter this treatment was carried out in tightly
covered drying dishes. This and replacement of the two
mentioned suspect L-amino acids in the next methionine
standard curve gave no improvement of the high blank
condition.

Recollection of previous suspicions of DL-aspartic
acid and L-glutamic acid seemed logical in light of the
fact that these two with DL-alanine are used in amounts
ten-fold that of the other amino acids. Hence even a
small percentage impurity could be expected to show up
to greatest detriment. These two amino acids were im-
mediately available from only one source, but fortunately
in adequate supply to permit purification. Recrystal-
ization appeared to be the quickest means, but in the
case of glutamic acid was preceded by refluxing with
aniline. This procedure converted any glutamic acid hydrochloride to the pure acid, by reason of the greater affinity of the HCl group for aniline than the amino acid. Following this, each of the two amino acids were dissolved in a minimum of hot water and evaporated by boiling to super-saturated solutions. After filtering hot, the resulting volumes were doubled with mixed 95% ethyl and 99% methyl alcohols, and crystallized at 0° C. The process was repeated three times in each case. A methionine standard curve using these products, and in addition a new supply of L-cystine, resulted in blank tubes which gave titrations of 0.2 ml. of 0.1 N NaOH. All methionine blank values have since been of that order, not exceeding 0.3 ml.

The similar problem with cystine was treated simultaneously up to this point, and although improved, this difficulty was of greater persistence. The gross source of initial contamination was L-glutamic acid, and to a lesser extent DL-aspartic acid, L-histidine, and DL-methionine were implicated in the previously described sequence. New sources of the latter two eliminated trouble from those amino acids, and recrystallization of aspartic acid freed it of detectable cystine. In spite of these steps and recrystallization of glutamic acid, blank titrations, however much reduced, remained
at objectionable levels of the order of 2 ml. of 0.1 N NaOH in cystine standard curves.

A further contamination study of the gamut of amino acids gave no indication of trouble from any source, except perhaps glutamic acid. In the face of this, while awaiting a new supply of the amino acid, some assays were carried out, but were of such an erratic nature as to be considered unreliable. At this point the thoughts came to mind of the metabolic interrelationships between cystine and methionine, and that the organism from the start or through mutation did not have an absolute requirement for cystine. To follow through however with the ultimate resolving of the contamination difficulty, standard curves run after securing commercial glutamic acid showed cystine blanks which were less than 0.1 ml. of 0.1 N NaOH, and have since been of that order. Unfortunately this product resulted in a new increase in methionine blanks, which has since necessitated maintaining the two different sources of glutamic acid, for use depending on the assay involved.

Prior to elimination of cystine and methionine contamination of the basal amino acids, it was felt that perhaps the sparing action of cystine for methionine, or possibly the reverse, might be factors in the undue blank response of the organism. Therefore with three standard
curves for each of the two amino acids, the basal amount of the opposite member of the pair was maintained and reduced by one-fourth and one-half. By this means it was supposed that if sparing was the case, blank values would show significant and perhaps proportional decreases, with displacement of the entire curves downward. It would also follow from such an eventuality that a hydrolysate containing both amino acids could not be validly assayed, regardless of whether or not the action could be compensated for in the standard curve. Results of this series showed no significant variance in either standard curve with the varied basal media. Alteration of response by such variations of the basal media, it was later realized, could only have been more generally interpreted to mean that the organism did not have an absolute requirement for the amino acid under consideration, rather than the more specific case of sparing action.

At this point methionine contamination had been cleared up, but that by cystine had persisted, although at the lower level of 2 ml. blank response. Other than the case where the replacement of one contaminated ingredient could completely reduce the blank to normal, most significance could be attached to tests whereby blank values were raised within the 8 ml. range of the upper portion of the curve. This as opposed to
operations which would lead to decreases in response of only fractional amounts of the 2 ml. acid production range below the blank values. To test again the possibility of a lack of absolute requirement for cystine by the organism by non-specific response to methionine, the medium was altered by increasing the methionine content of the basal medium.

Cystine standard curves were set up in which such increases amounted to half-again, and twice normal amounts of methionine. If such changes were to indicate lack of specific cystine requirement, results should have shown increasing blank values with additional basal methionine, and greater than ordinary responses to the lesser of standard cystine increments. The resulting cystine standard curves would therefore be progressively flattened out by displacement upwards of the lower portion towards the upper limits of acid production. This however was not the case, with no change in blank values nor any alteration of the general shape of the curves.

Test organism. The basis for choice of *Leuconostoc mesenteroides* P-60 as the assay microorganism has previously been mentioned. Early experience with high blanks led to experiments to be described here dealing with its culture and preparation of inocula. In addition a variation from the standard method of maintenance
of the stock culture was suggested and tested.

After correcting methionine contamination, but before elimination of high cystine blanks, the possibility of a lack of absolute requirement of the organism for cystine was examined by other means than earlier described. The chance existed that the organism as previously used had had a requirement for the amino acid, but that during more recent handling had through mutation acquired a sufficient degree of synthetic ability to be the cause of considerable acid production in the absence of cystine. The usual procedure in such a case has been to repeatedly sub-culture the organism in a medium much richer than normal in the substance in question. The reasoning here was that in the presence of a compound in amounts far in excess of that which the organism needs, it will lose the need, and therefore the function of any newly established mechanism of synthesis.

In the present case, the organism was first sub-cultured three times over a six day period in a medium with a ten-fold increase over the normal 1 mgm/tube cystine content. The resulting culture when used in subsequent standard curves, by comparison with an untreated original culture, did not show a lessened cystine blank. At the same time methionine standard curves were unaltered. Further sub-culturing of the organisms,
continuing with the treated and the original cultures, was repeated for a like period. Again no decrease in blank response was detected, which results could not therefore be interpreted in any fashion regarding the cystine requirement of the organism.

Unfortunately at this juncture, both the original and treated organisms resulting from the half-dozen subcultures gave maximum growth responses in cystine and methionine standard curves of little more than 3 ml. 0.1 N acid production. This compared to the necessary minimum of 10 ml. for reliable assays, which had previously been attained. In such an event, the usual remedy to produce more viability has been by several subcultures on succeeding days. Regardless of the fact, in the absence of any other explanation, that it was this very treatment of the original culture that seemed to have brought on the impasse, the procedure was resorted to. But thereafter this culture could not be revived even to the extent where growth of inoculum cultures was sufficient for use to prepare standard curves. Subsequently a new culture was obtained.²

During the contamination trouble, one drop of a 1:10 dilution of inoculum culture instead of the most often reported 1:2 dilution, had been used for

² Leuconostoc mesenteroides P-60 (Dunn's strain) was kindly supplied, again, by Dr. Cheldelin.
innoculation of assay tubes. This was done with the intent to minimize as much as possible any carry-over of cystine from the growing medium to assay tubes. Although there was no appreciable lessening of high blanks, it was noted that more uniform curves and closer checks of duplicate tubes were obtained using the more dilute inocula. With the thought of retaining its use, following elimination of contamination difficulties, a comparison of standard curves with the more concentrated inocula was made. Triplicate cystine and methionine standard curves were set up, which were inoculated with 1:2, 1:5, and 1:10 dilutions of the same culture. Practically identical half-maximum growth curves were obtained in the case of each amino acid, with slightly greater total acid production at the upper end of the range with the 1:10 dilution, compared to 1:2, in response to cystine. Since it is the lower portion of such curves that are used, such a difference was not regarded as significant.

