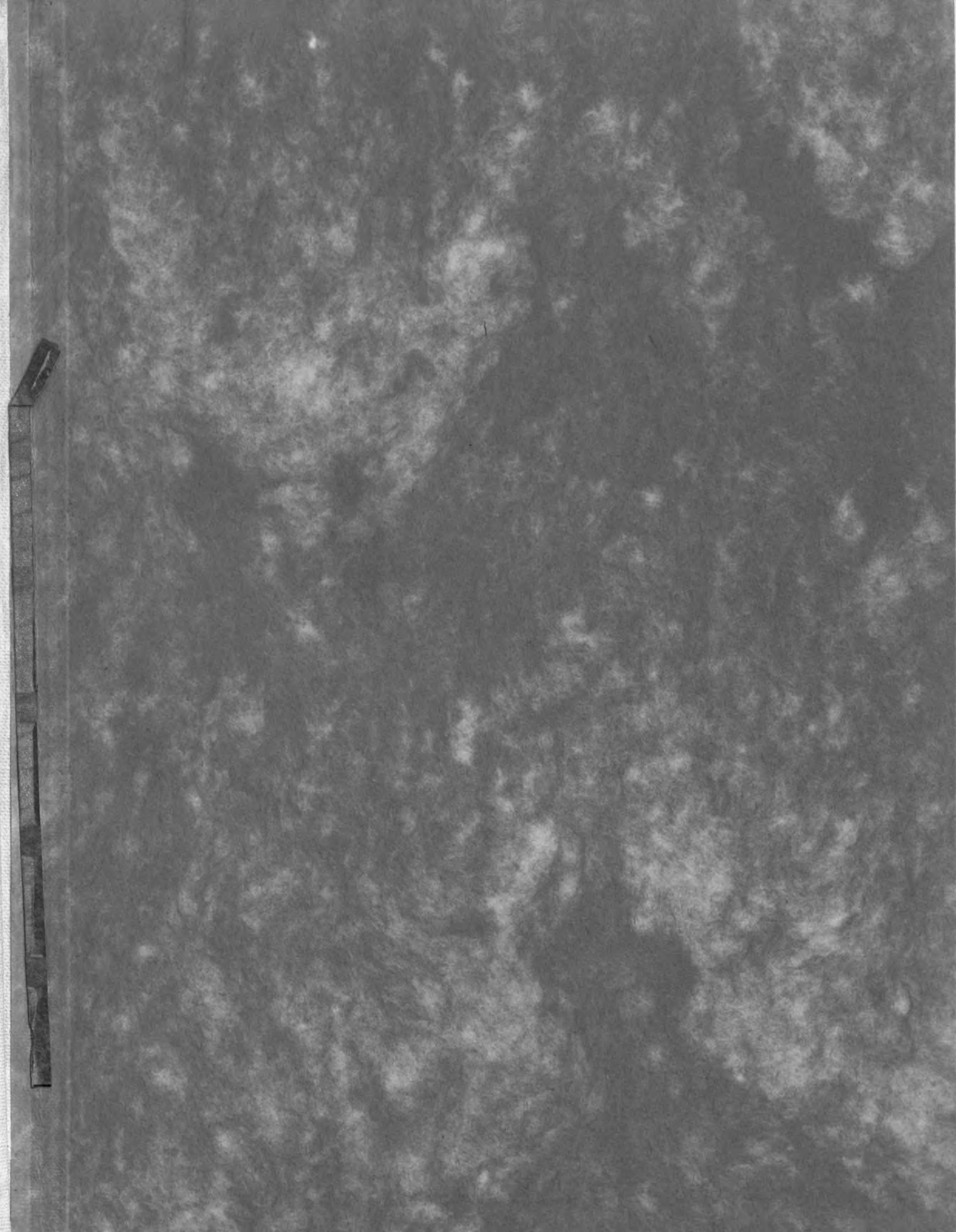
THE CAROTEN
SEVERAL HERBAC
GROWING SEAS
SUBSEQUENT
LABORATORY

Thesis for the D
MICHIGAN STA
Woodrow
19



THE CAROTENE CONTENT OF SEVERAL HERBAGES DURING THEIR GROWING SEASON AND LOSSES SUBSEQUENT TO VARIOUS LABORATORY TREATMENTS

By

Woodrow W. Snyder

Thesis

Submitted in partial fulfilment of the requirements
for the degree of Master of Science in the
Graduate School, Michigan State College
Department of Dairy Husbandry
June, 1939

THESIS

ACKNOWLEDGMENTS

The writer wishes to express his most sincere appreciation to Mr. L. A. Moore, Research Assistant in Dairy Husbandry, for kindly directing and planning the experimental work as well as arranging and correcting the manuscript; to Doctor Earl Weaver, Head of Department of Dairy Husbandry, for making this study possible; to Doctor C. F. Huffman, Research Professor in Dairy Husbandry, for his many helpful and inspiring suggestions; to Professor C. D. Ball of the Biological Chemistry Department for his generosity in giving helpful advice; and to the members of the Department of Dairy Husbandry for their cooperation.

TABLE OF CONTENTS

	Page
Introduction	1
Review of Literature	2
Relation of Carotene to Vitamin A	2
Biological Relation	2
Chemical Relation	5
Evaluation of Carotene in Plants	7
Extraction	7
Purification of Extract	12
Concentration of Final Extract	15
Stability of Carotene in Solvents	16
Stability of Carotene Solutions in Mixed Feeds	19
Stability of Carotene in Green Herbages	21
Effect of Enzymes	21
Effect of Field Drying	24
Effect of Artificial Drying	27
Effect of Drying Under Laboratory Conditions	28
Effect of Autoclaving	29
Stability of Carotene in Dried Hays	30
Stability of Carotene in Silages	34
Variations in Carotene Content of Plants During Growth	39
Early Development in Seedlings	39
Variation Druing the Growing Season	40
Effect of Soil Fertility on Carotene Content of Plants	43
Discussion of Review	45

Page

Results of Autoclaving and Subsequent Incubation 75

Results of Incubation in an Atmosphere of Carbon Dioxide 77

Buffer Curves for Alfalfa 79

Effect of Acids upon the Stability of Carotene in Alfalfa 79

Destruction of Carotene by Enzymes Contained in Potato Juice 83

Discussion of Results 85

Summary and Conclusions 93

Literature Cited 95

INTRODUCTION

It is now known that vitamin A as such exists chiefly in the animal kingdom, while carotene or provitamin A is found almost exclusively in plants. The importance of vitamin A in the ration of the bovine has been realized for about a quarter of a century. In order to meet the requirements of the dairy cow for maintenance and in order to fortify the milk produced it is essential to supply an abundance of provitamin A or carotene in the ration.

Although carotene is distributed widely throughout the plant kingdom the chief source for the dairy cow is from green plants such as pasture grasses in the summer months. However, the roughages fed during the winter months usually have a considerably lower carotene content because of destruction during the curing process and the following period of storage. The degree of destruction depends upon various factors which affect the stability of carotene under the conditions of curing and storing.

This thesis is concerned chiefly with the changes of the carotene content of several herbages during the growing season and considers especially the stability of the carotene as affected by the possible presence of oxidising enzymes. Although the plant tissue in this study was not cured or preserved by the usual farm methods, a uniform laboratory treatment was applied instead, in order to obtain a comparative measure of the stability of carotene for different herbages throughout their growing season. This comparative stability was sought in order to gain some information that might assist in furnishing the dairy cow with a roughage of high carotene content, especially for winter feeding.

REVIEW OF LITERATURE

Relation of Carotene to Vitamin A

Biological Relation

Carotene was first isolated in 1826 by Wackenroder (129) from the roots of carrots. He applied the term "carotin" and designated the pigment as specific for the carrot root. According to Palmer (85) Arnaud in 1885, isolated carotene from plant leaves and was the first to definitely associate this yellow plastid pigment with carotene from carrot roots. The first association between the carotenoids of plants and the yellow pigments in animals was revealed by Palmer and his coworkers (84) (87). Steenbock (112) while studying the vitamin A content of corn noted a high correlation between the content of yellow pigment and the vitamin A activity. In this and later reports Steenbock and associates (113) (114) (115) presented more evidence to indicate a definite relationship between the yellow pigments of corn and the vitamin A activity, noting that white corn was almost devoid of vitamin A. Definite evidence for an association between carotene and vitamin A was presented in experiments by Euler, Karrer and Rydbom (22) who prepared a highly purified form of carotene and reported a growth promoting effect upon rats in relatively small doses. They recrystallized their carotene preparation nine times and thus concluded that if growth in the rat was due to any other factor than carotene it must be effective in doses as small as 0.01 microgram which seemed improbable.

Kerppola (56) was able to obtain crystalline vitamin A from cod liver oil which gave the characteristic antimony trichloride color reaction.

After feeding purified carotene to rats, depleted of vitamin A, he was able to extract vitamin A from the rat livers as characterized by the antimony trichloride reaction, and thus assumed that carotene was probably provitamin A in plants. The work of Capper (13) has helped to confirm the idea that carotene may serve as a precursor of vitamin A.

In a series of experiments Moore (68 to 75 inclusive) presented conclusive evidence that carotene is utilized as a source of vitamin A by the rat as well as other animals including the dairy cow. Proof was presented to show that carotene is convertible to vitamin A in the animal body and that the conversion takes place in the liver. The liver serves as a regulator to supply the body needs of vitamin A.

Although there are about 60 known naturally occurring plant and animal carotenoids there are but four from plant sources that possess vitamin A activity. Bogert (10) has an excellent chapter on the carotenoids in which he pointed out that alpha-, beta-, and gamma-carotene as well as cryptoxanthin are the only known biologically active plant carotenoids. Many workers (55) (62) (117) present evidence that beta-carotene is the most common form of provitamin A in plants. Strain (117) noted that the chief carotene component of more than 20 different plant leaves was the beta-isomer. Many of the plants examined contained a very small amount of alpha-carotene of none of the alph-isomer at all. Mackinney (62) studied 59 plants species distributed in 40 botanical families and observed that the beta-isomer made up the major fraction of the leaf carotenes. Alpha-carotene was present in proportions varying from traces to 35 per cent of the total carotene content in 40 of the 50

species studied. From Mackinney's results it may be observed that the alpha-isomer is entirely lacking, or present in only small amounts, in most of the common plants used as roughage for cattle feeding.

Although much of the early data for the vitamin A value of plants is in terms of rat assays one can readily see that if a definite biological relationship were established between beta-carotene and its vitamin A value a quantitative chemical determination for carotene may serve as valuable a purpose as the rat assay.

Sherman and Munsell (104) have recommended a unit of vitamin A as that amount required to induce an average gain in weight of three grams per week for eight weeks in a standard test animal (rat) under the conditions described. Other units of vitamin A have been proposed by various workers but differ somewhat in the value assigned.

In order to arrive at a definite comparative value for vitamin A and carotene Goldblatt and Barnett (31) have shown that 0.5 gamma of pure beta-carotene was equal to a Sherman unit. By making both carotene and vitamin A determinations on the same feed samples, Fraps and co-workers (28) have found that one microgram of carotene was equal to 1.4 Sherman-Munsell units and one Sherman-Munsell unit was equal to 1.2 international units. These workers also concluded that in terms of international units one Sherman-Munsell unit was equal to 0.72 micrograms of pure beta-carotene.

At the present time the precise relationship between vitamin A and carotene is clearly expressed in terms of the international unit for vitamin A. By agreement (32) the international standard for vitamin A is a sample of pure crystalline beta-carotene, 0.6 micrograms of which equals one international unit.

Chemical relation

The chemical basis for the relation between carotene and vitamin A has been established by a number of workers, but more of the early work was done by Karrer and his associates. The first complete structural formula for carotene, proposed by Karrer and co-workers (50) has since been assigned to beta-carotene by Kuhn and Lederer (60). These later workers confirmed Karrer's report but separated an optically active isomer from the carotene extract of carrot roots. Both the optically active alpha-carotene and the optically inactive beta-carotene had the formula $C_{40}H_{56}$ and therefore Kuhn and Lederer assigned Karrer's structural formula to the optically inactive beta-carotene.

The structural formula for vitamin A has been developed by Karrer, Morf and Schopp (52). They proposed the formula on the basis of molecular weight (300-320), being optically inactive and forming geronic acid on treatment with ozone. Heilbron and associates (41) prepared crystalline vitamin A and confirmed Karrer's formula. Karrer, Morf and Schopp (53) completed the proof for the formula by means of an eight-step synthesis of crystalline perhydrovitamin A, beginning with beta-ionone. Perhydrovitamin A was proved identical to the completely hydrogenated natural vitamin A from fish liver oil.

Bogert (10) in his review pointed out that there are only four known phytocarotenoids possessing the beta-ionone structure. Those having the beta-ionone ring are cryptoxanthin, alpha-, beta-, and gamma-carotene. These four carotenoids serve as provitamin A and appear to be stero-isomers.

Karrer, Morf and Walker (54) observed that pure alpha-carotene treated with ozone yielded geronic acid and iso-geronic acid. This would indicate the presence of one beta-ionone and one alpha-ionone ring in the formula, since beta-ionone and alpha-ionone when treated similarly yielded geronic and iso-geronic acid, respectively.

The presence of the beta-ionone ring in the carotenoid is apparently essential for the pigment to serve as a precursor of vitamin A. According to Palmer (86) beta-carotene has two beta-ionone rings while alpha-carotene has one beta-ionone ring and one alpha-ionone ring. The alpha-ring is responsible for the optical activity of alpha-carotene. Gamma-carotene has only one beta-ionone ring, the other potential beta-ionone ring being open. Cryptoxanthin has one beta-ionone ring and one beta-oxidized ring. These four phyto-carotenoids all possess vitamin A activity which is due to the beta-ionone structure. From this relation it may be seen that beta-carotene yields two molecules of vitamin A while the other three each yield only one molecule.

Lycopene is a carotenoid with the formula of $C_{40}H_{56}$ which is common to alpha-, and beta-carotene as well. Lycopene is not active as a source of vitamin A since both potential beta-ionone rings are open.

Xanthophyll, sometimes called lutein, or more specifically leaf-xanthophyll has the formula of $C_{40}H_{56}O_2$. It is closely related to the biologically active carotenoids, however, xanthophyll possessed no biological activity since both potential beta-ionone rings contain an OH group. Bogert (10) pointed out that there are other closely related carotenoids that do not exhibit vitamin A activity because of some change in the beta-ionone ring.

Recently, Lederer and Moore (61) reported a zoo-carotenoid that possesses vitamin A activity which they isolated from the sex glands of the sea urchin. They applied the name "echinenone" and indicated that it was probably a monoketone of beta-carotene. Although its chemical structure has not been completely established it seems highly probable that this compound possesses the beta-ionone ring. It might be added here that besides beta-carotene, which is found in both plants and animals, this is the only carotene that has its origin in the animal organism.

Evaluation of Carotene in Plants

Most methods used for evaluating carotene in plants may be conveniently divided into three parts each of which will be considered separately. First, extraction of the plant tissues and obtaining a crude carotenoid extract will be considered; secondly, methods of purifying this extract; and thirdly, the methods used to determine the carotene concentration of the final solution.