The use of the more dilute inoculum was also retained since it was theoretically justified by Rahn (89, pp. 193-198) as follows. Danger of an initial growth lag, which might seem to be more apt to occur with a small rather than a large inoculum, was not serious, since actively growing organisms were used from new sub-cultures for each assay, whereas lag is a phenomenon of older
cultures. Relatively small decreases in inoculum size would have little effect on the ultimate maximum response, since for the greatest part, increase in numbers of bacteria occurs exponentially. This period of logarithmic growth is especially apparent in active organisms resulting from recent sub-cultures. There was also the assumption that due to their fission reproduction, all cells arising from a single bacterium are of the same age, and therefore the smaller the inoculum the more uniform should be the growth.

A question of inconsistency of most methods became apparent during the course of this work, in that the stock and inoculum cultures were usually grown in media that were enriched modifications of those used as basal for assay. The lack of consistency was most apparent in the many cases where the assay media were entirely defined through exclusive use of synthetic ingredients wherein growth was considered normal. But still, without an exception having been discovered, inoculum and stock media were enriched, usually with natural materials such as peptone and yeast extract. If as supposed this was necessary for maintenance of organisms in a more natural state, then by indirection it must be admitted that the synthetic media for assay were in some manner deficient, and should not support normal growth,
particularly in standard curves.

For this reason, the culture obtained was sub-cultured and thereafter maintained in entirely synthetic media, changed only by addition of agar for solidification. Liquid media for use in growth of inocula was similarly unchanged from the synthetic basal. Comparative standard curves for cystine and methionine over the 72 hour incubation period using synthetic and naturally cultured organisms were not significantly different. Actually, slight improvement in total acid production was noted in the case of both standard curves using the synthetically grown culture. In the case of cystine a ten percent extension of linearity of the lower portion of the curve was obtained. Repeated standard curves with use of the synthetically maintained organism have been uniformly reproducible.

In an attempt to detect any untoward effects of synthetic maintenance of the organism, bacterial growth curves as described by Rahn (89, p.192) were run for this organism and the culture from which it was taken. Such curves would also indicate an hourly range of age at which the organisms were most actively proliferating, namely, the period of logarithmic growth. Use of cultures within this time for inoculation of assays should therefore show the best acid production. Incubation of
inocula has been variously reported to require from 8 to 18 hours. Although the original organism had been successfully used during the mid-portion of this time, it was important to learn if synthetic culturing necessitated any change.

The results showed that a lag phase persisted for about four hours in the case of both cultures, but that thereafter the synthetically cultured organism grew more slowly than the original, although ultimately to almost the same maximum. However, the former also appeared to enter the logarithmic growth phase sooner and, occurring from 8 to 24 hours of age, was of greater duration than the 10 to 18 hour period of the original. The new culture therefore should have a wider range of time of usefulness as inocula. The maximum growth of the original culture occurred at 24 hours of age, while that of the synthetic culture was not reached until 36 hours, and it also showed a greater tendency to maintain the high level of growth. This last factor indicated that the synthetic cultured organism would be most apt to show greatest maximum acid production. The over-all results therefore seemed to justify its use for assay purposes on a basis of at least equal and perhaps better performance than an organism maintained in an enriched medium.

Techniques. Early attempts to assay alfalfa
yielded values for its cystine and methionine content which were disturbingly lacking in uniformity. This variance occurred in several forms, both in absolute and recovery values. Results could not be duplicated on re-assay of the same hydrolyzate, and there was considerable inconsistency within a single assay, even between replicate tubes. Most disconcerting however were recovery values so completely lacking in regularity as to defy interpretation. From the outset it was hoped that these difficulties could be overcome in some manner, especially since the best of values obtained were somewhat less than expected and did not support the working hypothesis. The first general approach towards solution of the problem was through the several following changes and refinements of standard microbiological assay procedures. Values obtained from these variations performed on a single hydrolyzate are compared in Table IV.

The first assay attempted was of a rather unusual alfalfa sample with the higher than normal crude protein content of 28 percent. It had been obtained by picking only the top portions of a stand in early stages in maturity, for use in rations for rats whose protein intake was restricted to that one source. This particular sample was used in the present instance on the assumption that the high protein content would most easily
TABLE IV

The Cystine and Methionine Contents of Alfalfa and Casein as Determined by Different Microbiological Procedures. (Assay values expressed as per cent of sample.)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Determination</th>
<th>1</th>
<th></th>
<th></th>
<th>2</th>
<th></th>
<th></th>
<th>3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>R.</td>
<td>T.C.</td>
<td>R.</td>
<td>T.C.</td>
<td>R.</td>
<td>T.C.</td>
<td>T.C.</td>
<td>Turbid</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>T.C.</td>
</tr>
<tr>
<td>H4403</td>
<td>Assay (%)</td>
<td>0.23</td>
<td>0.18</td>
<td>0.16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Cystine Recovery (%)</td>
<td>92</td>
<td>58</td>
<td>55</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>85</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Assay (%)</td>
<td>0.23</td>
<td>-</td>
<td>0.29</td>
<td>0.37</td>
<td>0.40</td>
<td>0.32</td>
<td>0.32</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Methionine Recovery (%)</td>
<td>56</td>
<td>-</td>
<td>103</td>
<td>90</td>
<td>63</td>
<td>70</td>
<td>117</td>
<td>59</td>
</tr>
<tr>
<td>Casein</td>
<td>Cystine Assay</td>
<td>0.20</td>
<td>0.32</td>
<td>0.33</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>1.94</td>
<td>- 2.63</td>
<td>2.45 2.45</td>
<td>2.34</td>
<td>2.78</td>
<td>2.17</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

1. Comparison of values obtained by regular increment (R) and toxic compensation (T.C.) methods.
2. Rerun of cystine values with low blanks. (First column cystine values from high blank experiments.)
3. Comparison of values obtained from measurement of acid production (72 hours) and turbidity (20 hours).
4. Values obtained by turbidimetric measurement of toxic compensation method with glucose sterilized separately from assay tubes.
lend itself to determinations of the two sulfur-containing amino acids. At best, quantities of these are present in small amounts in alfalfa, but with high protein could be expected to be proportionally higher. Secondly, there was the ultimate plan to use such a sample in feeding experiments, with and without methionine supplementation, to parallel the work of Haag (34, pp.363-370) concerning cystine. All work with alfalfa described hereafter was restricted to this sample, H4403, unless stated otherwise.

The early inconsistent alfalfa assay results were obtained by use of the usual technique, whereby regular increments of a hydrolyzate were added to the basal medium, the response to which was compared to standard growth curves. Recovery values were determined using such figures as a base for subtraction from assay values of the same sample to which known amounts of crystalline cystine or methionine were added for hydrolysis. The hydrolytic treatment used was that advocated by Henderson and Snell (40, p.24), namely, an 0.5 gm. sample with 20 ml. of 3 N HCl autoclaved at 15 lbs. in a sealed tube for 6 hours. The methionine content was varyingly determined as being between 0.28 and 0.41% of the sample, with recovery values ranging from 56 to 104 per cent. The cystine content seemed to vary for the same sample.
between 0.12 and 0.24%, and added cystine gave analyses of from 58 to 92 per cent recovery.

Some of these assays were performed before cystine contamination was eliminated, but others were obtained with low blanks which were no less variable. The lack of homogeneity of recovery values can at least partly be attributed to reasonable doubts as to the validity of the assumption on which their calculation is based. For if they are to be accurate, it must be supposed that the rates of destruction of the amino acids in combination in the sample, and those free forms which are added, are the same. Here in addition, where neither the base nor combined values could be reasonably duplicated, their difference expressed as recoveries were not significant. But no better method has been proposed to date.

Investigations were then carried out in an attempt to remedy the variations in base values. These latter were at first thought attributable in whole or in part to the possible presence of some material in the assay sample tending to poison the test organism. Such an effect was indicated by two observations, the first of which was manifested as a lag in growth, such that in extreme cases none was visible up to 24 hours. However, seemingly normal amounts of acid were produced by the end of the usual 72 hour incubation period. Secondly,
there was the lack of agreement of values with the various increments of sample, tending in many cases to decrease with hydrolyzate additions.

A method proposed to permit assay for amino acids in samples containing toxic materials, whose effects are such as to preclude use of the regular increment method, is that of Thompson and Kirby (114, pp. 127-130). As previously described this can be termed a toxic compensation method. Results so obtained are compared with those from the regular increment method in 1 of Table IV. As can be seen, the assay values for cystine and methionine contents were about the same as before, although the percentage of the former dropped slightly. Recovery values were greatly different, that for methionine improving such as to appear complete, while as regards cystine the situation worsened considerably.

The methionine values having been favorable on first trial of the toxic compensation method, an attempt was made to duplicate them, again by comparison with the regular increment procedure. These results are shown in the first two columns of part 2, Table IV, as measured by acid production. Assay values were higher in both cases than previously, favoring slightly the toxic compensation procedure. But the recovery values were once again perplexing by being reversed in favor of increments
of hydrolyzate.

One of the admitted limitations of the toxic compensation method is that only concentrations may be used, response to which remains within the linear initial portion of standard curves. In the present case, acid production was linear only up to about 3 ml. in response to either cystine or methionine. It was felt therefore, that differences between fractional parts of this amount would not be as significant as desirable. Greater validity and improved constancy of results might be expected if the linear response range could be extended. One method of doing so is to use turbidimetric measurement of growth, which values are read at the end of about 30 hours incubation and are usually linear over the first 50% of the growth range.

In spite of these advantages of shortened time, and increased linearity, (plus instrumental elimination of human errors inherent in titrating to indicator endpoints) turbidity response measurements had previously been avoided. This was principally for the reason that such inhibitions as were to be avoided, exerted their greatest effect on growth during short incubation, but could oftentimes be overcome when more time was allowed. Hence, 72 hour acid production appeared to be preferable for most reliable results. Nevertheless, turbidity
assays for methionine and its recovery were set up both by the regular and toxic compensation methods. Each gave the same value of 0.32% methionine in the sample, but with conflicting 70 and 117% recoveries, respectively.

Although the methionine content values were somewhat less by turbidimetric than acid production criteria, the recovery value obtained by the toxic compensation method was heartening enough to warrant a repetition of that part of the experiment. This was done and included a cystine assay. The same unreliability persisted, for although the base value of methionine was duplicated, the recovery indicated this time was only 59%. In round numbers, earlier cystine values were also duplicated, with an improved, but still deficient recovery.

Sometime later a further variation in method was suggested by the work of Camien and Dunn (16, p.565). They recently demonstrated an increase of ten times in the sensitivity of the cystine method by separate sterilization of glucose and aseptic addition to the sterile assay tubes. It was thought that this procedure, by permitting assay of a ten-fold more dilute hydrolyzate, might be a solution. This however was not immediately the case as shown by results listed in the last column of Table IV.

In summing up these results of various assay procedures applied to alfalfa, the only conclusions that
could be reached were in regard to the values for the content of cystine and methionine. The recovery figures varied so illogically as to be worthless, except as an indication that they in themselves constituted a problem, and to cast doubt on the base determinations. For the latter however it appeared that the toxic compensation procedure, utilizing acid production as the criterion of response, gave more consistent results than any other combination which included the regular increment set up or turbidimetric measurement.

This conclusion was supported by assays of a sample of casein which were performed concurrently with those of the alfalfa related above. It is admitted that such a material is much less complex than alfalfa, but when prepared and assayed by the similar varying methods, results obtained using the toxic compensation and acid response procedures gave best results. This was judged on the basis of a comparison of the results in Table IV, to literature reports of the cystine and methionine contents of casein of 0.35 and 3.4%, respectively, as summarized by Hawk, et al. (37, p.109).

Duplication of the work of Camien and Dunn (16, p.565) was of great potential value, for with increased response to smaller amounts of cystine the effect of toxic elements, if present, could be decreased by the
necessarily greater dilution of hydrolyzates. The reason given for the previous high standard range was that during heat sterilization the reducing group of glucose reacted with a large part of the cystine. Thus, effectively removing it by assuming that the resulting product was not available to the organism. The technique of separate sterilization and later aseptic addition of glucose, was an added inconvenience and had to be performed with care to avoid bacterial contamination. Another possible solution was the use of a non-reducing sugar available to *Leuc. mesenteroides P-60*, which could again be sterilized in the medium. According to the Bergey classification (13, p.347) this organism will produce acid from glucose, fructose, galactose, mannose, xylose, arabinose, and sucrose; generally produce acid from lactose, raffinose, salicin, and mannitol; and rarely produces acid from dextrin, starch, inulin, sorbitol, rhamnose, and glycerol. The first six named are reducing sugars and hence could not be used to any greater advantage than glucose. However, of the others, sucrose, raffinose, mannitol, dextrin and starch are non-reducing and were available immediately for test. In addition lactose was tried.

Cystine standard curves were set up with glucose sterilized separately and in the medium for comparison
with others containing the six available sugars. Of these, in a 20 hour incubation period for turbidimetric measurement, only the first and last named permitted so much as a slight amount of growth, and hence none could be of use in assay media. This variance with the Bergey system was previously noted by Caten, et al. (18, p.37) and it was explained that the Leuc. mesenteroides, strain P-60, was probably misnamed as it was more nearly like Leuc. citrovorum described in Bergey's manual (13, p.348). It may also be said that the latter system is largely a qualitative one, and the slight growth noted was perhaps indicative of some acid production, which although not sufficient for assay purposes, might serve to make it fit the original classification.