Extraction

Willstatter and Stoll (132) in 1913 presented a method for the quantitative extraction of carotene, xanthophyll and the two chlorophylls from plant leaves, which has served as a starting point for most subsequent methods. In this early method the fresh plant leaves were ground in a motar with sand while being covered with 40 per cent acetone. The pigments were completely extracted with pure acetone and recovered from this extract by using ethyl ether. The ethyl ether solution containing the four pigments was divided into two equal parts, one of which was used

to determine the chlorophylls, while the other was used to determine the carotenoids. In order to obtain a solution containing only the yellow pigments the chlorophylls were saponified, which was accomplished by adding a concentrated methyl alcohol solution of KOH followed by strong agitation. The saponified materials and other impurities were removed by washing the ether solution with water containing a small amount of methyl alcoholic KOH and later with water in a seperatory funnel. The ether was then reduced in volume and the carotenoids taken up in petroleum ether from which the xanthophyll was removed by washing with 85, 90 and 92 per cent methyl alcohol, respectively.

Schertz (99) critically examined the Willstatter and Stoll method (132) and suggested several important changes. In this modification a little dry sodium carbonate, to neutralize the plant acidity, was added to the green leaves which were then thoroughly ground and extracted with acetone. All of the caretenoids were later extracted from the plant tissue by using ethyl ether. The combined etheral-acetone extract was then put into a seperatory funnel and washed with a one per cent sodium carbonate solution to remove flavones and anthocyanins. In order to saponify the chlorophylls a solution of methyl alcohol saturated with KOH was added to the ether solution and the solution allowed to stand in an ice box over night or it was thoroughly shaken for 15 minutes. After removing the saponified materials the "run-off" was re-extracted with ethyl ether to prevent the loss of yellow pigments. The remaining part of the procedure was similar to that of the Willstatter and Stoll method (132).

Sprague and Shive (111) were interested in determining the carotenoids

of corn leaves and found it convenient to extract the dry plant tissue directly with petroleum ether instead of ethyl ether as used by Schertz (99), thus simplifying the procedure without impairing its accuracy.

Miller (64) presented evidence to show that the Willstatter and Stoll procedure (132) was quantitative only when modified to make for more complete extraction. He extracted the ground plant tissue with acetone, then with ethyl ether to remove most of the color, but for complete extraction he used ethyl ether in a continuous Soxhlet extractor.

Deleano and Dick (16) presented one of the first methods for extracting the carotenoids without completely extracting the chlorophylls. In this method the ground leaves were extracted with absolute alcohol until colorless extracts were obtained, and then the carotenoids were completely removed from the alcohol solution by several extractions with petroleum ether in a seperatory funnel. The combined extracts were then treated with KOH in methyl alcohol (approximately a 20 per cent solution) and allowed to stand for complete separation, after which the petroleum ether layer was washed with water to remove the alkali and pigment impurities.

In the methods considered thus far, all pigments have been extracted from the plant tissue before the chlorophylls, and other saponifiable materials, were saponified. Guilbert (33) modified the Schertz method (99) by accomplishing the saponification before extracting the carotenoids. This method could not be used for a quantitative determination of chlorophylls. However, it is very applicable where it is desired to determine only carotene. About 20 ml. of freshly prepared ethyl alcohol

saturated with KOH was added to a finely ground sample (one to five grams) and refluxed for 30 minutes on a steam bath or hot plate. Extraction of the carotenoids was accomplished by using ethyl ether. The yellow pigments were later transferred to petroleum ether from which solution the xanthophyll was removed by washing with 85 and 90 per cent methyl alcohol.

Ijdo (47) was the first to modify the Guilbert method (33), and did this by using less alcoholic KOH and extracting the saponified material directly with petroleum ether instead of ethyl ether. It was claimed that by reducing the amount of alkali added the alcohol concentration was reduced to about 90 per cent, by the moisture present in the plants, at which concentration the carotene was more easily extracted. Less xanthophyll was also removed by the petroleum ether, thus saving in extraction time as well as the time required to remove the xanthophyll impurity from the carotene extract.

Peterson, Hughes and Freeman (91) modified the Guilbert method (33) by extracting the saponified material directly with petroleum ether instead of ethyl ether. They also found Skelly-solve, a commercial grade of petroleum ether, just as satisfactory. This method is similar to the earlier modification of Guilbert's method except that Peterson, Hughes and Freeman did not use less alkali in the saponification procedure as described in Ijdo's modification (47). This method is also similar to that of Peterson and Hughes as given in the Munsey report (76), except that the latter method recommends the use of a commercial grade of petroleum ether.

Fraps and Kemmerer (26) also modified the Guilbert method by using aldehyde free ethyl alcohol saturated with KOH for the saponification procedure. The remainder of the method was similar to that of Peterson and Hughes (139).

Very recently Brooke, Tyler and Baker (12) modified the Peterson and Hughes method, which seems more adaptable to routine analysis, by using a convenient shaker for the seperatory funnels. They used fewer extractions, a smaller amount of petroleum ether for each extraction, with longer shaking periods, and thus it was not necessary to reduce the petroleum ether extract in volume during the procedure, since the final volume did not exceed 100 ml. In this procedure also, most of the alkali was removed by the methyl alcohol washings (instead of water) while removing the xanthophyll.

Russell, Taylor and Chichester (98) claimed a recovery of 95 to 97 per cent of added carotene in a method in which the green plant material was ground with sand, under acetone, and then allowed to stand for 48 hours in a solution of acetone. The pigments were completely extracted from the triturated plant tissue by using more acetone, and the carotenoids recovered from the combined acetone extracts by using petroleum ether in a seperatory funnel. The petroleum ether solution of carotene was purified by washing with 89 per cent methyl alcohol, to remove the xanthophyll, then adding a 25 per cent solution of KOH in absolute methyl alcohol, and washing with water to remove the saponified chlorophyll as well as the alkali.

Wiseman, Kane and Cary (134) found that grinding hay samples in a ball mill at room temperature, over night resulted in losses of carotene, sometimes as high as 47 per cent. This loss was prevented by suspending the hay in absolute alcohol during the grinding. Wiseman and Kane (133) combined this procedure with a method for preventing losses from samples while being carried from the field to the laboratory. This latter procedure could be added to any method and deserves special mention here. The fresh

cut plant is immediately immersed into absolute alcohol, which has been cooled to -70° C. by using solid carbon dioxide. This frozen sample may be carried to the laboratory and analyzed immediately or held in an ice box over night.

Many other methods have been described and used by various workers. Holmes and Leicester (45) used normal NaOH to saponify the saponifiable materials in fresh plant tissue at room temperature and then extracted the tissue directly with chloroform. Murri (79) suggested a simplification of most methods, such as the modification of the Willstatter and Stoll method (224), by omitting the stages of saponification of the saponifiable materials in the ether extract and also the distribution of the carotenoids between two immiscible solvents, such as using methyl alcohol to remove the xanthophyll from the final carotene extract.

Purification of the carotenoid extracts

In the method of Willstatter and Stoll (132) the petroleum ether solution was washed with separate and successive portions of 85, 90 and 92 per cent methyl alcohol until all of the xanthophyll had been removed, thus leaving a relatively pure carotene extract. This procedure has been followed by many analysts. In the Guilbert method (33) 85 and 90 per cent methyl alcohol was used and thus the subsequent modifications also prescribe this procedure. At higher concentration of methyl alcohol some of the carotene may be removed and lost in the washings.

Russell, Taylor and Chicester (98) claim that methyl alcohol of greater strength than 89 per cent will cause emulsions as well as extract some of the carotene from the petroleum ether solution. Moon (65) studied

the various concentrations of methyl alcohol used in seperating the xanthophyll from the carotene solution of petroleum ether and stated that 90 to 92 per cent gave the most satisfactory results.

Another procedure used for purifying the carotene extract is based on the Tswett principle (122) of chromatographic adsorption. In early work by Tswett it was found that by using a column of thoroughly dried, finely precipitated calcium carbonate, as well as certain other compounds, adsorption of the carotenoids into different bands would result when a solution containing several of these pigments was drawn through the column. Strain (116) has found that metalic oxidies were the best adsorbing agents of the many different kinds tried, and that a purified magnesium oxide proved to be an exceptionally good and especially applicable to carotenoid separation.

It should also be pointed out that certain precautions must be taken if the chromotographic adsorption procedure is followed. Gillam and co-workers (29) (30) claimed that by repeated chromatographic adsorption on aluminum they were able to convert pure beta-carotene into a new pigment, pseudo-alpha-carotene (void of vitamin A activity). Pure alpha-carotene was also converted to a new neo-carotene (void of vitamin A activity). Later work by Holmes and Corbet (44) indicates that certain catalysts which are undoubtedly responsible for this conversion may be present in the solution before adsorption or in the material used in the adsorption column. The use of nitrogen during the adsorption procedure prevented the oxidation of carotene.

At the present time the adsorption column serves an important place in detecting pigment impurities in the final petroleum ether solution of

carotene. Kane and associates (49) were unable to determine definitely the amount of carotene present in alfalfa silage because of certain pigment impurities resembling those described by Kuhn as being formed by the action of acids on xanthophyll. In two other reports from the same laboratory by Shinn and co-workers (105) and Wiseman and colleagues (136), it was demonstrated that these pigment impurities may account for a relatively high percentage of the apparent carotene value where the regular petroleum ether methyl alcohol phase separation method was used. The impurities were least in fresh green plants and leaf meal, as well as hays with high carotene content, while with materials such as the acid silages these pigment impurities accounted for as much as 30 per cent of the carotene value.

By using the adsorption column, Quackenbush, Steenbock and Peterson (94) were able to isolate five new pigments from acidified alfalfa silage, that were not present in the alfalfa plant. Three of the pigments occupied a position in the column between beta-carotene and xanthophyll and were found to possess no vitamin A activity. They have shown that at least two of these new pigments, which make up the largest proportion of the five demonstrated, were formed by the action of acid on leaf-xanthophyll. These two pigments have solubilities similar to that of beta-carotene and undoubtedly account for the apparently high carotene values reported by various workers (39) (57) (58) (90) for acidified silages.

Very recently Hegsted, Porter and Peterson (40) made an addition to the Peterson, Hughes and Freeman method (91) by using a diacetone solution, consisting of 100 parts of diacetone and 6 parts of water, on the final

petrolic carotene extract of the older method. By subjecting the final extract from both methods, to chromatographic analysis it was found that the new method was more specific for carotene. Errors for the old method amounted to 20 to 35 per cent for A.I.V. silage and 10 to 20 per cent for molasses silage, while error for the new procedure amounted to six per cent for A.I.V. silage and three per cent for molasses silage. These workers also found that the use of a diacetone solution on carotene extracts from hays, green material and corn silage did not lower the apparent carotene content, so that its use probably does not greatly increase the accuracy of results for these feeds.

Determinations of carotene concentration in the final extract

Willstatter and Stoll (132) prepared a petroleum ether solution of pure carotene and compared this in a colorimeter with the yellow color of the final extract of unknown carotene content and from the comparative value calculated the carotene content for the orignal plant leaves. These workers noted that the petroleum ether solution of carotene gradually lost its yellow color upon standing and thus substituted more stable color standards. Alizarin or potassium dichromate solutions of known strength were used whose comparative carotene value had been determined with a fresh preparation of the less stable petroleum ether solution of pure carotene. Many workers (16) (24) (33) (110) have found it convenient to use this method. Palmer (85) has constructed a curve showing the quantitative relation between a 0.2 per cent potassium dichromate solution and 5 x 10^{-5} molar solution of carotin. From this curve the carotene value for an unknown solution may be calculated. Ferguson (24) has worked out a curve showing the micrograms of

carotene in an unknown solution corresponding to the readings obtained from the colorimeter using a specific concentration of potassium dichromate. He worked out a similar curve corresponding to the yellow color units observed on the Lovibond Tintometer. Fraps (from Munsey 76) has shown a convenient talbe for calculating carotene when using a 0.1 per cent potassium dichromate solution in a colorimeter which has been a standardized with a 0.001 per cent solution of pure carotene.

Fraps (25) has stated that the colorimeter offers a convenient and rapid method for routine work, especially when more expensive equipment is not available. He further stated that reading the color values by means of a photoelectric colorimeter with a proper filter is just as rapid and a little more accurate. Moore (166) has used the Evelyn micro photoelectric colorimeter with a filter that transmits maximum light at a wave length of 440 millimicrons and has found this instrument satisfactory for carotene determinations when properly calibrated. The spectrophotometer should be more accurate than the colorimeter especially when other coloring materials are present besides carotene. This instrument was used by Wiseman and coworkers (136) for determining the amount of pigmented impurities in their final petroleum ether extract from hays and silages. They found this instrument very accurate and satisfactory.

Stability of Carotene in Solvents

The most common solvent used at the present time for extracting carotene from plant tissue is petroleum ether and thus the stability of carotene in this solvent should be considered briefly. Willstatter and Stoll (132) have found that pure carotene dissolved in petroleum ether lost some of its yellow color when allowed to stand at room temperature for

several days, and also that the loss of color was greatly reduced when the solution was stored in an ice box. They likewise found that carotene was more stable in petroleum ether than ethyl ether. Schertz (99) has stated that pure carotene was unstable when dissolved in ethyl ether but apparently was perfectly stable in alcohol or petroleum ether when kept in a cool dark place. He thus suggested that no appreciable loss of carotene would result from storing the petroleum ether extract of carotene in an ice box for one or two days, but very noticeable losses resulted when the extract was allowed to remain exposed to air and sunlight at room temperature.

Baumann and Steenbock (4) studied the stability of carotene in 24 different organic solvents which are frequently used to dissolve lipids. The carotene was dissolved in these solvents and held at 4° C. for five months during which time occasional tests were conducted to determine the amount of carotene that was lost due to storage under these conditions. In benzene the loss was 23 per cent and in petroleum ether 46 per cent while in ethyl ether, chloroform, cyclohexane, acetone, carbon disulfide, toluene, and pyridine more than 75 per cent of the pigment was destroyed during the five month storage period. More than 80 per cent of the added carotene was lost when hydroquinone was added to solutions of carbon disulfide, benzene, pyridine, toluene, and cyclohexane, while in petroleum ether with hydroquinone, 35 per cent was lost and with acetone, chloroform and ethyl ether with hydroquinone, 60 per cent or more of the added carotene was destroyed when stored for five months at 4° C. These workers pointed out that the stability of carotene in organic solvents showed no obvious relationship to the structure of the solvent molecule, and also that the carotene was more stable in methyl and ethyl alcohol than in the other solvents mentioned

above, while at the same time carotene was less soluble in methyl and ethyl alcohol.

There seems to be a difference of opinion as to the stability of carotene in some of the edible oils. Scheunert and Schieblich (100) stored a 0.1 per cent solution of international standard carotene in oxygen free sesame oil for 18 months in a cool dark place without loss of vitamin A activity. Baumann and Steenbock (4) stored carotene in sesame oil, not free of oxygen for three months, and reported a loss of 15 per cent at 4° C. and 81 per cent at room temperature. Turner (123) reported complete destruction of carotene when he stored a 0.2 per cent solution in ethyl laurate at room temperature for 30 days or at 100° for 12 days; however, by adding hydroquinone to a similar sample and storing at 100° for six months only 25 per cent of the carotene was lost. Baumann and Steenbock (4) suggest that cottonseed oil has an antioxident in it to prevent oxidation of carotene, since they were able to store a sample of carotene in cottonseed oil at 4° C. for two months with a loss of only 10 per cent, while the addition of hydroquinone did not prevent this natural loss. They found refined cottonseed oil outstanding among the common edible oils as a stabilizing solvent for carotene.

McDonald (63) studied the stability of carotene in three highly purified esters (ethyl butyrate, ethyl laurate, and ethyl palmitate) one liver oil (medicinal cod liver oil) and three refined vegetable oils (maize oil, peanut oil, and Wesson oil). Carotene in the three esters as well as in peanut oil stored in partially filled, tightly stoppered brown bottles at 37°, 24°, and 5° C. or in evacuated tubes at 37° C. was destroyed very

rapidly. The loss of carotene in cod liver oil under similar conditions was also rapid at 37° and 24°, but it was considerably retarded at 5° or in the absence of oxygen at 37° C. Solutions in maize oil and Wesson oil in stoppered bottles at 37° and 24° were unstable, while at 5° they kept well for four weeks and showed a small loss in eight weeks. In evacuated sealed tubes at 37° these later solutions showed a loss of 15 per cent of the carotene in eight weeks.

Baumann and Steenbock (4) reported that ethyl laurate and diethyl sebacate when fortified with hydroquinone were equal to highly refined cottonseed oil as a stable solvent for carotene, however, when unfortified they were very poor. Carotene dissolved in olive oil lost 28 per cent when stored for two months at 4° C. while the addition of hydroquinone reduced the loss under the same conditions to 20 per cent. Hydroquinone had no effect upon the natural loss of carotene from corn oil since a sample stored for two months at 4° C. with the antioxident lost 23 per cent while without the antioxident the loss was 20 per cent. Under similar conditions hydroquinone reduced the loss of carotene from cocoanut oil from 24 per cent to 10 per cent.

Hydroquinone is a powerful antioxident. As noted above it is helpful in preventing the loss of carotene from some of the common edible oils, while in others it serves no beneficial purpose. Holmes (43) was granted a patent for the use of phospholipins, such as soybean lecithin, caphalin, cuorin, sphingomyelein, etc. for stabalizing materials such as halibut liver oil, carotene in oils and irradiated ergosterol.

Stability of Carotene Solutions in Mixed Feeds

It is sometimes desirable to increase the vitamin A activity of mixed

feeds by adding carotene concentrates. Fraps and Kemmerer (26) have shown that when various fish oils were added to mixed feeds, from 79 to 100 per cent of the vitamin A disappeared after four weeks when stored at either 7° C. or 28° C. The use of hydroquinone delayed the loss in the first week or two but the loss at the end of five weeks was practically the same as it if had not been used.

Fraps and Kemmerer (26) also studied the stability of carotene when added in oil to mixed feeds. They used two samples of carotene. Sample one was a commercial preparation of carotene in oil used by a manufacturer of commercial feeds. Sample two consisted of pure crystalline carotene dissolved in refined cottonseed oil. Both of these preparations were mixed with several feeds at the desired concentration. The feeds used were white cornmeal alone, white corn meal with yeast, dried skim milk and wheat shorts. The same mixtures were stored both at refrigerator and room temperature and the loss of carotene determined during the storage period. The presence of skim milk powder, wheat shorts or yeast did not increase the stability of carotene to any practical extent. The commercial preparation of carotene was slightly more stable than that dissolved in cottonseed oil which they claim was undoubtedly due to the presence of small amounts of chloroform used to dissolve the carotene before adding it to the cottonseed oil. When these mixed feeds were stored at 7° C. there was a loss of two to three per cent carotene in four weeks of storage, three to six per cent in eight weeks, and five to 14 per cent in 12 weeks. At 28° there was a loss of seven to 27 per cent carotene in four weeks of storage, 12 to 53 per cent in eight weeks, 17 to 67 per cent in 12 weeks, and 24 to 70 per cent in 16 weeks. They concluded that if carotene in oil is added to mixed feeds to supply vitamin A potency, there should be a liberal allowance for losses during storage.

Stability of Carotene in Green Herbages

Effect of enzymes

Hauge and Aitkenhead (38) claimed that enzymes present in the alfalfa plant are the important factors responsible for the loss of vitamin A content in the curing process. Douglass and co-workers (18) in discussing the causes of the loss of vitamin A from alfalfa indicated that bacteria, molds and enzymes or several of these factors acting together may play an important part. These workers did not lose sight of the fact that ultra violet rays, and oxygen or ozone may also be involved when hay is cured under adverse field conditions. Guilbert (34) in commenting on this subject, stated that carotene is known to be readily oxidized, and at least since the time of Capranica (1877) has been recognized as a light, sensitive compound. He adds that these facts would appear to cast some doubt upon the conclusions of Hauge and Aitkenhead (38) which minimized the importance of sunlight and oxidation. The process is probably one of oxidation regardless of conditions.

In order to substantiate the theory of enzymatic destruction of carotene in alfalfa, Hauge (37) conducted a more detailed study. He used alfalfa (10 - 12 inches) of high vitamin A potency. The alfalfa was cut early in the morning and taken immediately to the laboratory, where it was separated into special treatments. Sample one was autoclaved in the presence of live steam, at 17 pounds pressure, for one hour and then dried on screens by direct exposure to the sun's rays. Sample two was sterilized as in sample one, cooled, placed in a closed jar to retain moisture, and then incubated at 37° for 24 hours, after which it was dried on screens by exposure to the sun. Sample three was sterilized, cooled and 100 ml. of fresh potato juice,

which contains oxidative enzymes, was then added to 500 grams of the alfalfa. This was thoroughly mixed, enclosed in jars and held at 37° for 24 hours, after which it was dried on screens in the sun. Sample four was frozen at about -25° C. to rupture the cellular structure of the plant tissue and liberate the enzyme. It was then defrosted and held at 4° C. for 24 hours, after which it was sterilized and dried. Sample five was treated similarly to sample four except the holding temperature was increased to 37° C. Samples six, seven and eight were placed in jars without preliminary treatment and held at 4°, 25°, and 37° C., respectively for 24 hours after which they were sterilized and dried. All the samples were thoroughly dried, ground in a Wiley mill and stored in glass jars for biological assays. His results were given on a chart in terms of Sherman-Munsell vitamin A and were rearranged in tabular form as shown below.

Table 1. Loss of Vitamin A Value From Alfalfa Due to Enzyme Activity.

Sample Number	Treatment	Vitamin A Units per gram of Alfalfa	Vitamin A Lost in Per cent
		Air-dry basis	
1	Autoclaved - as check	160	
2	Autoclaved and incubated	150	6.3
3	Same as 2 - with added enzymes	70	56.3
4	Frozen, enzymes activity at 4° C.	115	28.1
5	Frozen, enzymes activity at 37° C.	20	87.5
6	Enzyme activity at 4° C.	120	25.0
7	Enzyme activity at 25° C.	80	50.0
8	Enzyme activity at 37° C.	30	81.3

Bolin (11) studied the stability of carotene in green grasses and green alfalfa stored at 5° F. for 10 months. All the samples used in his study were collected during the four weeks in July, and included alfalfa, Kentucky blue grass, orchard grass, brome grass, and meadow fescue. All

the green plants were cut at two weeks growth and then at weekly intervals for three successive weeks. The cut samples were packed in dry ice, sent to the laboratory, one-half used for immediate analysis and the other half packed in mason jars, put into shipping containers (to prevent action of light) and then stored at 5° F. for 10 months. His results demonstrated that the alfalfa lost a large percentage of its carotene while the grasses lost little or no carotene when stored in the fresh state under these conditions. This fact seemed to indicate that a carotene destroying factor active at 5° F. was present in alfalfa and absent from the grasses studied. The stage of maturity seemed to affect the loss of carotene under these conditions. The alfalfa when cut at two weeks growth, contained 21.7 mg. of carotene per 100 gms. of dry matter when stored at 5° C. and only 8.01 mg. after 10 months. The alfalfa cut at three weeks growth contained 28.3 mg. as stored and only 7.51 mg. after storage, while at four weeks growth their values were 25.0 mg. and 8.0 mg. and at five weeks 15.1 mgs. and 10 mgs., respectively. The average percentage loss for all these samples was 62.7 for the 10 months of storage. He further noted that a sample of artificially dried alfalfa stored under the same conditions lost only one per cent of its original corotene.

Moore (67) studied the incurred losses of carotene from green plants by exposing finely ground plant tissue (ground through a food chopper) to room temperature for two hours. Under these conditions fresh alfalfa lost 35 per cent of its original carotene on the wet basis, while fresh Alsike clover lost 32 per cent, fresh sweet clover lost 46 per cent, and fresh timothy lost only 26 per cent of its carotene. In another study Moore (67) found that maturity was a factor in the loss of carotene from alfalfa when

the fresh plant was ground and incubated at 37° C. for four hours. His results are given in the following table:

Table 2. Effect of Stage of Maturity on the Stability of Carotene in Fresh Green Alfalfa When Incubated For Four Hours at 37° C.

Heights in inches	Carotene Content Fresh Gammas/Gm on Dry Basis	Carotene Content After Incubation Gammas/Gm on Dry Basis	Per Cent Loss
3	372	200	46
11	518	270	48
14	383	180	53
22	379	172	55
24	357	147	59

Effect of field drying

Green grasses and legumes are usually high in carotene content when cut for hay, however, after field curing there is considerable loss. Bethke and Kick (8) reported that exposing alfalfa to sun, rain, or dew over a period of several days resulted in a marked loss of vitamin A. Hauge and Aitkenhead (38) compared field drying with artificial drying and noted that field curing was much more destructive to vitamin A than the artificial drying. Smith and Briggs (107) observed that alfalfa leaves allowed to lie exposed in the field while drying lost considerably more vitamin A than a sample cured in a dark well ventilated room. A loss of 20 to 30 per cent resulted by exposing the cut hay for 2-3/4 hours in mid-day, while no greater loss was noted when exposed for 6-3/4 hours after 2 P. M. A 75 per cent loss of vitamin A was found when the hay was allowed to lie exposed in the field over night, 24 hours from the time of cutting the loss amounted to 84 per cent. Alfalfa severely bleached as a result

of one week exposure to sun and rain retained only four per cent of that present in the sample cured in the dark.

Douglass, Tobiska and Vail (18) presented data to show the marked effect of sun and rain on the vitamin A potency of alfalfa hay. Vitamin A was lost soon after cutting and this loss was greatly increased under rainy weather conditions. Samples cured in diffused light retained more vitamin A than the field cured hay under good drying conditions. The following table gives a summary of their results:

Table 3. Loss of Vitamin A From Alfalfa Hay When Cured in Sun, Rain, and Diffused Light (Units of Vitamin A per gram).

	Curing Method			
	Diffused light	Sun shine	1 inch rain	2 inch rain
Early bloom first cutting	60	36	16	9.5
Early bloom second cutting	32	18	20	13.0
Early bloom third cutting	64	28	12.5	12.5
Early bloom third cutting (in cock)	-	28	-	22.0
Early bud third cutting	24	42	32	23.8

These workers also noted that under rainy conditions less vitamin A was lost when the hay was cocked rather than when left in the swath.

Russell, Taylor and Chichester (97) reported that in the field-curing process there is a progressive loss of carotene, especially during the hours of day light. During the first 24 hours over 80 per cent of the original carotene content of alfalfa hay was lost. The degree of loss depended upon the length and conditions of exposure in the field. Guilbert (66) stated that the exposure of hays to sunlight and air caused a rapid decrease in carotene. He added that a well field-cured product may closely approximate dehydrated meal in vitamin A activity.

Dexter and Moore (17) studied the losses of carotene from alfalfa during the field drying process. Second cutting alfalfa hay was cured in the swath, windrow, and in cocks and the carotene content determined immediately and at intervals while curing. The following table gives their results:

Table 4. Effect of Curing Alfalfa Hay on the Carotene Content.

Hours after cutting	Swath gammas/gr dry basis	Windrow gammas/gr dry basis	Cock gammas/gr dry basis
0	270	270	270
2	225	225	
5	210	210	
10	160	175	
19	152	180	
25	147	165	165
29	125	175	
72	85	135	120
120	65	110	120

Krauss and Washburn (59) also found high losses of carotene due to field curing. Second cutting alfalfa, cured in the windrow during excellent drying weather lost 55.8 per cent of its original carotene. They reported still higher losses for first cutting alfalfa cured in the windrow and cock for four days when accompanied by a shower on the second day. In this latter case the windrow cured hay lost 87.1 per cent and the cock cured hay 83.2 per cent of the original carotene.

Bartlett and associates (3) reported that the carotene lost from grass dried in the field amounted to 79.8 per cent when the grass was dried to 16.2 per cent moisture. This drying period extended over 96 hours, during which time there were 30 hours of sunshine. There was a progressive

loss of carotene during the curing process and it is interesting to note that one hour after cutting the grass had lost 12 per cent of its carotene.

Fagan and Ashton (23) observed that herbages allowed to wilt under practical field conditions lost from 10 to 27 per cent of the carotene after 24 hours, and 24 to 31 per cent after 48 hours. They also demonstrated that partial field drying or wilting for prolonged periods may destory as much as 66 per cent of the carotene. Rain accentuates this loss of carotene still further.

Effect of artificial drying

Russell (96) observed that alfalfa leaves dried by artificial heat, by the Mason process, contained at least seven times as much vitamin A as the leaves from hay cured in the field under adverse conditions. Hauge and Aitkenhead (38) also noted that artificial drying preserved the vitamin A activity much better than did the field drying. Scheunert and Schieblich (101) detected little or no loss of vitamin A in artificial drying of alfalfa, while the air dried alfalfa lost more than two-thirds of its vitamin A activity. Russell, Taylor and Chichester (97) stated that machine dried alfalfa had two to ten times more vitamin A value than field cured hay. They were unable to notice any loss due to machine drying.

Hilton, Hauge and Wilbur (42) did not detect any significant losses of vitamin A due to artificial drying, however, they did observe marked differences between artificially dried and field cured samples of alfalfa and soybeans when cut at different stages of maturity. Young alfalfa cut at an early stage (10 to 12 inches high) and cured in the field contained 80 per cent less vitamin A than did the corresponding sample artificially

dried. For alfalfa cured in the bloom stage this difference was 71.4 per cent. Young soybeans (12 to 15 inches high) cured in the field gave a hay product that contained only 33.3 per cent less vitamin A than the sample artificially dried. For soybeans cured at a more mature stage (green bean in pods) this difference was 73.3 per cent.

Watson (130) stated that the carotene content of fresh grass is not materially affected by artificially drying, however, some loss was noted. His data indicates an inverse ratio of drying temperatures to the loss of carotene in artificial drying. This is due to the fact that at the low temperature a longer drying period is required. Guilbert (34) reported a loss of 7.4 per cent of the carotene due to mechanical dehydration of alfalfa. There was an additional loss of 11.4 per cent of carotene before the alfalfa was delivered to the dehydrator, thus a total of 18 per cent of the original carotene was lost in the operation. Fagan and Ashton (23) pointed out that drying grasses in two types of mechanical driers decreased the carotene widely, the average loss being 33 per cent. They likewise noted high losses of carotene during wilting in the field and before drying in the mechanical driers.

Effect of drying under laboratory conditions

Vail, Tobiska and Douglass (124) studied the variation in vitamin A value for alfalfa hays cured under different laboratory conditions. The check samples were slowly dried in diffused light. After completely cured all the samples were subjected to rat assays and the results recorded in terms of vitamin A units per gram . The following table gives their results.