To make use of the gain in sensitivity of the cystine method, the procedure of separate sterilization of glucose was adopted for use, although somewhat late in this study. A full 10 ml. of acid production was thereafter attained at the upper concentration of a 0 to 20 γ standard range of L-cystine, whereas previously 50 γ had been required. The increased sensitivity was conclusively demonstrated by comparison of two standard curves of from 0 to 5 γ, one sterilized with glucose, the other without. In the latter an acid production of 7 ml. was reached, and consistently so thereafter, while
otherwise, only a bare 1 ml. was attained.

Two further points remained to be checked as regards the change, first the effect if any on methionine standard curves. Also, the authors suggested a medium pH of 6.3, with the proposal that the lability of cystine on heating was lessened from that in the neutral media ordinarily used. Consequently, cystine and methionine standard curves were prepared with glucose sterilized concurrently and separately, in both cases at pH's of 6.3 and 6.9. Quite unexpectedly, methionine supported lesser growth with separate glucose sterilization at pH 6.9, being only 75% of the other three maxima, which were identical. Therefore the media pH of 6.3 was adopted, both for convenience in its simultaneous preparation for the two assays, and since the cystine standard curve showed more extended linearity at this pH. It should be noted however, that comparison of the four cystine standard curves indicated that of the two factors, the lessened pH appeared more contributory to increased sensitivity than the effect of heating in the absence of glucose.

**Sample Preparations**

Assay results from early hydrolyzates of alfalfa sample H4403 were not considered good for reasons of inconsistency and poor recovery. A third disconcerting
result was the realization that the highest cystine and methionine percentages obtained fell far short of being sufficient to support the hypothesis that these two amino acids should account for the organic sulfur in the sample as calculated from chemical analyses.

For the alfalfa sample H4403, crude protein content was determined to be 28.17%, or a total nitrogen percentage of 4.51, by the AOAC Kjeldahl procedure (29, p.27). Total sulfur, as determined by the AOAC magnesium nitrate method (29, p.127) was found to be 0.261%. The method of analysis for inorganic sulfur was essentially that of Cook (19, p.26) and the value found was 0.013%. Some trouble was experienced with the latter determination due to mechanical difficulties of filtration. These were reported to have been largely overcome by use of 2% trichloracetic acid as the medium by which the sulfates were leached from the plant on sitting overnight on a steam bath. In the present use however, a flocculent gel persistently appeared at this point, and again during overnight steam bath digestion of the barium sulfate precipitate finally produced. Elimination of this interference was accomplished by substituting several hours mechanical stirring for heat treatment in both the sample steeping and precipitate digestion periods.

The above figures then gave an organic sulfur
content of 0.248% by the difference between total and inorganic sulfur determinations, and an organic sulfur/total nitrogen ratio of 0.055 was calculated. On the other hand, the first assay values obtained were cystine, 0.17%, representing 0.064% sulfur, and a methionine content of 0.36%, representing 0.078% sulfur. The total organic sulfur contributed to the sample by the combined amounts of these two amino acids found was therefore 0.142% or slightly more than half that determined to be present. As an approximation of the individual amounts of the two amino acids that were expected, the following calculations were made. The assumption was made that a true picture had been established by subsequent assays (Table IV) that the relative amounts of methionine and cystine present were in a ratio of 2 to 1, or each contributed equal amounts of sulfur. It was found that to give the lowest value of 0.050 in the organic sulfur/nitrogen ratio range, the minimum amounts of methionine and cystine present would have to be of the order of 0.64 and 0.32%, respectively.

In spite of the good results obtained by Henderson and Snell (40, p.24) with their method of hydrolysis of purified protein, it was felt that perhaps this treatment was not applicable to alfalfa. This view was supported by the fact that of all the conditions of hydrolysis
cited in previous references, few were exactly the same. It seemed likely that the variance may have existed dependent on the material investigated. Possibilities in this case appeared to be that the hydrolysis used was too drastic and resulted in destruction of the sulfur-containing amino acids, as suggested by consistently low cystine recovery values. Or, hydrolysis was incomplete under too mild conditions. Either case might be shown to be clear-cut by varying hydrolysis conditions as was done in the following experiments.

**Hydrochloric acid hydrolyses.** Using as a basis for comparison the Henderson and Snell (40, p.24) hydrolysis of an 0.5 gm. sample with 20 ml. of 3 N HCl in a sealed tube by autoclaving 6 hours, the time, acid strength and volume factors were varied. These trials included both more and less drastic conditions. The hope in these variations was that an hydrolyzate might be produced from which more consistent assays could be obtained, and that would give higher values than previously found. The variations were thought numerous enough that they would at least indicate how to proceed further toward the same ends.

The 0.5 gm. sample and sealed-tube autoclave treatment factors were constant throughout, as were two of the three variables in turn. Hydrolyzates were
prepared having been heated with 20 ml. of 3 N HCl for
4, 6 and 8 hours. Three others were heated for 6 hours
in 20 ml. of HCl of 2, 3, and 4 N strength, and a third
set was treated for 6 hours with 15, 20, and 25 ml. of
3 N HCl. The results of cystine and methionine assays
of these hydrolyzates are shown in the 2nd through 10th
rows of Table V.

TABLE V

The Cystine and Methionine Contents of One
Sample of Alfalfa Variously Hydrolyzed with HCl by
Autoclaving in Sealed Tubes.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid</td>
<td>Acid Time of Hydrolysis</td>
</tr>
<tr>
<td>Strength</td>
<td>Volume Hydrolysis</td>
</tr>
<tr>
<td>3</td>
<td>20 ml. 6 hrs.</td>
</tr>
<tr>
<td>3</td>
<td>20 4</td>
</tr>
<tr>
<td>3</td>
<td>20 6</td>
</tr>
<tr>
<td>3</td>
<td>20 8</td>
</tr>
<tr>
<td>3</td>
<td>20 6</td>
</tr>
<tr>
<td>4</td>
<td>20 6</td>
</tr>
<tr>
<td>3</td>
<td>15 6</td>
</tr>
<tr>
<td>3</td>
<td>20 6</td>
</tr>
<tr>
<td>3</td>
<td>20 6</td>
</tr>
<tr>
<td>3</td>
<td>25 6</td>
</tr>
<tr>
<td>3</td>
<td>20 4</td>
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<tr>
<td>2</td>
<td>25 6</td>
</tr>
<tr>
<td>2</td>
<td>20 4</td>
</tr>
<tr>
<td>3</td>
<td>20 6</td>
</tr>
</tbody>
</table>

* Assay values expressed as percent of sample H4403.

** Hydrolyzates identically prepared at different times.
In checking over these values it was seen that no marked increase was attained for the content of either amino acid such that the highest total would account for much more than half the organic sulfur.

Interpreting the values in terms of highest cystine content found, it seemed that hydrolysis limited to 4 hours gave the only improvement over the original procedure. Changes of acid volume appeared definitely disadvantageous, as did increased hydrolysis time. Results of change of acid strength were inconclusive but perhaps could be said to give no benefit. With highest methionine results as the criterion, it appeared that 2 N acid strength gave a slight improvement, while 4 N was contraindicated. Time of hydrolysis seemed most appropriate at the original 6 hour period.