Table 5. Vitamin A Value for Alfalfa Hay Cured in the Laboratory.

Cutting	Treatment	Units of Vitamin A per gram	Difference in per cent
First	Diffused light	56	check
"	Sun lamp	28	-50
"	Oven dried	80	+46
Second	Diffused light	60	check
"	Sun lamp	48	-20
"	Oven dried	107	+78
"	Crushed and air dried	87	+45
Third	Diffused light	64	check
"	Sun lamp	44	-31
"	Oven dried	127	+98
"	Crushed and air dried	120	+87.5

Carotene in plants seems to be fairly stable to heat when the tissue is dried under reduced pressure. Guilbert (34) reported that little or no loss of carotene could be detected when fresh green alfalfa was dried in Vacco for three hours at 100° C. Similar results were reported by De (15).

Effect of autoclaving

Hauge and Atkenhead (38) assumed that no loss of caortene (vitamin A potency) resulted from autoclaving for one hour at 17 pounds pressure. Guilbert (34) stated that presumably no loss of carotene would occur in an autoclave from which the air could be rapidly evacuated, however, he used an autoclave in which evacuation required 15 to 20 minutes and thus noted a loss of 33 to 67.5 per cent of the carotene from four samples of alfalfa. Moore (67) noted a small loss of carotene when alfalfa was autoclaved when 17 pounds of pressure was reached within a few minutes.

Stability of Carotene in Dried Hays

Fraps and Treichler (27) found that alfalfa leaf meal stored at room temperature lost 20 per cent of its carotene in 8 months and 50 per cent in 11 months. Taylor (118) reported that machine dried alfalfa lost 50 to 67 per cent of its carotene during storage in a barn for seven months. The greatest loss occurred during the first three months.

Smith (106) pointed out that the loss of carotene from bailed alfalfa varied directly with the storage time and temperature. During the first three months of storage when the outside temperatures were 83° to 101° F. the destruction of vitamin A amounted to 50 per cent. No further destruction occurred during the next three winter months. With the rise in temperature in the spring the rate of destruction was increased again so that after 12 months of storage the bailed hay contained only 25 per cent of its original carotene.

Kane, Wiseman and Cary (48) found that the rate of carotene loss was similar in alfalfa, timothy and clover hays when stored under comparable conditions. They also indicated a close association between stability of carotene in these hays and the storage temperature. All their hay samples were stored in a dark unheated barn. When the outside temperature was 7.2° C. or less an average of three per cent of the carotene was lost per month. With the outside temperature between 7.2 and 18.9° C. the loss per month was increased to 6.5 per cent, and as the temperature increased the destruction of carotene increased. Similar results were reported by Wiseman and co-workers (135). These later workers stated that during the first summer after cutting the loss of carotene was 21.2 per cent while during the second summer the destruction took place at the rate of 11.6 per cent per month. It was also noted that the percentage loss of

carotene under their storage condition was much greater than the percentage loss of natural green color.

Fraps and Kemmerer (26) pointed out that alfalfa kept at a temperature of 35° C. lost carotene rapidly at first, and then quite slowly, indicating that some of the carotene may be easily destroyed, while a portion may be so protected that it is much less easily destroyed.

Woodman and Shepherd (137) studied the losses of carotene from chopped and unchopped alfalfa hay. Two lots of hay were stored in June. One-half of the first lot was finely chopped into 1/4 inch lengths while the other half was stored as long hay. One-half of the second lot was coarsely chopped into 3/4 inch lengths while the other half was stored as long hay. At the time of storage the first lot contained 27 per cent moisture while the second lot contained 25 per cent and thus all the hays heated rather extensively. Lot one contained 46 parts per million of carotene when stored and three months later the long hay contained 2.9 parts per million, while the finely chopped hay was reduced to 0.9 parts per million. Lot two contained 74 parts per million of carotene when stored and when removed three months later, the long hay retained 4.9 and the coarsely chopped hay 3.8 parts per million of carotene. All the hays in the experiment lost large amounts of carotene as well as green color due to the extensive heat development during storage.

This work was continued by Shepherd and Woodman (102). First cutting alfalfa hay was used and when stored it contained only 15.8 per cent moisture. One part of the hay was chopped into 3/4 inch lengths while the other part was stored as long hay. When stored the carotene

content was 76 parts per million and after five months storage this value dropped to 20.7 parts per million for the chopped hay and to 31.9 parts per million for the long hay. The green color was destroyed at a lower rate than the carotene.

Taylor and Russell (120) reported that chopped, artificially dried alfalfa stored at barn temperature lost 50 per cent of its carotene during three months of storage in late summer and early fall. No further loss occurred during the winter but an additional 25 per cent carotene was destroyed during the following summer. They claimed that both exclusion of air and low storage temperature are important factors in carotene preservation. They were able to store finely ground artificially dried alfalfa at approximately 0° C. for 20 months with little or no loss of carotene.

Vail and associates (124) found that chopped alfalfa hay stored in air tight tin containers, at moderate temperature in a dark room, lost 28 per cent of its vitamin A potency after 14 months, 31 per cent after two years, and 68 per cent after three years storage. They also pointed out that ground hay stored in cloth sacks lost 40 per cent of its carotene in nine months while another sample stored in a paper sack lost only 25 per cent during the same time.

Wilder and Bethke (131) studied the amount and rate of carotene loss from dehydrated alfalfa leaf meal when stored in paper sacks and gunny sacks at different temperatures. Portions of the meal were stored in a room at outside temperature, in a heated room, and in a refrigerator. Carotene analyses were made on all samples at monthly intervals for a

period of one year. Results showed a progressive loss of carotene in all samples with a close correlation between storage temperature and carotene destruction. Losses ranged from 27 per cent for the samples stored in the refrigerator to 80 per cent for the samples stored in the heated room. The type of container had no effect either on the rate or amount of destruction of carotene in the samples.

Bethke and Wilder (9) added alfalfa leaf meal and cod liver oil to two vitamin A deficient mixed feeds to study their stability. The first mixed feed consisted of white corn, wheat middlings, casein, irradiated yeast and minerals. The second ration was similar to the first except that meat scraps and dried skim milk were substituted for the casein. These feeds were stored at room temperature and fed to rats and chicks after three and six months of storage. The results showed that there was a similar loss of vitamin A from cod liver oil and carotene from alfalfa leaf meal; however, the loss was much greater in the meat scraps-dried milk ration than in the casein ration. The loss of vitamin A or carotene was not complete even after six months storage.

Fraps and Kemmerer (26) diluted alfalfa meal with starch at the rate of one to nine and studied the loss of carotene. When stored at room temperature for four months the alfalfa meal itself lost 51.5 per cent of its carotene while the diluted sample lost 59 per cent. At 6° C. the alfalfa meal lost 36.4 per cent and the diluted sample 44.4 per cent of the carotene.

Stability of Carotene in Silages

Virtanen (125) stated that when cattle roughages were preserved by the A.I.V. method the carotene content remained unchanged after many months, or if anything a slight increase was noted. Richardson (95) pointed out that on the dry basis, alfalfa preserved by the A.I.V. method had practically the same carotene value as either fresh alfalfa or machine dried alfalfa.

Peterson and co-workers (90) reported an increase in carotene content for both A.I.V. alfalfa and soybean silage as compared with the fresh plant material. Peterson and associates (89) noted that acid treated silage was higher than the silage without acid, however, both silages were higher in carotene than the plant from which the silage was made. Alfalfa, when put in the silo, contained 90 micrograms of carotene, and after 6-1/2 months of storage the apparent value was 156 micrograms. Peterson and colleagues (89) (90) were of the opinion that these apparent increases in carotene were probably due to the action of acid on the plant material to increase the extractability of the carotene.

Krauss and Washburn (58) found that alfalfa in the field contained 180 gammas of carotene per gram (dry); after cutting and hauling to the silo and then chopping, this value dropped to 130 gammas (dry). Eleven determinations were made as the acidified silage was fed. Values for the silage from the upper part of the silo approximated that of the field sample, while the values for silage from near the bottom were much higher, which resulted in an average value of 219 gammas of carotene per gram of the dry acidified silage. Hay from the same field, exposed to rain, con-

tained only 22 gammas per gram (dry).

Hathaway, Davis and Brauer (35) used the rat assay technique to study the vitamin A value of A.I.V., molasses, and ordinary silage. The A.I.V. silage contained only slightly more vitamin A than did the molasses silage. The untreated silage was decidedly inferior to either A.I.V. or molasses silage in vitamin A value.

Taylor (119) reported that the carotene content of grass silage was about twice that of moisture-free alfalfa hay which had been stored for the same length of time. His results indicated that the carotene content of silage was a good index to the vitamin A potency regardless of the chemical changes that occurred to give an apparent increase in carotene during the ensiling process.

Krauss and co-workers (57) used the temporary silo, of the paper-lined snow-fence type, to study the different methods of ensiling second cutting alfalfa. The untreated silage contained 153.2 parts per million of carotene when put into the silo and 86.0 parts per million as taken out. The molasses silage contained 144.3 parts per million when ensiled and 236.8 parts per million as taken out, while the A.I.V. silage contained 144.6 parts per million when put in and 71.2 parts per million of carotene as fed from the silo.

Hayden and colleagues (39) believed that acid preserved carotene and thus favored the A.I.V. method of preserving legumes in the silo. A summary of three years work is given in the following table.

Table 6. Carotene Preservation in Acid Silage

	Gammas of Carotene per Gram of Air-Dry Alfalfa		
	1934-35	1935-36	Summer of 1936
Green crop in field		188.5	186.0
Crop as sampled at silo	105	130.5	
Upper half of silage as fed	39.5	176.9	262.6
Lower half of silage as fed	35.5	269.4	267.6
Average pH of silage	4.35	3.65	3.78

Churchill and Horwood (14) claimed that the degree of acidity apparently had little or no influence upon the preservation of either protein or carotene when alfalfa was preserved by the addition of molasses. They indicated that fineness of grinding, proper moisture, and tight packing in an air tight silo are important factors.

Shepherd and Woodward (103) filled a 14' x 17' silo with partially cured first cutting alfalfa and produced a good quality silage without adding water or any other material. The alfalfa contained 56 per cent dry matter when put in the silo; it was finely cut and weighted down thus preventing spoilage. The pH of the silage ranged from 4.61 to 4.78. The carotene content of the alfalfa when stored was 63.6 parts per million and when removed from the silo it still retained 47.75 parts per million on a dry weight basis.

Richardson (95) converted green corn into A.I.V. silage and observed no appreciable loss of carotene. Regular corn silage did not retain quite as much carotene but the other constituents were about equal to those of A.I.V. corn silage and thus there seemed no advantage in preserving corn by this process. In order to have a good high quality inexpensive alfalfa silage he suggested using 50 pounds of molasses per ton of alfalfa and storing in towers, pits or stacks. Bender and co-workers (7) reported

that two per cent molasses added to green grasses and legumes of high moisture preserved the carotene very effeciently.

Watson (130) pointed out that carotene was well preserved in low-temperature silage, with or without added sugar, and to a marked extent in the A.I.V. fodder. He stated that the addition of acid to the fodder mass reduced fermentation to a very low level and thus the development of high temperatures was prevented. Page and Watson (83) stated that one of the advantages of ensiling grasses was the retention of carotene in the finished material. This was observed in low temperature silage, with or without added molasses and also in A.I.V. silage. When the temperature had gone much over 100° F., the carotene disappeared rapidly. Drew and co-workers (19) observed that carotene destruction was very excessive in a stack silo where high temperature fermentation was so great that the grass silage became dark brown in color. In a concrete silo, on the other hand, the temperature was confined to reasonable limits, due to proper moisture and sufficient compaction, which resulted in a high degree of carotene conservation.

Peterson (88) prepared pea vine silage, including five lots of stacked material and two lots preserved in silos, one with and one without mineral acid. He indicated that the carotene was well preserved in the two silos as well as one of the stacks, however, the other four lots of stacked silage were relatively low in carotene due to high temperature fermentation.

Woodward and Shepherd (138) studied several methods of making silage from grasses and legumes and,reported the carotene values for the crop before ensiling as well as the silage taken out of the silo. Their carotene values with calculated losses due to ensiling are given in the following table:

Table 7. Losses of Carotene from Herbages in the Silo, Exclusive of Spoiled Layer on Surface.

Material and Treatment	Carotene in Dry Matter: As put in: p.p.m.	Carotene in Dry Matter: As taken out: p.p.m.	Loss in per cent
Orchard grass, with a little cover and Lepesdeza, nothing added	332	268	19.3
Partially dried, nothing added		199	
6% 2-Normal acid added	332	294	12.4
Second cutting Kentucky blue grass			
Nothing added	268	245	8.6
Partially dried, nothing added	184	146	20.7
10% 2-Normal acid added	268	249	7.1
3 % molasses and 3 % water added	268	252	59.7
First cutting alfalfa - 1935			
Nothing added	164.2	175.2	-6.7
10% 2-Normal acid added	164.2	167.8	-2.2
3% molasses and 3% water added	164.2	112.0	31.8
First cutting alfalfa - 1936			
Nothing added	174.2	207.7	-19.2
9% 2-Normal acid added	174.2	218.2	-25.2
3% molasses and 3% water added	174.2	152.9	12.2
Partially dried, nothing added	100.9	49.5	50.9
Partially dried and 14% 2-Normal acid	100.9	63.5	37.1
Partially dried and 3% molasses and 3% water added	100.9	50.5	50.0
Soybeans, cut into different lengths			
1/4 inch lengths, nothing added	100.9	67.0	33.6
3/4 " " " "	110.1	82.9	24.7
1/4 " " partially dried, nothing added	45.8	20.2	55.8
1/4 inch lengths, 3% molasses and 3% water added	91.7	62.4	32.0

Recently, Taylor and Russell (120) reported that different silages varied widely in the rate at which carotene was lost during storage. They noted that fresh alfalfa contained 0.0176 per cent carotene (dry basis) while the A.I.V. alfalfa silage after three months contained 0.0218 per cent carotene and after five months 0.0203 per cent. Fresh corn as put in the silo contained 0.0050 per cent carotene while regular corn silage contained 0.0060 per cent and the A.I.V. corn silage 0.0070 per cent. After five months storage the A.I.V. corn silage lost approximately 20 per cent of its original carotene while the A.I.V. alfalfa silage lost practically

none of its carotene in the same length of time.

Variations in Carotene Content of Plants During Growth

Early development in seedlings

Euler and Hellstrom (21) studied the formation of carotene xanthophyll and chlorophyll in barley seedlings. The seeds were germinated in the dark for nine days and then exposed to light for five days. Determinations were made daily for various pigments beginning on the sixth day. The xanthophyll content increased regularly from the sixth to the ninth day, but thereafter showed a decrease when the plants remained in the dark, however, when the plants were brought to the light after the ninth day a further regular increase was noted. Carotene and chlorophyll were not found in measurable quantities before the plants were exposed to light. During the exposure period the increase in carotene closely paralleled the increase in chlorophyll, however, both of these pigments increased more rapidly than xanthophyll. Norris (81) found that young etiolated sunflower seedlings were more suitable for studying pigment development than other seedlings. He irradiated sunflower seedlings and found that the carotene content was lowered during the first hour of irridiation and that the original carotene was not regained until four to five hours after irradiation. There was a very rapid rise during prolonged irridation. By controlling oxygen tension he showed that carotene developed independent of xanthophyll.

Beck (5) studied the development of plant pigments in sunflower seedlings grown in the dark. Both carotene and xanthophyll increased with

the age of the plant, although the rate of development decreased with time. In a later report Beck (6) stated that etiolated plants must be irradiated before chlorophyll can be formed, thus he suggested that carotene and xanthophyll may be precursors of chlorophyll. He found more carotene in seedlings from young seeds than from older ones.

Variations during the growing season

Murneek (77) (78) studied the carotene and xanthophyll content of plants when the length of daylight exposure was varied. He selected a short photo-period of seven to eight hours and a long period of 14 to 15 hours. He found both carotene and xanthophyll in greater concentrations in the short day plants. Barnes (2) reported that length of day did not affect the carotene content of carrots, however, the shape, size, as well as rate of growth were affected. He stated that temperature was an important factor for carotene development and that maximum carotene was developed at 15 to 21° C. Smith (109) observed that greenhouse grown tomatoes contained less carotene than the same variety grown outdoors. She indicated that maximum light favored maximum production of carotene in the tomatoes.

Virtanen and co-workers (128) stated that the total quantity of carotene of a plant increased rapidly up to the time of blooming and then diminished continuously until the ripening of the fruit. In a later report Virtanen (126) stated that carotene and vitamin C attained a maximum value just before or at the beginning of flowering, and both may be increased by proper and adequate fertilization or they may be decreased by the same factors that retard growth.

Hauge (36) assumed no loss of vitamin A activity due to artificial drying of alfalfa and thus reported that young alfalfa, 10 to 12 inches high, contained almost twice as much vitamin A as did the alfalfa in full bloom. Hilton, Hauge and Wilbur (42) made the same assumption and thus found that young alfalfa, 10 to 12 inches high, contained 90 units of vitamin A while in the bloom stage it contained only 70 units. Under similar conditions they found that soybeans, 12 to 15 inches high, contained 54 units of vitamin A while the more mature plant as cut for hay contained only 30 units.

Myburgh (80) reported the carotene content of veld grass (grown in South Africa) when cut in the spring, summer, autumn and winter. The analyses were made on the fresh material and his results are given below in terms of milligrams of carotene per 100 grams of dry grass.

Table 8. Carotene Content of Veld Grass.

Season of cutting	Carotene content mg/100 gm.
Cut in spring	0.11 - 0.61
" " summer	1.85 - 2.64
" " autumn	0.98 - 4.66
" " winter	0.23 - 0.26

Smith and Stanely (108) determined the vitamin A value of the blue grama range grass at different stages of growth. In Arizona growth of this grass is stimulated by the summer rains in late July, thus it grows rapidly and matures quickly, and by the middle of October begins to dry up, except for a few green basal leaves. The plant lies in the dormant stage during the winter. When sampled August second the grass was a

potent source of vitamin A, by September 20 it was only one-half as rich, and when sampled in November, 100 times as much of the grass was required to produce the same rate of gain in rats as that cut in August.

Atkeson and colleagues (1) observed that pasture plants were relatively high in carotene content during the early summer, but with wide variations. Carotene decreased during the hot mid-summer months and after the fall rains most plants re-established their carotene content similar to early summer values, although big blue stem and Buffalo grass were notable exceptions. Their results in the following table represent carotene determinations made on grasses taken from pure stands when six to fifteen inches high.

Table 9. Carotene Content of Some Pasture Plants at Different Periods Throughout the Growing Season.

Plant studied	Date Sample Taken	Carotene Mg/100 gms. Dry basis	Plant studied	Date Sample Taken	Carotene Mg/100 gms. Dry basis
Rye	5-22	25.1	Red top	6-10	11.3
Rye	11-28	30.6	" "	6-22	6.8
Big blue stem	5-23	19.2	" "	6-26	5.4
" " "	6-21	22.1	" "	11-6	24.1
" " "	7-8	8.4	Alfalfa	6-22	29.2
" " "	7-21	8.0	"	7-6	22.2
" " "	11-4	0.4	"	7-22	8.3
Canadian Brome	5-23	53.3	"	11-6	14.3
" "	7-11	7.7	Dakota Brome	6-23	17.8
Kentucky blue grass	5-28	10.2	" "	7-23	6.8
" " "	6-26	6.8	" "	11-4	33.4
" " "	7-9	8.0	Local Brome	6-25	15.6
" " "	7-20	4.1	" "	7-11	8.5
" " "	11-4	21.4	" "	11-13	16.7
Little blue stem	5-28	12.2	Orchard grass	6-25	15.2
" " "	6-28	15.6	" "	7-9	12.2
" " "	7-8	8.1	" "	7-22	7.5
" " "	7-21	4.3	" "	11-13	15.4
Buffalo grass	6-10	28.2	Wheat	11-28	17.9
" "	6-23	15.9	Barley	11-28	37.6
" "	7-6	6.0			
" "	7-20	4.8			
" "	11-6	0.9			

Esselen and co-workers (20) studied the effect of maturity of the maize plant on its vitamin A content. Their results showed a definite increase in carotene as the maize plant reaches full growth, followed by a decrease as the plant matures. The values they reported are given here:

Table 10. Effect of Maturity of Maize Plants on Vitamin A Content.

Variety	Vitamin A Units per Gm Dry Matter		
	25 cm. High	Full grown	Mature
Canada flint	55.7	89.4	33.9
Lanc. sure crop	50.0	75.8	34.6
Bantam evergreen	44.0	90.0	9.2

Effect of Soil Fertility on Carotene Content of Plants

It was shown by Virtanen and Hansen (127) that peas grown under sterile conditions, at pH 6.5 and using potassium nitrate to supply nitrogen, and those grown under natural conditions contained equal amounts of carotene, which was 0.13 milligrams per gram of dry matter. They also observed that the source of nitrogen is of some influence, since somewhat lower values were obtained when sulfate of ammonia was used instead of potassium nitrate. It was concluded that plants synthesize carotene without the assistance of soil bacteria. Virtanen and associates (128) found that a relatively larger amount of carotene was present in plants grown at the optimum pH (which for peas and wheat was assumed to be 6:5) than when they were grown at a higher or lower acidity. They noted the greatest carotene content in plants when the nitrogen supply was sufficient to promote maximum growth. In fact, they postulated as a general principle that maximum growth and maximum carotene content go hand in hand, and thus they regarded carotene as an essential growth factor in plants.

Ijdo (46) grew spinach on analyzed soil and quartz sand with solutions of known composition to study the effect of fertilizers on the carotene content. He observed that carotene increased with an increase of nitrates in the soil to a point where nitrogen toxicity appeared in the plants. Deficiencies of nitrogen caused a much greater drop in carotene content than deficiences of magnesium, potassium or calcium. In another report Ijdo (47) pointed out that potassium salts, in excess, caused a marked decrease in carotene and an increase in ascorbic acid. A relationship between carotene and chlorophyll was suggested since fertilizers that increased carotene, also increase chlorophyll.

Virtanen (126) pointed out that factors such as low pH, excessive concentration of phosphates, potassium or sodium chlorides, which retard growth in plants also lowered the carotene and vitamin C content. It was observed by Ott (82) that carotene and vitamin C in carrots increased to an optimum with potassium fertilizers, however, excessive amounts of potassium reacted adversely on carotene and vitamin C. Potassium sulphate gave more desirable results than potassium chloride. The formation of carotene was influenced much more by the type of fertilizer than was the formation of vitamin C.

Pfutzer and Pfaff (93) studied the carotene and vitamin C content of a variety of vegetables as influenced by fertilizers. They found that full fertilization gave an increase in carotene content and an increase in plant yield, however, vitamin C remained fairly constant. Additional neon illumination in the greenhouse increased the carotene and vitamin C content as well as plant yield for parsley, chives, and spinach. Pfaff and Pfutzer

(92) conducted a more complete study and found that well nourished plants contained more carotene, chlorophyll and ascorbic acid than under nourished plants. The amount of carotene and chlorophyll were greatly increased when nitrogen and magnesium were supplied on nitrogen and magnesium poor soils, respectively. High lime fertilization of spinach, resulted in decreases of both carotene and chlorophyll which could be offset by the addition of magnesium. It was also noted that nitrogen did not have any influence upon the ratio of carotene to chlorophyll.

Thomas and Moon (121) applied different fertilizers at monthly intervals, to pasture grass, and noted the carotene content and yield of carotene per acre. Sulphate of ammonia produced marked increases in carotene content and in total yield of carotene per acre. Sulphate of iron had no effect upon the carotene content or yield of carotene, but seriously reduced the yields of dry matter. Carbonate of lime did not produce any marked effect upon carotene or dry matter. There was a marked and fairly constant increase in carotene under all conditions throughout their experimental period which extended from June 10 to September 2.

Discussion of Review of Literature

The literature reviewed showed that beta-carotene is the chief carotene component of the plants commonly used as roughage for the bovine. A definite chemical and biological relation has been established between carotene and vitamin A so that a quantitative determination of beta-carotene serves as a good index for the vitamin A value of green herbages.

Of the various methods considered in this review for the evaluation of carotene in plants there are several that have special merit. The Peterson and Hughes method (76) has received considerable recognition and

is being widely used by research workers. The Ijdo method (47) was published previous to the Peterson and Hughes and appears to be somewhat shorter than the later procedure. Both methods are modifications of Guilbert's method (33). The recent modification of the Peterson and Hughes method by Brook, Tyler and Baker (12) is shorter than most methods and could probably be used very successfully in research laboratories as well as for commercial routine analysis. Most of the present day methods do not consider the destruction of carotene that may take place before the green plant is analyzed. By freezing the tissue, as suggested by Wiseman and Kane (133), the Peterson and Hughes method, or modification of their procedure, could be improved in order to prevent the loss of carotene and thus give more accurate results. Another important point to consider, in regard to the methods, is the pigment impurities in the final petrolic ether extract. The use of a diacetone solution, as suggested by Hegsted, Porter and Peterson (40), would greatly increase the accuracy for carotene values for feeds in general and especially for acidified silages.

The yellow color of the final petrolic ether extract may be read in a colorimeter by comparison against a standard dye solution, or in a photoelectric colorimeter; or amount of light absorption at various wave lengths may be measured in a spectrophotometer. The colorimeter is a convenient and rapid method for routine work, however, in order to avoid the limitations of visual determination of color, the photoelectric colorimeter, with proper filters, may be used to good advantage. When considering cost and accuracy, it seems that the photoelectric colorimeter is one of the best and most applicable instruments for evaluating carotene in the final extract. The spectrophotometer is more accurate, especially when other coloring

materials besides carotene are present, however, it is very expensive and thus its use is limited to a small number of laboratories.

Carotene is more stable in petroleum ether than in ethyl ether or many other fat solvents. This observation has induced the more extensive use of petroleum ether for extracting carotene from plant tissue. Petroleum ether solutions of carotene lose color when exposed to light and room temperature and thus the final carotene extract from plant tissue should be read immediately after completing the purification procedure or this solution should be stored in a cool dark place until read.

Cottonseed oil seems outstanding among the common edible oils as a stabilizing solvent for carotene probably because of the presence of an antioxident. Most other edible oils must be fortified with an antioxident in order to prevent the natural loss of carotene. Hydroquinone is a powerful antioxident and has been used rather extensively to prevent this natural loss of carotene, however, other antioxidents have been suggested.

Large losses of carotene are usually observed when green plants are cut for hay and allowed to dry by exposure to sunlight and air. Under unfavorable weather conditions, such as rain and dew, this natural loss of carotene is greatly accentuated. When hay is dried at high temperatures in mechanical driers this natural loss is greatly reduced. If green plants are autoclaved and then allowed to dry in direct sunlight and air this loss of carotene is minimized. These facts indicate that some heat liable factor present in the plant may be due in part to the high losses of carotene during the normal curing of hay. Direct evidence has been presented to show that oxidative enzymes may be the primary factor affecting this instability of carotene in green plants. This point is extremely interesting and has been studied in the experimental work for this thesis.

Hay crops stored for a year or more usually have a low carotene content. The rate of carotene destruction varies directly with the storage temperature. Finely ground or chopped hay as well as high-moisture hay loses more carotene than a good quality long hay. By diluting finely ground leaf meal with mixed feeds the normal loss of carotene from the meal is somewhat increased.

Carotene seems to be well preserved when green grasses and legumes are ensiled, as a matter of fact acidified silages many times appear to have a higher carotene content than the plant from which the silage was made. This higher value is due largely to the action of acid upon leaf-xanthophyll which produces pigments that have very nearly the same solubility as does carotene, and thus these pigment impurities are read as part of the carotene color in the final petroleum ether extract.

The total quantity of carotene in a plant increases rapidly up to the time of blooming, or shortly before blooming, and then diminishes continuously as the plant matures. Factors that affect plant growth also affect the carotene content of the plant. In other words, rapidly growing plants have a higher carotene content than slowly growing plants. Plant growth is increased by proper fertilization and favorable climatic conditions whereas improper fertilization and drought decrease plant growth. These factors affect the carotene content in the same manner as they affect plant growth.

OBJECT

The object of this experimental work was to study the change of carotene content of several herbages during their growing season. In addition, the fresh plant tissue was incubated, after various laboratory treatments, in order to observe the stability of carotene as affected by the possible presence of oxidizing enzymes.

EXPERIMENTAL PROCEDURE

Herbages Studied

The herbages used in this investigation included the following: alfalfa, a mixture of Grimm and Harrison varieties; Canadian brome grass; corn leaves, from Michigan hybrid corn 561; oats, Iagold variety; soybeans, Manchu variety; sudan grass; and biennual white sweet clover. These herbages were grown on soil of average fertility maintained by the Michigan State College, except the sweet clover which gree voluntarily on a vacant lot near the College. The alfalfa and brome grass were taken from a field with a good stand of an alfalfa-brome mixture. The sudan grass was taken from a pasture in which a small plot was set aside for this purpose. The soybeans were obtained from an experimental plot maintained by the Farm Crops Department. The oats and corn leaves were taken from fields of these crops which were to be harvested when mature. The voluntary growth of sweet clover used in this study was about equal to that of a normal sweet clover pasture. These sources of herbages were selected since they represented the best available supply and also because

they were not being pastured or harvested during their early growing season.

Sampling of Herbages

Samples for carotene analysis and subsequent laboratory treatment were collected once a week from the start of the growing season until the crops were harvested or cut for hay. Plants of normal heights were selected at random and their average maximum height ascertained. The appearance of blossoms or development of seeds was noted at the time of sampling, so that the stage of maturity could be determined from these observations. The plants were cut about an inch above the soil on which they were grown and then wrapped in heavy paper and immediately taken to the laboratory.

Carotene Analysis

The method used for extracting carotene from the plant tissue was based on the Guilbert method (33) as modified by Peterson, Hughes and Freeman (91). The ethyl alcohol used in this procedure was made aldehyde free by adding eight to ten grams of aluminum filings to a liter of 95 per cent ethyl alcohol and distilling. The proper amount of alcohol was then saturated with KOH for use in the following procedure.