Interpretations of best values obtained for each individual variable were considered for the next set of hydrolyses. As regards cystine the combination most apropos seemed to be 20 ml. of 3 N acid, treated for 4 hours. The best set of conditions for release of methionine were taken to be 25 ml. of 2 N HCl, hydrolyzed for 6 hours. A choice of a single hydrolysis that would satisfactorily permit assay of both amino acids was a more difficult one to make, and might result in loss of the small gains made. These were such slight improvements
however, that a compromise procedure could give just as valid results. The best set of circumstances for this purpose was selected as 20 ml. of 2 N HCl, hydrolyzed for 4 hours. These three combinations of conditions were next performed, and in addition the original hydrolysis was repeated for comparison. Recoveries were included in each case.

The results of the second group of comparative hydrolyses are also included in Table V, in the last four rows thereof. It will be noted that in no case were the previous assumptions as to best conditions for each amino acid borne out. The previous high value for methionine was not attained, and was most nearly approached under conditions assumed to be optimum for cystine. The previous high value for the latter amino acid was reached or exceeded in three of the four variations, with the exception being under those conditions assumed best. Recovery values again did not lend themselves to satisfactory interpretation.

All values in Table V were determined with high cystine blank basal media, by the regular increment method, with measurement of acid produced as the criterion of growth response. Those determinations involving the last described set of hydrolyzates were repeated by the same method for cystine content when low blank
reagents were secured. They were again repeated for both amino acids by the toxic compensation, acid production procedure, with similarly unsatisfactory results.

As a check on the effect of the hydrolysis procedure on the pure amino acids alone, known amounts of cystine and methionine were each subjected to the original treatment used, at the time of the last sample hydrolysis variations. Assay of methionine so treated showed only 68% recovery by the regular increment method, but 109% using the toxic compensation procedure. Similarly cystine was recoverable in amounts of 58 and 101% by the same two methods; respectively. This seemed to indicate that the difficulties of hydrolysis and recovery with alfalfa lay in faulty base values although possible destruction of the pure amino acids in the presence of alfalfa still could not be discounted.

The partial success of the toxic compensation method provided some evidence of an inhibitory substance present in hydrolyzates. A further indication was the observance of growth lags in assay tubes, which however were largely overcome by continued incubation. In some of these cases acid production was increasingly depressed at higher concentrations of hydrolyzate, and the amounts of settled cells were unexpectedly low. This presented a complex problem in the case of alfalfa hydrolyzates,
but where the pure amino acids had been treated alone and ultimately accounted for, the only other material present after neutralization was common salt. Calculations were made on the basis of 20 ml. of 3 N HCl used for hydrolysis, neutralized with NaOH, and dilution to 110 ml. as had been the procedure. These showed that as much as 30 mgm. NaCl/ml. might be present in assay solutions, which used in amounts up to 5 ml. might contribute 150 mgm. of salt in the highest assay level tubes.

With this possibility in mind it was thought well to test the effect of such amounts of NaCl on cystine and methionine standard curves. To do so, standard solutions were taken to which were added 30 mgm./ml. of NaCl, and used to compare with ordinary standard curves. Ranges were set up of from 0 to 50 \text{L-cystine} and 0-100 \text{DL-methionine}, and were duplicated including salt concentrations of 0-150 mgm., the latter intended as an approximation of the salt range supplied by increments of hydrolyzates. Lags in initial growth were noted as previously described, particularly at higher levels. In the case of methionine, identical curves were obtained up to 40 \text{L-methionine} and that plus 120 mgm. NaCl, but thereafter the salt concentration markedly depressed response. The cystine and salt standard curve showed an immediate depression which resulted in maximum acid
production at the 30% L-cystine level instead of at the normal point beyond 50%.

In the initial portion of the cystine curve utilized for assay comparison, an inhibition of at least 15% was shown, which increased as the cystine and salt concentrations approached their maxima. For methionine assays however, the effect was negligible due to lack of inhibition in the critical portion of the curve. Elimination of the salt effect on cystine assays appeared necessary. As it happened, the increased sensitivity of the assay later permitted sufficient dilution of the hydrolyzate, and consequently its salt content. In addition it was felt that any residual effect could be overcome through use of the toxic compensation method.

It was seen that sodium chloride had contributed very little to the previous inconsistent assays of alfalfa hydrolyzates for methionine, at least when permitted to incubate for the period of time requisite for full acid production. It might then also be supposed that salt was not the entire answer as regards unreliable cystine assays. After adoption of the more sensitive cystine method, the following experiments were set up to detect and attempt to measure any inhibitory effect of the hydrolyzate itself. The general plan of such experiments projected assays of an hydrolyzate for each of
the amino acids alone, and in each case in the presence of a constant amount of the pure amino acid added just prior to assay. From the combined amount thereafter determined was subtracted that found in the hydrolyzate at each level assayed, assuming the difference to represent the amount added which had been recovered. Each assay was set up in duplicate, and one measured turbidimetrically at the end of 18 hours to detect early effects, and the other by titration of acid produced in 72 hours to detect any changes with lengthened growing time.

As regards cystine, dilutions of the hydrolyzate were used which represented a range of from 0.25 to 20 mgm. alfalfa, and were assayed alone and in the presence of 10^6 L-cystine at each level. The short term inhibition of recovery of this cystine was shown to be 0 to 2% by 0.25 to 1 mgm. of hydrolyzed alfalfa. This inhibitory effect was measured as at least 40% by 2 mgm. and jumped to 99% in the presence of 4 mgm. These results were most drastically and surprisingly reversed following the longer incubation period. With the increasing amounts of hydrolyzate, the organism appeared to be increasingly stimulated. This was indicated by the fact that the differences between responses to hydrolyzate increments plus added cystine, and the hydrolyzate alone were more
than sufficient to account for the added cystine. This stimulatory effect progressed from 1% at the lowest level of hydrolyzate up to 26% in the presence of 10 mgm. of hydrolyzed alfalfa. Between this level and the next of 20 mgm. a switch occurred to 22% inhibition.

Results for methionine recovery under similar circumstances indicated initial stimulatory effects even with turbidimetric measurement. This amounted to nearly 10% with 1 mgm. of alfalfa hydrolyzate, but above this point a change-over to inhibition came about, gradually increasing to 100% before 10 mgm. hydrolyzed alfalfa had been added. After the more extended incubation periods, the stimulatory effect increased gradually to more than 30% at a level of 3.5 mgm. of alfalfa hydrolyzate, but was thereafter decreased.

As far as further assays were concerned, without attempting to eliminate or characterize the causes of stimulation and inhibition, only one recourse seemed to be available. It appeared that the method could only be used satisfactorily for determinations wherein the sample aliquots were restricted to those amounts whose adverse effects were not significantly large. This range for both cystine and methionine assays was largest when the acid production response was measured. For cystine it appeared that determinations might be made on amounts of
hydrolyzate representing 1.5 mgm. of alfalfa at the maxi­
mum. Methionine assays were even more drastically limit­
ed to amounts less than 0.5 mgm. These conclusions were
based on only the single series of determinations. They
might be altered by further checks of the same sample,
and quite conceivably so when other alfalfa samples are
assayed.

**Sulfuric acid hydrolyses.** The notable lack of suc­
cess attained in assay of alfalfa when hydrolyzed with
hydrochloric acid, as conventionally used for sample
preparation for microbiological assay, led to considera­
tion of other acid procedures. Chemical analyses for
these two amino acids are thought by Block and Mitchell
(10, p.254) to be somewhat more reliable than microbio­
logical assays. They reported in their review that chem­
ical methods gave comparatively higher values for alfal­
fa. Acid hydrolytic treatment of samples for these
methods were quite different, and it was thought that
their adoption might prove advantageous in the present
instance.

After extensive investigations, Hess and Sullivan
(41, p. 637) reported that optimum conditions for con­
current liberation of cystine and methionine were attain­
ed through use of sulfuric acid. Their method was in­
itially adopted for sulfuric acid hydrolysis of H4403
alfalfa. It consisted of 12 hours reflux treatment, in an atmosphere of flowing nitrogen, of a half gram sample with 6 ml. of 6 N H\textsubscript{2}SO\textsubscript{4}. In the hope that increased temperature and pressure of autoclave heating might reduce the time factor, this was done concurrently for comparative purposes. The procedure developed was to place the sample and acid in a pyrex tube which was then partially drawn out. The container was flushed for five minutes with nitrogen, and the vial sealed.

Recovery hydrolyzates for both procedures, and both cystine and methionine were run. At this late date two errors in judgement as to the preparation of the hydrochloric acid recovery hydrolyzates were noted, and hereafter corrected. The amounts of pure amino acids added to samples previously had been such as to require at least half again as great a dilution of recovery hydrolyzates as of those of the sample alone. Hence, although both the cystine and methionine contents of each were brought to the same level, the other hydrolysis products of hydrolysis of alfalfa were much more dilute in recovery hydrolyzates than in those for straight assay. This undoubtedly was largely contributory to invalid recovery values previously described. The second error probably resulted in an unnecessarily large amount of hydrolysis work. This came about through the previous practice of preparing recovery hydrolyzates of each of the two amino acids
separately. Therefore recovery hydrolyzates hereafter
reported contained L-cystine and DL-methionine added
together, the former at a level of 0.1% of the sample
and the latter at 0.5%. These amounts were small enough
to permit equal dilution of the assay and recovery hydro-
lyzates.

The results of the first sulfuric acid hydrolyses
are presented in Table VII. It will be noted that methi-
onine values, especially by reflux were improved but that
cystine content and recovery remained low. To give a
basis for comparison, and assuming that the treatment
was too drastic, the sulfuric acid was used twice again,
in decreasing normality and with lessened time of heating.
These conditions and results thereof are shown in Table
VII. The assays were performed by the regular incre­
ment method after 72 hour incubation for acid production.

It was heartening to note a more definite picture
than could previously be detected, as concerned methio-
nine released and affected by these hydrolyses. There
appeared to be a definite correlation, up to a point not
definitely established, between increasing acid strength
and time, and most effective release of methionine by
refluxing. At the same time recovery values appeared
reasonably consistent. The picture was not as clear
with autoclave treatment, and it may have been that
**TABLE VII**

The Cystine and Methionine Contents of One Sample of Alfalfa When Variously Hydrolyzed with H\textsubscript{2}SO\textsubscript{4}

<table>
<thead>
<tr>
<th>Variables</th>
<th>Acid Heating Strength</th>
<th>Time of Hydrolysis</th>
<th>Determination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cystine Assay Recovery</td>
</tr>
<tr>
<td>Reflux</td>
<td>6 N</td>
<td>12 hrs.</td>
<td>0.12%  78% 0.43%  97%</td>
</tr>
<tr>
<td></td>
<td>4 N</td>
<td>6 &quot;</td>
<td>0.12  96  0.32  109</td>
</tr>
<tr>
<td></td>
<td>2 N</td>
<td>3 &quot;</td>
<td>0.04  82  0.14  109</td>
</tr>
<tr>
<td>Autoclave</td>
<td>6 N</td>
<td>6 hrs.</td>
<td>0.09  56  0.37  72</td>
</tr>
<tr>
<td></td>
<td>4 N</td>
<td>2 &quot;</td>
<td>0.12  75  0.37  86</td>
</tr>
<tr>
<td></td>
<td>2 N</td>
<td>1 &quot;</td>
<td>0.05  81  0.21  91</td>
</tr>
</tbody>
</table>

* Assay values expressed as percent of sample H4403.

Excessive pressure and temperature were the cause. It was also reasonably clear that autoclave hydrolysis for release of cystine was unsatisfactory. In the case of reflux hydrolysis of alfalfa for cystine analysis optimum conditions under these circumstances seemed to be those which were midway between the extremes. These then were tentatively designated as the best conditions of the three, on which to base future work on preparation of a single sample for assay for cystine and methionine.

**Enzyme digestions.** The previous high point for cystine content had not been reached by sulfuric acid hydrolysis, and recovery values remained inconsistent. Even with methionine results much improved in some respects, the combined content of sulfur-containing amino
acids had still not been determined as sufficient to account for organic sulfur. One distinct alternative method of hydrolysis remained, namely, utilization of the action of proteolytic enzymes. It must be conceded that they have not been widely reported to be successful in such major undertakings as digestion of alfalfa.

The fundamental basis of the following experiments lay in the work of Hiller and Van Slyke (45, p.258), and their findings concerning the deproteinization action of trichloroacetic acid. It was found that nitrogenous material of protein origin which was soluble in a 5% concentration of this acid consisted largely of amino acids and short chain poly-peptides. Larger proteinaceous entities were coagulated and retained on filtration. Use was made of this information to estimate the extent of proteolysis during the course of enzyme digestions.

The techniques worked out for attempted digestion of protein in intact alfalfa may be ascribed to the assistance of Remmert (95). The digestions for the most part were carried out in a 0.1 M KH₂PO₄ buffer medium. This solution was made up to nine-tenths volume for stock. Portions were adjusted to the optimum pH of the enzyme used and made to final volume. Trichloroacetic acid stock was prepared at 10% strength by weight, and was used volume for volume with digests to give final
concentrations of 5% of the acid. It was found that the largest amount of alfalfa which could be conveniently handled was 10 gm. per 100 ml. of digest. The suspensions therefore contained 2.5% crude protein. Enzymes were used at levels of 2% of the sample.