In the laboratory, a representative portion of the original plants was ground through a food chopper and thoroughly mixed in order to obtain a representative sample for analysis. A two to five gram sample was quickly weighed out and immediately covered with alcoholic KOH in a mortar. The sample was placed in the alcoholic KOH as soon as possible to prevent enzymatic or oxidative destruction of carotene. A small amount of fine

clear quartz sand was added and the tissue thoroughly ground with a pestle, in order to destroy the cellular structure of the plants. The finely ground sample was then transferred to a 250 ml. Erlenmeyer flask by means of a rubber policeman, using sufficient alcoholic KOH to make the transfer complete. Approximately 40 to 50 ml. of the alkali solution were added to the tissue depending upon the size of the sample used. The chlorophylls and other saponifiable materials, were saponified by attaching the Erlenmeyer flask to a reflux condenser and slowly boiling the sample on a water bath for 30 minutes. The flask was occasionally rotated during the heating in order to prevent the tissue from sticking to the sides, as well as to prevent it from lumping.

The saponified sample was then cooled to room temperature and about 25 ml. of petroleum ether added. This was shaken for approximately a minute and the liquid decanted into a separatory funnel. The residue in the flask was re-extracted with several 20 ml. portions of petroleum ether, and each extract decanted into the separatory funnel. At this point it was usually necessary to break up the residue in the flask by using 5 ml. of ethyl alcohol. The residue was then re-extracted with petroleum ether until colorless extracts were obtained.

Approximately 20 ml. of water were added to the combined extracts in the separatory funnel and the lower aqueous alkali layer re-extracted with petroleum ether in a second separatory funnel until colorless extracts were obtained. Emulsions were often encountered during this latter extraction procedure, largely due to excessive shaking, and were broken by adding 2 ml. of ethyl alcohol. A total of 200 to 300 ml. of petroleum ether were required to completely extract the carotenoids from the sample.

After the extraction was complete, both the residue and alkali solution were discarded. The combined petrolic ether extracts were freed of alkali by washing with water until the washings remained colorless upon the addition of phenolphthalein as an indicator.

The petroleum ether solution was drained into a flask and the volume quickly reduced to 30 to 40 ml. by suction and holding the flask in a stream of warm water. This small volume of solution was quantitatively transferred to the separatory funnel for purification. The xanthophyll was removed by first washing with several portions of 85 per cent methyl alcohol. These washings were transferred to the second separatory funnel to which was added a small volume of petroleum ether in order to recover any carotene that may have been removed by the washing. The methyl alcohol layer was then drained from the second funnel and the petroleum ether retained. This methyl alcohol washing procedure was continued by gradually and successively increasing the strength of methyl alcohol to 90 per cent until the washings were colorless. By keeping the methyl alcohol concentration below 90 per cent and allowing sufficient time for complete separation of the methyl alcohol and petroleum ether layer very little carotene was recovered in the second separatory funnel. Nevertheless this practice was followed as a precautionary measure. The petroleum ether solutions were combined; washed once more with 90 per cent methyl alcohol, transferred to a volumetric flask (usually 100 ml.) and made up to volume. A small amount of anhydrous sodium sulfate was added to remove traces of water and help clear up any turbidity that may have developed. This petroleum ether solution of carotene was stored in a refrigerator until it was perfectly clear before the carotene

concentration was determined. One to two hours was usually sufficient, although this solution was often held for two or three days until more samples were extracted.

The concentration of this carotene extract was determined by using the Evelyn micro-photoelectric colorimeter as discussed by Moore (66). He calibrated the instrument by preparing carotene solutions of varying concentrations in petroleum ether and recording the galvanometer readings for the particular concentration and depth used. From this data he prepared calibration curves on one cycle semi-logarithmic paper. Tables were set up from these curves which gave the carotene value for the unknown solution in terms of the galvanometer readings. This table was used to calculate the carotene content of the original sample, after taking into consideration dilution and size of sample used.

Moisture Determination

Moisture in all samples was determined at the same time that the carotene analysis was made in order to calculate the results in terms of micrograms of carotene per gram of dry matter. For the moisture determination, five grams of the macerated tissue were weighed out and dried for three or four hours in a constant temperature drying oven, at 100° C. The loss in weight was considered as the moisture content of the plant tissue.

Incubation of Samples

A portion of the sample as collected from the field was cut into one-half inch lengths by using a sharp knife. This tissue was thoroughly mixed and a representative sample loosely packed in a 400 ml. beaker,

covered with a watch glass, and incubated at 37° C. for 44 hours. After the incubation period, the sample was analyzed for carotene and moisture, and the amount of carotene calculated on the dry weight basis. This value was compared with the carotene content of the fresh sample in order to calculate the loss due to incubation under these conditions. The incubated samples could not always be analyzed immediately when taken from the incubator and in this case were stored at -15 to -20° C. until the following day. This incubation procedure was followed during the entire course of the study.

In order to study the effect of maceration of the plant tissue upon the loss of carotene during incubation, a portion of the macerated tissue as ground through the food chopper for carotene analysis was loosely packed in a 400 ml. beaker, covered with a watch glass and incubated at 37° C. A few preliminary results indicated that a four hour incubation period was sufficient and convenient to detect high losses of carotene. These samples were analyzed for carotene and moisture immediately or held at -15 to -20° C. until the following day. The carotene was again calculated on the dry weight basis and the loss determined by comparing the results with those for the fresh sample. This procedure was begun in early July and continued during the remainder of the study.

Autoclaving

The following procedure was carried out in order to study the effect of autoclaving as well as subsequent incubation upon the stability of carotene in plants. Samples for autoclaving were collected at the same time that the regular samples were collected. These samples were placed in

an autoclave and covered with a metal pan turned upside down, in order to prevent the plant tissue from becoming excessively moist due to water dripping from the sides of the autoclave. About five minutes were required to get the pressure up to 15 pounds. This pressure was maintained for one hour, after which the plants were ground through a food chopper. About 100 grams of this macerated tissue were put into a beaker and incubated at 37° C. for four hours similar to that of the regular incubation procedure. Another portion of the macerated tissue was used for immediate analysis following the same procedure as outlined for the determination of carotene of fresh plants. This autoclaving and subsequent incubation procedure was used as a check on the results reported in the literature and was conducted on only a limited number of samples because of the time involved in making the extra carotene analysis.

Incubation In An Atmosphere of Carbon Dioxide

In order to study the loss of carotene due to incubating the macerated plant tissue in the absence of oxygen, an apparatus was set up by which incubation could be carried out in an atmosphere of carbon dioxide. This apparatus was constructed in the following manner. A desiccator with a stop cock and glass tube attachment was placed in the incubator. Heavy walled rubber tubing was fastened to the glass tube and extended to the outside of the incubator where it was connected to a glass T connection. From this connection one piece of rubber tubing was extended to a column of mercury while another part led to a three-way stop cock. Connections were made from this latter three way stop cock to a Cenco-hyvac pump and also a carbon dioxide generator. Another three way stop cock was inserted between the

last three way connection and the hyvac pump. Precautions were taken to make the system air tight.

The samples used for this part of the study were obtained from a portion of the sample as collected from the field for the fresh analysis. These plants were ground through a food chopper and a portion of the macerated tissue was loosely packed in a 400 ml. beaker and set in the desiccator. The top of the desiccator was securely fastened and then the system evacuated to 29 inches of mercury and filled with carbon dioxide. This operation was repeated three times in order to insure more complete removal of the air from the apparatus. After the system was filled with carbon dioxide the third time at atmospheric pressure, the stop cock on the desiccator was closed. This entire procedure required about one-half hour and thus the samples were held for an additional three and one-half hours at 37° C. in order to be comparable to the regular macerated and incubated sample.

Carotene and moisture analyses were made on the samples as soon as removed from the incubator, and the results calculated on the dry basis. These carotene values were then compared with the results for the fresh sample as well as the results for the samples macerated and incubated during the regular procedure. The stability of carotene as affected by incubation in an atmosphere of carbon dioxide, was determined from these comparisons.

Buffer Curves for Alfalfa

Buffer curves had to be prepared during this study, in order to determine the amount of certain acids to add to a sample of macerated plant

tissue to get a desired pH. This information was needed since certain acids were to be added to macerated samples in order to observe the effect of pH as well as different acids upon the stability of carotene in alfalfa. Four acids were used in this study which included lactic, hydrochloric, sulfuric and phosphoric. The normality of each acid was determined by titrating with 0.098 normal NaOH using phenolphthalein as the indicator. The milli-equivalents of NaOH required to titrate one milliliter of acid was taken as the normality of the acid.

In order to determine the buffer curves on the acid side, these acids were added in increasing amounts to a series of separate 50-gram portions of macerated alfalfa. No carotene values were determined on these first samples, although they were placed in the incubator at 37° C. for four hours in order to be comparable to the later samples.

After the incubation period, the macerated tissue was pressed in a hydraulic press and the expressed sap used for pH determination. The pH was determined by using a Beckman glass electrode pH meter. The pH values were then plotted against the amount of acid added and the resulting curves used to determine the amount of acid to be added to a 50 gram sample of alfalfa in order to get the desired pH in the subsequent procedure.

Addition of Acids to Macerated Alfalfa During Incubation

It was desired to incubate macerated samples of alfalfa at pH 4.0, 3.5, 3.0 and 2.0 using different acids to obtain these pH values. The acids used were 0.95 normal hydrochloric, 1.05 normal sulfuric, 2.84 normal phosphoric and 9.44 normal lactic. The amount of each acid required to get the desired pH for that particular sample was determined by referring to

the buffer curve for that respective acid. Four beakers for each acid, or a total of 16 beakers were arranged with the proper amount of acid added and the total volume of liquid adjusted to 20 ml.

For this study a large sample of fresh alfalfa was macerated and 50 gram portions added to each of the above beakers. The samples were thoroughly mixed with the acid by using a heavy stirring rod and then incubated at 37° C. for four hours. They were stirred at hourly intervals during the incubation procedure in order to assure more complete mixing of the acid with the macerated tissue. After incubation the samples were stored at -15 to -20° C. for a day or two until the analyses for carotene and moisture could be made. Carotene and moisture determinations were made on each of the samples following the regular procedure and the remaining portion of the sample was used to determine pH. The carotene values were calculated on the dry weight basis and compared with the results for the fresh samples and also the results for a macerated and incubated sample without the addition of acid.

Addition of Potato Juice to Carotene in Solution

Potato juice is known to contain oxidizing enzymes (37) therefore in studying the effect of oxidizing enzymes upon the stability of carotene in solution, the potato juice may serve as a source of these enzymes. It was desired to prepare a solution of carotene that was miscible with potato juice in order to carry out this experiment. By adding carotene dissolved in oil to a gum solution and then emulsifying the mixture, a solution of carotene was prepared that was miscible with potato juice. Gum tragacanth was found superior in this respect to gum arabic, gelatin or saponin.

The carotene solution was prepared by dissolving four grams of gum tragacanth in about 900 ml. of water, recently distilled and boiled to remove the oxygen. Twenty milliliters of cottonseed oil containing approximately 200 micrograms of carotene per milliliter were added and the entire mixture thoroughly shaken. The solution was made up to a liter and then forced through a hand emulsifier at least five times. In this condition the oil remained well dispersed for two weeks, although the experimental work was carried out the same day the solution was prepared.

The potato juice was obtained by cutting potatoes in small pieces and pressing the tissue in a hydraulic press. The juice was freshly prepared the same day it was used. This juice was then added, in amounts varying from 0.1 to 5 ml., to 10 ml. of the carotene emulsion in 50 ml. test tubes, and the total volume adjusted to 15 ml. This mixture was then incubated at 37° C. to observe the effect of the added potato juice on the stability of carotene. Five milliliters of a saturated solution of KOH, and 10 ml. of ethyl alcohol were added to each 15 ml. sample, immediately after incubation. Each tube was then attached to an air cooled condenser and the solution slowly boiled in a water bath for about 10 minutes, after which the samples were quickly cooled by holding in cold running water.

The extraction of carotene was principally a phase separation based on a procedure used for extracting carotene from blood (66). This was accomplished by adding 10 ml. of petroleum ether, tightly corking the tubes and returning to the cold running water. The tubes were shaken vigorously for about one minute and again placed in the cold water. This shaking procedure was repeated twice more during the next 10 or 15 minutes keeping the tubes in cold water during the entire procedure. After the last shaking the tubes were nearly filled with cold tap water to facilitate the separation

of the petroleum ether layer. The tubes were then stored in a refrigerator for at least one or two hours after which the carotene concentration was determined by using the photoelectric colorimeter.

RESULTS

Carotene Content of Alfalfa During Growth

Data on the carotene content of three crops of alfalfa during the growing season of 1938 are presented in Table I. These results show that the carotene content of young alfalfa before blooming was much higher than at the stage of maturity at which it was cut for hay. There was a definite trend toward a decrease in carotene as the alfalfa matured. The first sample collected in the spring, on April 25, when the alfalfa was five inches high contained 432 micrograms of carotene per gram of dry matter and when it was cut for hay June 20 this value was only 171 micrograms. This decrease in carotene was not gradual for the first crop.

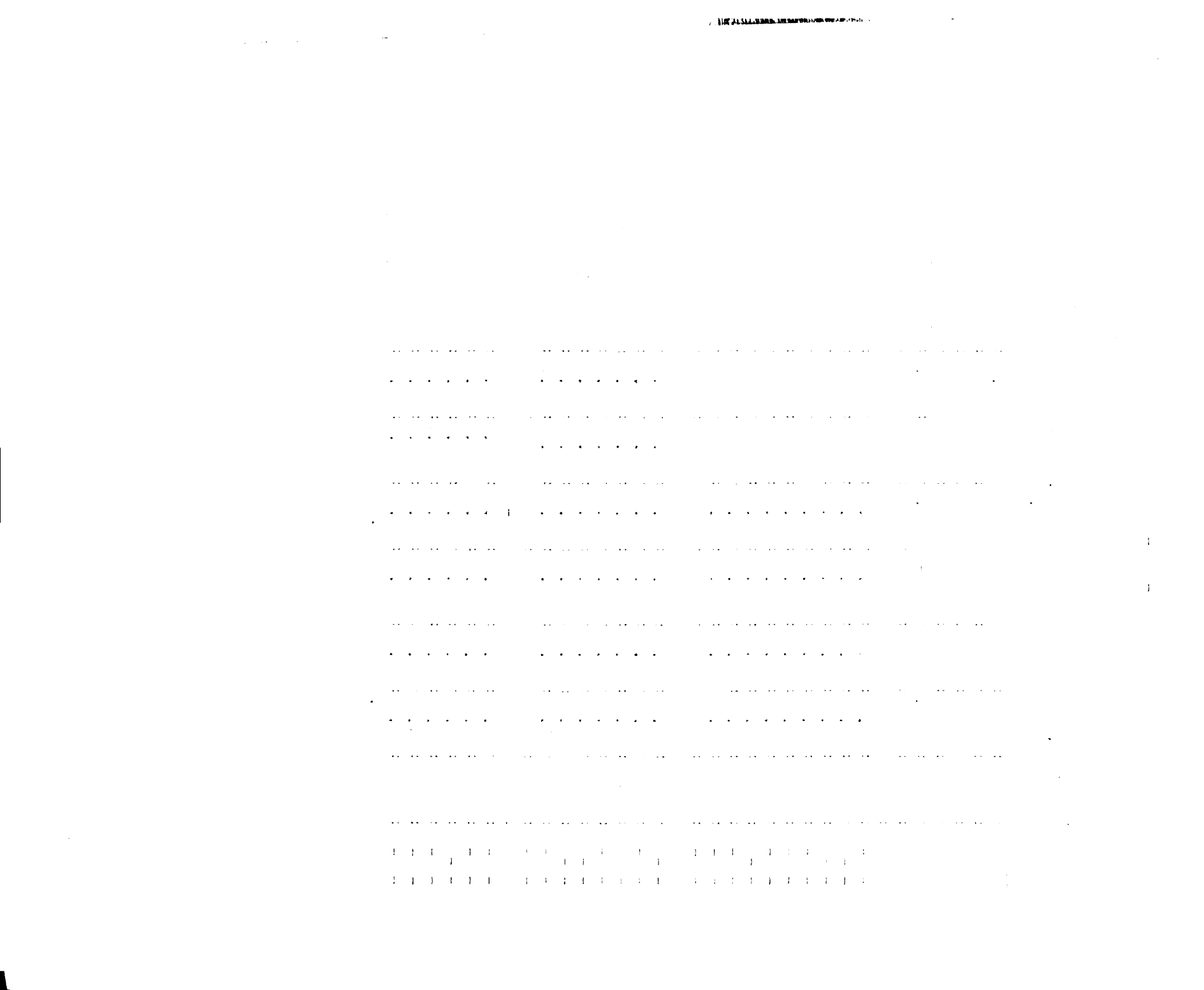
The first sample of the second growth was taken 17 days after the first crop was cut for hay and at that time the carotene content was 319 micrograms. This value dropped to 132 micrograms by August 16, when the second growth was cut for hay. The results, for the second crop show that the decrease in carotene was progressive as the plants matured.