The digestion procedure consisted of steam sterilization of alfalfa and buffer for 20 minutes in 100 ml. graduated centrifuge tubes, which were cotton stoppered to prevent boiling over. Dry enzyme preparations were added and the digests made to volume with steamed buffer. Five drops of benzene were added as a preservative. After closing with steamed rubber stoppers and shaking, suitable aliquots were removed, two for direct Kjeldahl total nitrogen determinations (29, p.27) and another for addition to an equal volume of 10% trichloroacetic acid. The latter was allowed to stand with frequent shaking for 20 minutes, while the remainder of the digest was again tightly stoppered and put to incubate in a 37°C water bath. A rocking cradle provided constant agitation. At suitable intervals of time, usually at 16, 24, 40 and sometimes 72 hours, additional aliquots were removed to mix with trichloroacetic acid. These were filtered after standing, and duplicate samples of the filtrate taken for determination of their total nitrogen contents.
The latter acid soluble nitrogen values were taken as the approximate portion of the original crude protein which had been digested. They were calculated as percent of the original total nitrogen of the digests and plotted against time removed. The zero time acid soluble nitrogen content of the alfalfa digests repeatedly amounted to about 25% of the total nitrogen. This was thought to largely represent non-protein nitrogen, which was indicated by the "true" protein values of Cook (19, p.iii). It followed then that the total nitrogen, less the zero hour acid soluble nitrogen taken as a blank, approximately represented protein nitrogen. Therefore, subsequent acid soluble nitrogen determinations, less the same blank value, were calculated as percent of the initial subtraction difference. This gave a measure of the protein nitrogen which had been digested. It too was plotted against the various times of aliquot removal.

The enzymes used were well known and characterized for their proteolytic abilities. They are listed in Table VIII as used here, alone and in combination, along with the end-results of the digestion experiments performed. Of the first group of four trials, combined Takamine protease and trypsin appeared to have accomplished the most thorough digestion, although none gave complete hydrolysis.
TABLE VIII

The Extent of Digestion of One Sample of Alfalfa by Various Enzymes.

<table>
<thead>
<tr>
<th>Enzyme Treatment</th>
<th>Acid Soluble Nitrogen</th>
<th>% of Total N</th>
<th>% of &quot;Protein&quot; N</th>
<th>Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Takamine protease*</td>
<td>76.2</td>
<td>65.7</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Trypsin</td>
<td>77.3</td>
<td>68.8</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Takamine protease &amp; trypsin</td>
<td>83.0</td>
<td>75.0</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Polidase-3***</td>
<td>61.3</td>
<td>53.2</td>
<td></td>
<td>72</td>
</tr>
<tr>
<td>Takamine protease &amp; trypsin 72.6</td>
<td>60.0</td>
<td></td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Trypsin; followed by, Takamine protease</td>
<td>70.5</td>
<td>58.1</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Pepsin; followed by, Takamine protease</td>
<td>46.8</td>
<td>27.7</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Taka-diaastase*** predigestion 39.2</td>
<td></td>
<td>X</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Trypsin; followed by, Takamine protease &amp; trypsin</td>
<td>80.4</td>
<td>67.7</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Trypsin; followed by, (1) 68.8</td>
<td>56.2</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Takamine protease</td>
<td>86.7</td>
<td>31.3</td>
<td></td>
<td>24</td>
</tr>
<tr>
<td>Trypsin; followed by, (2) 65.9</td>
<td>53.2</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Takamine protease</td>
<td>79.6</td>
<td>72.7</td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

* Takamine Laboratories, Clifton, N.J.
*** Schwarz Laboratories, New York
### Parke-Davis, Detroit.

(1) Digestion for content assay.

(2) Digestion for recovery assay. (L-cystine and DL-methionine added in amounts of 0.1 and 0.5% of alfalfa.)

(1) & (2) Average results of duplicate digestions, less average of duplicate enzyme auto-digests.
Taking the best of the first group as a basis for comparison, it was repeated, and three further trials were set up. These differed in that the digestions were performed in two stages. Following the first step in each case, the remaining amount of alfalfa was calculated, on the assumption that a homogeneous suspension had been attained by shaking at the time of removal of aliquots. The pH was readjusted, and the digest steamed again. Addition of the second enzyme and benzene was followed by making to the 100 ml. mark with sterile buffer, and the resulting concentration of alfalfa was calculated. Taking this as a new zero time, aliquots were again withdrawn for total and acid soluble nitrogen determinations, and the latter were repeated as before.

The pH optimum for pepsin was so low that its action was carried out in 0.1 M sodium citrate buffer adjusted to the proper pH. At the conclusion of the digestion the pH was increased to the optimum for Takamine protease. After steaming, the digest was made to volume with sterile phosphate buffer of the same pH. Taka-diastase was used for predigestion in one trial with the thought that it might break up any close association of carbohydrate and protein.

As may be seen by Table VIII, the separate action of trypsin and Takamine protease appeared superior to
that of their concurrent use. The former combination also seemed more effective than the others tried, and was therefore chosen for an attempted preparation of a sample for assay. This appeared to be premature in the light of incomplete digestion, but the primary consideration was the hope of being able to show some improvement over previous hydrolyses. If the cystine and methionine contents of alfalfa could be demonstrated to be higher than found with acid treatment, attempts to improve the digestion would follow. If no increase could be obtained, then this approach would be dropped due to unwieldiness and the time consuming nature of the procedure.

The digestions for assay were each prepared in duplicate and included recoveries and blanks. The completed digestions were filtered, and portions of the filtrates were diluted for assay. Acid soluble and total nitrogen determinations of this filtrate showed the former to be 92.3% of the total. Also, the filtrate acid soluble nitrogen was 30% of the total nitrogen of the digest.

Upon assay of the enzyme hydrolyzate, there was no growth response to the largest aliquot used, which indicated that there was less than 0.001% cystine and 0.005% methionine released. It was presumed that these amino acids were present in the filtrate, but in
forms unavailable to the test organism, probably as polypeptides.

The residues after filtration of the digests were thoroughly washed and run for total and inorganic sulfur, which gave measure of the amount of organic sulfur which was not digested. The organic sulfur remaining in the alfalfa appeared to be 33% of the amount previously found in the untreated sample. This indicated that the organic sulfur fraction had been about two-thirds hydrolysed and should appear in the digestion filtrate.

The enzyme digest filtrate appeared to contain about 80% of the total nitrogen of the original sample, and yet showed essentially no cystine or methionine by assay. It has been suggested that this may have been in polypeptide and hence unavailable forms. To test this premise, aliquots of the filtrate, calculated to represent a half gram sample of alfalfa, were made 4 N with respect to sulfuric acid in 12 ml. total volume. These were then refluxed under nitrogen for three hours. Assays of these hydrolyzates were not quantitatively valid, but were performed to give an estimation of the cystine and methionine of the alfalfa proteins which had been partially released by enzyme digestion.

Values found were 0.10% cystine and 0.26% methionine
in the sample. It has been calculated that the organic sulfur and total nitrogen contents of the enzyme digest filtrate were only 67 and 80% respectively, of those of the sample. Therefore, the assay results of the combination enzyme and acid treated sample were remarkably like those obtained from previous acid hydrolyzates.

Lowry and Thiessen (62, p.152-153) found that the action of selected proteolytic enzymes resulted in release of amino nitrogen from a heat-induced glucose-casein complex. It was therefore hoped that enzyme treatment of an acid hydrolyzate might result in a further release of cystine and methionine. A previously assayed sulfuric acid hydrolyzate was therefore adjusted to the proper pH, diluted with an equal volume of phosphate buffer, and subjected to the combined action of trypsin and Takamine protease for 24 hours. Assay of the filtrate of this digest showed approximately 0.1% cystine and 0.3% methionine. These results were unchanged from those of assays of the hydrolyzate following acid treatment alone. Hence the additional treatment did not support the premise on which it was based.

Sulfur Fertilization and the Composition of Alfalfa Proteins

In spite of the questionable success of previous parts of the study, a limited attempt was made to
correlate the work with that previously done in this laboratory. A set of three of the samples used by Cook (19, p.1) was selected as representative of his investigation. Sulfur fertilization had been shown to increase the yield of alfalfa and its content of crude protein, and of total, inorganic, and organic sulfur. Organic sulfur, total nitrogen ratios had approximated the constant range proposed.

Some of the previous analytical work on these samples was repeated, the results of which are shown in Table IX. The total nitrogen and inorganic sulfur values, by the same methods, were essentially the same as before. With the exception of the check plot, whose value was also duplicated, the total sulfur values reported here are significantly higher. This may be due to the present use of the AOAC magnesium nitrate method (29, p. 127), as compared to the earlier application of the Benedict-Denis method by Cook (19, p.20). The latter reported that there was evidence of incomplete recovery of methionine sulfur by the method used. In this case, the higher total sulfur contents of the fertilized samples led to higher organic sulfur values. This in turn did not allow calculation of organic sulfur, total nitrogen ratios within the range expected. Recalculation of the same data indicated that sulfur fertilization
TABLE IX

Nutritionally Important Nitrogen and Sulfur Constituents of Some Third Cutting Alfalfa in Response to Sulfur Fertilization

<table>
<thead>
<tr>
<th>Determination</th>
<th>Plot Description</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H614</td>
</tr>
<tr>
<td></td>
<td>Check</td>
</tr>
<tr>
<td>Total N, %</td>
<td>2.32</td>
</tr>
<tr>
<td>Crude Protein (N x 6.25), %</td>
<td>14.6</td>
</tr>
<tr>
<td>Total S, %</td>
<td>0.124</td>
</tr>
<tr>
<td>Inorganic S, %</td>
<td>0.001</td>
</tr>
<tr>
<td>Organic S, % of Sample</td>
<td>0.123</td>
</tr>
<tr>
<td>Organic S, % of Protein</td>
<td>0.65</td>
</tr>
<tr>
<td>Organic S/Total N</td>
<td>0.053</td>
</tr>
<tr>
<td>Cystine Content % of Sample</td>
<td>0.05</td>
</tr>
<tr>
<td>% of Protein</td>
<td>0.33</td>
</tr>
<tr>
<td>S % of Organic S</td>
<td>10.6</td>
</tr>
<tr>
<td>% Recovery</td>
<td>74.9</td>
</tr>
<tr>
<td>Methionine Content % of Sample</td>
<td>0.17</td>
</tr>
<tr>
<td>% of Protein</td>
<td>1.12</td>
</tr>
<tr>
<td>S % of Organic S</td>
<td>29.3</td>
</tr>
<tr>
<td>% Recovery</td>
<td>105.</td>
</tr>
<tr>
<td>Cystine plus Methionine S % of Organic S</td>
<td>39.9</td>
</tr>
</tbody>
</table>
resulted in an increase in the organic sulfur content of the alfalfa protein.

Portions of these samples were hydrolyzed by the $\text{H}_2\text{SO}_4-\text{N}_2$-reflux method in 4 N acid for 6 hours. The assay and recovery results, and calculated values are also given in Table IX. The contents of the amino acids were much lower than those of H4403, which was to be expected since their crude protein contents were much lower. This was a reflection of harvesting normal samples representative of whole mature plants, which had not been the case with the sample previously assayed.

The methionine values appeared to be valid in all cases, when judged by the criterion of recovery. This was consistent with good recovery results from the previous hydrolysis of H4403 under the same conditions. The samples apparently responded to sulfur fertilization by slightly increased contents of methionine. These results were more marked when calculated on the basis of percent of crude protein. Cystine results once more were invalidated by low and inconsistently varying recovery values. It was quite apparent that the total sulfur contributed by the combined amount of sulfur-containing amino acids found again failed to account for all of the organic sulfur determined.
GENERAL DISCUSSION

The results for methionine appear to be quite satisfactory as judged by consistency among themselves, recovery of added methionine and the known nutritive value of alfalfa proteins. The results for cystine are not satisfactory as judged by the recovery of added cystine. The results for combined cystine and methionine are disturbing from two points of view. They account for less than 50% of the organic sulfur in alfalfa. Neither do they appear to be in harmony with the accepted biological value of alfalfa crude protein.

Taken as a whole, the results obtained in this investigation suggest several important generalizations. For complex materials such as alfalfa, in contrast to relatively pure proteins, the problem of protein hydrolysis without amino acid losses still remains to be solved. This is especially true of cystine. This difficulty appears to be inherent in both chemical and microbiological methods.

A second generalization is that under practical conditions the determination of methionine only, yields information of limited application in the field of nutrition. This appears to be frequently overlooked because of the essential nature of methionine and the
difficulties encountered in the determination of cystine.

As a third generalization, it should be pointed out that resort to the analysis of purified alfalfa proteins is not promising for several reasons. One is the large fraction of non-protein nitrogen found in alfalfa. More important is the need for strong alkalies in extraction and purification procedures. These are especially objectionable in the case of cystine.

It would therefore appear that until methods for the hydrolysis of complex materials such as alfalfa are very substantially improved, many problems, such as the one under discussion in this thesis, will remain in part unsolved.
SUMMARY AND CONCLUSIONS

An attempt was made to adapt microbiological assay procedures to the determination of cystine and methionine in alfalfa. An all-purpose, synthetic medium was selected. Initial difficulty with high blanks was ultimately found to be due to contamination of several basal amino acids. The test organism, *Leuconostoc mesenteroides* P-60, was selected on the basis of its reported specific requirement for both of the sulfur-containing amino acids. Uniform reproducibility of standard curves justified the choice of medium and organism.

Numerous assay procedures and varied hydrochloric acid-autoclaved hydrolyzates of alfalfa gave inconsistent analyses and recovery values. Compensation for toxicity improved the former situation but not the latter. Increased sensitivity of the assay for cystine by sterilization of glucose separately from the medium permitted greater dilution of samples and toxic effects.

One of several combinations of conditions of sulfuric acid-reflux hydrolysis of alfalfa permitted more consistent assay and recovery of methionine.

Numerous enzyme digestions were attempted, the best of which did not permit assay for either of the two amino acids.
Assay of several alfalfa samples of known sulfur fertilization history showed increased methionine content which paralleled increasing organic sulfur. Insufficient sulfur-containing amino acids were found to support the premise that they should account for all of the organic sulfur.

It has been suggested that the failure to account for all of the organic sulfur as cystine and methionine may largely be due to difficulties inherent in the determination of cystine in complex materials such as alfalfa.
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