The carotene content of the first sample of the third crop was 367 micrograms and five weeks later when in the three-fourths bloom stage this value was still 284 micrograms. The third crop showed a gradual decrease in carotene as the plants matured except for one sample taken September 21

Table I. Carotene Content of Alfalfa, in Micrograms Per Gram of Dry Matter, During the Growing Season, and Losses Subsequent to Incubation.

Date sampled	Height	Dry matter	Fresh sample	Tissue incubated at 37° C. For 44 hours Cut in 1/2" lengths		For 4 hours Macerated		Remarks
	Inches	Pct.	Micro-grams	Micro-grams	Pct. Loss	Micro-grams	Pct. loss	
				First crop				
4-25-38	5	20.8	432.0	310.0	28.2			
5-3-38	8	20.4	397.0	257.0	35.3			
5-9-38	11	22.9	269.0	203.0	24.5			
5-16-38	13	24.2	278.0	228.0	18.0			
5-23-38	18	19.0	295.0	210.0	28.8			raining
5-29-38	23	20.0	233.0	175.0	24.9			"
6-6-38	24	22.0	188.0	151.0	19.7			few blossoms
6-13-38	28	25.7	150.0	132.0	12.0			early bloom
6-20-38	31	27.1	171.0	141.0	17.5			half bloom
6-20-38								cut for hay
				Second crop				
7-7-38	11	19.4	318.7	224.6	29.5	137.6	56.8	
7-11-38	13	19.1	301.9	204.2	32.4	117.9	60.9	
7-18-38	17	26.7	272.7	166.8	38.8	111.5	59.1	few blossoms
7-25-38	20	32.3	221.5	187.5	15.4	88.5	60.0	early bloom
8-2-38	21	32.4	212.8	125.8	40.9	78.5	63.0	half bloom
8-9-38	22	32.9	169.9	117.2	31.0	65.1	61.7	2/3 bloom
8-16-38	22	31.8	116.0	116.0	12.7	62.5	53.7	3/4 bloom
8-17-38								cut for hay
				Third crop - different field				
8-23-38	15	21.1	366.7	290.3	20.8	169.8	53.7	
8-29-38	18	21.6	358.2	324.3	9.5	158.2	55.8	
9-5-38	20	24.6	340.6	171.4*	49.7*	154.0	54.8	few blossoms
9-13-38	21	28.8	311.7	176.8	43.3	198.7	36.3	early bloom
9-21-38	22	27.5	321.1	208.5	35.1	197.5	38.5	half bloom
9-26-38	22	29.9	283.6	227.9	19.6	174.2	37.9	3/4 bloom

* Incubator at 47° C. for last 18 hours.



at which time the value was slightly higher than that for the previous week.

It should be noted that the carotene content of the first crop was slightly higher than that of the second crop and also that the values for the third crop were higher than either the first or second crop. The results for the third crop may not be directly comparable to the other values since these samples were collected from a different field.

Loss of Carotene in Alfalfa Due to Incubation

The results in Table I show that when alfalfa is macerated by grinding through a food chopper and then incubated at 37° C. for four hours it loses much more carotene than when a portion of the same sample is cut in half inch lengths and incubated at 37° C. for 44 hours. It should be noted that the losses due to maceration and incubation for the second crop were higher than the same losses for the third crop,and also that the percentage loss for the third crop was less after the plants reached the early bloom stage.

When a portion of the sample was cut in half inch lengths and incubated for 44 hours the results show no similarity to those of the macerated sample and incubated for four hours since the former lost less carotene and also the values fluctuated much more.

The loss of carotene in alfalfa due to incubation for 44 hours increased in most cases, during the early growth. This was followed by a decrease as the plants reached the advanced stages of maturity. When the data are more complete, the results indicate an additional drop and rise, before the drop at the blooming stage.

Carotene Content of Brome Grass During Growth

Results obtained for the carotene content of two crops of brome grass during the growing season are presented in Table II. These results show that the carotene content decreased as the plants matured, although the decrease was not progressive.

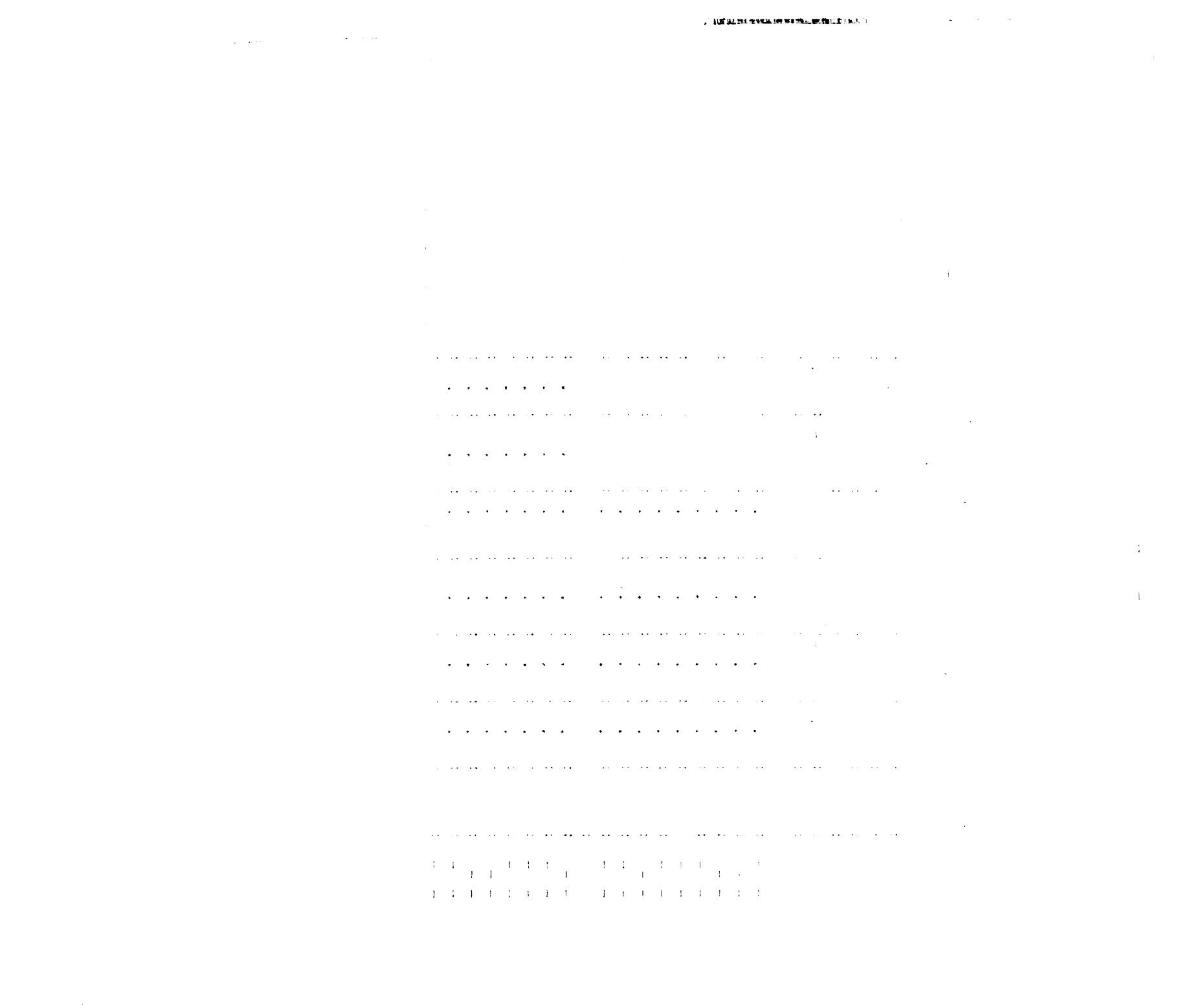
Sampling was begun April 25 at which time the brome grass contained 453 micrograms of carotene per gram of dry matter. When the grass was cut for hay June 6, the carotene content was only 141 micrograms. Fifteen days later the second growth reached a height of seven inches and the carotene content was 466 micrograms. The carotene increased to 518 micrograms by the following week, but thereafter the value decreased. When the second growth was cut for hay the carotene content was still 317 micrograms. This value was more than twice the value of the first crop when cut for hay.

Loss of Carotene in Brome Grass Due to Incubation

The results for the loss of carotene due to incubation of brome grass are given in Table II. When the plants were cut in half inch lengths and incubated at 37° C. for 44 hours the loss of carotene, in some cases, was less than two per cent while the maximum loss was about 25 per cent. The greatest loss, for both crops, was noted in samples collected at the beginning of growth. Although the subsequent samples lost less carotene, the results do not show a uniform decrease. The general trend for the percentage loss of carotene seemed to be a decrease at the beginning of growth followed by an increase and then a decrease as the brome reached the stage at which it was cut for hay.

Table II. Carotene Content of Brome Grass, in Micrograms Per Gram of Dry Matter, During the Growing Season and Losses Subsequent to Incubation.

Date Sampled	Height	Dry matter	Fresh sample	Tissue incubated at 37° C. For 44 hours Cut in 1/2" lengths		For 4 hours Macerated		Remarks
	Inches	Pct.	Micro-grams	Micro-grams	Pct. loss	Micro-grams	Pct. loss	
				First crop				
4-25-38	3	23.8	453.0	342.0	25.0			
5-2-38	5	22.8	377.0	302.0	19.9			
5-9-38	11	23.6	284.0	278.0	2.1			
5-16-38	15	22.5	311.0	298.0	4.2			
5-23-38	21	19.8	293.0	269.0	8.2			raining
5-29-38	26	17.1	306.0	301.0	1.6			"
6-6-38	34	22.0	157.0	140.0	10.8			few heads out
6-13-38	36	30.2	144.8	110.7	23.5			heads out
6-20-38	45	32.0	140.6	124.2	11.5			cut for hay
				Second crop				
7-5-38	7	15.1	466.0	348.1	25.3	289.1	38.0	
7-11-38	10	13.8	517.6	494.2	4.5	306.5	40.8	
7-18-38	15	18.0	418.5	369.2	11.7	195.1	55.7	
7-25-38	19	24.7	412.9	406.9	1.5	157.5	61.9	
8-2-38	20	25.5	390.8	292.6	25.1	148.5	62.0	
8-9-38	23	29.6	305.2	273.8	10.3	130.4	57.3	few heads
8-16-38	23	24.4	317.1	292.9	7.6	117.5	62.9	raining
8-17-38								cut for hay



By macerating the plant tissue and incubating the sample at 37° C. for four hours the loss of carotene was from 38 to 63 per cent. This loss was much higher than the loss for unmacerated tissue, when incubated for 44 hours. The maceration technique was used for the second growth only. These results show that the loss of carotene increased as the plants matured, although one sample collected when the heads were appearing, showed a slightly lower loss than the previous sample.

Carotene Content of Corn Leaves During Late Growth

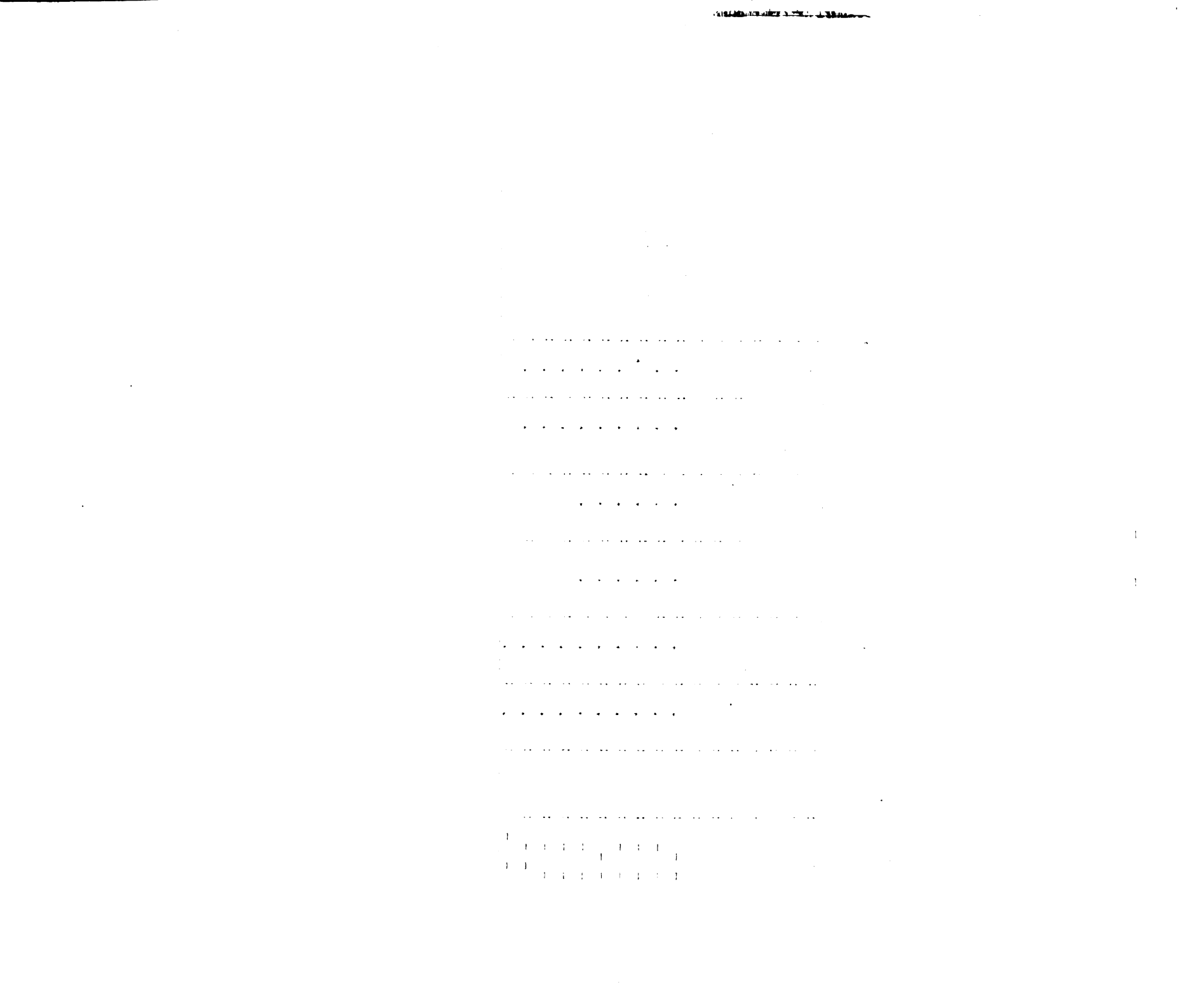
The results for the carotene content of corn leaves during the late growing season are presented in Table III. The first sample was collected as the tassels appeared on the corn, and at this time the carotene content was 482 micrograms per gram of dry matter. This value increased during the next two weeks to 646 micrograms, after which time there was a progressive decrease in carotene as the plants matured. When the corn was mature enough for husking the carotene content of the dried leaves was only 25 micrograms.

Loss of Carotene in Corn Leaves Due to Incubation

The results for the loss of carotene from corn leaves due to incubation are given in Table III. The data show that the loss of carotene was 2 to 14 per cent when the leaves were cut in half inch lengths and incubated at 37° C. for 44 hours. Much higher losses were noted when a portion of the same corn leaves was macerated by grinding through a food chopper and incubated for only four hours. This latter loss amounted to 34 to 48 per cent with the greatest loss for the earliest samples. Although the percentage loss was less after the ears reached the milk stage this was not a uniform decrease.

Table III. Carotene Content of Corn Leaves, in Micrograms Per Gram of Dry Matter, During the Late Growing Season, and Losses Subsequent to Incubation

Date sampled	Height of plant	Dry matter	Fresh sample	Tissue incubated at 37° C. For 44 hours Cut in 1/2" lengths		For 4 hours Macerated		Stage of maturity
	Inches	Pct.	Micro-grams	Micro-grams	Pct. loss	Micro-grams	Pct. loss	
8-9-38	105	25.4	481.6	413.1	14.2	260.2	46.0	tassels out
8-17-38	110	22.9	615.7	602.4	2.2	322.8	45.9	pollen ripe
8-24-38	112	27.5	646.1	590.9	8.5	339.0	47.5	early milk
8-30-38	115	25.6	561.2	490.4	12.6	298.4	46.8	early milk
9-5-38	118	29.8	539.1	515.6	4.4	326.4	39.5	late milk
9-13-38	118	24.2	516.5	480.5	7.0	331.2	35.9	dough stage
9-21-38	118	27.7	361.0			238.0	34.1	glaze stage
9-26-38	116	31.3	243.3			140.8	42.1	grain hard
10-3-38	118	59.4	68.4			42.2	38.3	corn ripe
10-11-38	118	90.0	25.4					dead ripe



Carotene Content of Oats During Growth

The results for the carotene content of the entire oat plant during the growing season of 1938 are given in Table IV. When the oats were six inches high the carotene content was 525 micrograms and when harvested this value decreased to 13 micrograms. The results show a definite downward trend in carotene content as the plants matured, although the decrease was not uniform from week to week. The most notable drop in carotene was during the early growing season until the heads appeared, during which time the original 525 microgram value was lowered to 100 micrograms.

Loss of Carotene in Oats Due to Incubation

When the oat plants were cut in half inch lengthsand incubated at 37° C. for four hours the loss of carotene varied from no detectable loss up to about 30 per cent. The greatest loss was observed in the first sample when the oats were six inches high. This loss gradually decreased to less than three per cent when the oats were 18 inches high after which an increase in the percentage loss was noted. During the time the seeds were developing, two successive samples showed no detectable loss of carotene due to the 44 hours incubation, while the following sample showed a loss of 29 per cent, when the seeds were in the late dough stage.

When a portion of the oat plants were macerated and incubated for four hours, the loss of carotene was much greater than when the unmacerated tissue was incubated for 44 hours, as shown in Table IV. The loss from the macerated tissue was about 50 to 70 per cent, with the greatest loss occurring when the grain was in the late dough stage. The maceration and incubation procedure was begun at a late date so that only four values are represented.

Table IV. Carotene Content of the Oat Plant, in Micrograms Per Gram of Dry Matter, During the Growing Season, and Losses Subsequent to Incubation

Date sampled	Height	Dry matter	Fresh sample	Tissue incubated at 37° C. For 44 hours Cut in 1/2" lengths		For 4 hours Macerated		Maturity
	Inches	Pct.	Micro-grams	Micro-grams	Pct. loss	Micro-grams	Pct. loss	
5-11-38	6	14.3	525.0	365.0	30.4			
5-18-38	8	14.9	442.9	362.2	18.2			
5-25-38	10	14.7	449.0	414.0	7.8			
6-1-38	15	14.0	375.0	365.0	2.7			
6-8-38	18	16.4	219.5	213.9	2.6			
6-15-38	20	17.5	260.0	239.0	8.1			
6-22-38	30	19.8	140.5	116.0	16.9			head in boot
6-29-38	34	24.5	100.4	87.5	12.8			head out boot
7-7-38	39	29.4	66.1	66.5		33.5	49.3	milk stage
7-11-38	39	30.7	54.3	55.7		21.7	60.0	dough stage
7-18-38	39	40.0	14.7	10.5	28.6	4.4	69.3	late dough
7-25-38	39	48.7	12.6			4.0	68.3	ripe
7-27-38								harvested

Carotene Content of the Soybean Plant During Growth

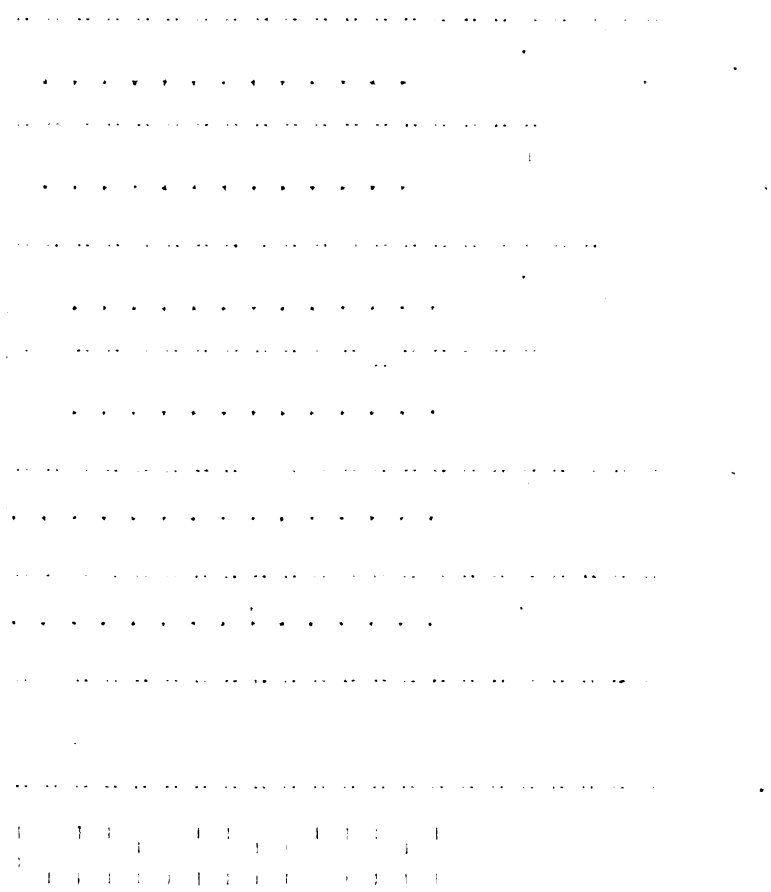
The results for the carotene content of the soybean plant during the growing season are given in Table V. The first values were obtained when the plants were nine inches high at which time the carotene content was 279 micrograms per gram of dry matter. This value gradually increased to 432 micrograms when the plants were 22 inches high and the bean pods were developing. From this stage of maturity on, there was a gradual and progressive decrease in carotene as the plants matured, so that by the time the leaves were yellow the carotene content of the plant was only 35 micrograms.

Loss of Carotene from the Soybean Plant Due to Incubation

The results for the loss of carotene from the soybean plant due to incubation are presented in Table V. These results show that the percentage loss of carotene is relatively low for both the macerated and unmacerated samples, when the plants are in the early stage of growth. The results also show that the plants during early growth lost more carotene, during the 44 hour incubation periods, than when the tissue was macerated and incubated for four hours. This condition was reversed as the plants matured. In both the long and the short incubation procedure, the loss of carotene was highest during the early development of the seeds, although as the seeds reached the more mature stage, the percentage loss was decreased. There was an increase in the loss of carotene from the macerated samples after the seeds were ripe and the leaves became yellow.

Table V. Carotene Content of the Soybean Plant, in Micrograms per Gram of Dry Matter, During the Growing Season, and Losses Subsequent to Incubation

Date sampled	Height	Dry matter	Fresh sample	Tissue incubated at 37° C. For 44 hours Cut in 1/2" lengths		For 4 hours Macerated		Remarks
	Inches	Pct.	Micro-grams	Micro-grams	Pct.	Micro-grams	Pct.	
6-29-38	9	22.5	279.0	264.5	5.2			
7-5-38	14	20.3	288.8	243.7	15.6	264.4	8.4	few blossoms
7-12-38	17	21.3	299.5	199.3	33.5	234.7	21.6	early bloom
7-19-38	21	22.5	357.8	195.9	45.3	230.6	35.6	few pods
7-26-38	22	22.6	432.2	244.3	43.5	189.0	56.3	more pods
8-2-38	27	26.4	406.6	199.3	50.9	168.3	58.6	few beans
8-9-38	30	25.3	334.7	155.3	53.6	155.4	53.6	small beans
8-16-38	34	24.5	248.7	137.1	44.9	91.6	63.2	beans developing
8-23-38	35	29.0	196.9	128.0	35.0	76.0	61.4	" "
8-29-38	36	29.5	193.6	175.8	9.2	99.1	48.8	" "
9-5-38	36	30.3	163.4	115.6	29.3	97.8	40.2	beans developed
9-13-38	36	34.5	30.5	23.8	22.0	22.6	25.9	yellow leaves
9-21-38	37	34.5	35.4	26.9	24.0	23.8	32.8	beans mature
9-26-38	36	38.4	21.0			13.1	37.6	leaves dropping
10-3-38	36	66.8	7.9					stalks dry



Carotene Content of Sudan Grass During Growth

The results for the carotene content of two crops of sudan grass during the growing season are given in Table VI. The first sample was collected July 7 when the plants were 18 inches high and at which time the carotene content was 455 micrograms per gram of dry matter. There was a progressive decrease in the carotene content during the next three weeks at which time the value was only 198 micrograms. At that time the plants had reached a height of 48 inches and were cut and removed from the plot to allow for a second growth.

The first sample of the second growth was collected when the plants were 10 inches high and the carotene content was 362 micrograms per gram of dry matter. After another week's growth this value increased to 404 micrograms, but from this point on, the results show a progressive decrease as the plants matured. When the plants were well matured and the leaves began to yellow, the carotene content was only 72 micrograms per gram of dry matter.

Loss of Carotene From Sudan Grass Due to Incubation

The results for the loss of carotene from sudan grass during the incubation procedure are shown in Table VI. In all cases the macerated tissue, when incubated for four hours, lost more carotene than did the unmacerated tissue incubated for 44 hours. For the first crop the loss of carotene from both the macerated and unmacerated samples increased as the plants matured. In this second crop, the results for the unmacerated tissue do not show any definite trend, the percentage loss from the macerated tissue increased progressively as the plants matured to the seed development stage, after which time a definite decrease in this loss was noted.

Table VI. Carotene Content of Sudan Grass, in Micrograms per Gram of Dry Matter, During the Growing Season and Loss Subsequent to Incubation.

Date sampled	Height	Dry matter	Fresh sample	Tissue incubated at 37° C. For 44 hours Cut in 1/2" lengths		For 4 hours Macerated		Remarks
	Inches	Pct.	Micro-grams	Micro-grams	Pct. loss	Micro-grams	Pct. loss	
				First crop				
7-7-38	18	15.6	454.7	360.0	20.8	269.0	40.8	
7-12-38	34	17.1	316.9	257.3	18.8	175.0	44.8	
7-19-38	43	20.4	218.5	167.8	23.2	114.3	47.7	
7-26-38	48	25.0	198.4	121.7	38.7	88.1	55.6	heading
7-29-38								grass cut
				Second crop				
8-29-38	10	20.1	362.1	245.6	14.6	212.8	41.2	
8-17-38	20	15.8	403.8	237.7	41.2	216.4	46.4	raining
8-24-38	36	17.9	317.9	231.7	27.1	157.3	50.5	heads in boot
8-30-38	43	18.4	229.5	196.7	14.3	101.5	55.8	heads out boot
9-5-38	46	24.0	179.3	140.8	21.5	71.6	60.1	seeds developed
9-13-38	47	26.7		95.6		57.5		seeds ripe
9-21-38	48	25.3	112.5	95.8	14.8	70.7	37.2	leaves yellow
9-26-38	50	36.1	71.8	57.6	19.8	43.6	39.3	grass cut

Carotene Content of Sweet Clover During Growth

Carotene analysis for sweet clover was made during the first growth only since there was no available supply thereafter. These results are presented in Table VII. The carotene content of the young plants increased progressively to a rather low maximum of 244 micrograms per gram of dry matter when the plants were 18 inches high. After that there was a decrease as the plants matured and when in full bloom the carotene content was only 125 micrograms.

Loss of Carotene in Sweet Clover Due to Incubation

None of the sweet clover was macerated and incubated for the four hour period, however, the results for the unmacerated and incubated samples are given in Table VII. The loss of carotene was relatively low, with all values except two falling below 14 per cent, when the plants were cut in half inch lengths and incubated for 44 hours. One sample at the beginning of growth lost 32.2 per cent, while another sample, taken when the sweet clover was in full bloom, lost 22.2 per cent. These were the only two high percentage loss values following the long incubation procedure. It should be noted that there was a decrease in the loss of carotene as the plants matured up to the appearance of blossoms after which there was an increase.

Table VII. Carotene Content of Sweet Clover, in Micrograms per Gram of Dry Matter, During the First Growth, and Losses Subsequent to Incubation at 37° C. for 44 hours.

Date sampled	Height	Dry Matter	Fresh sample	Incubated sample cut in 1/2" lengths		Remarks
	Inches	Pct.	Micro-grams	Micro-grams	Pct. loss	
4-27-38	7	14.6	218.0	196.0	10.1	
5-4-38	12	12.8	242.0	164.0	32.2	
5-11-38	18	16.4	244.0	211.0	13.5	
5-18-38	20	16.0	175.0	151.8	13.3	
5-25-38	24	16.0	216.0	206.0	4.6	
6-1-38	30	17.4	208.0	182.0	6.0	
6-8-38	37	19.2	177.0	172.0	2.9	few blossoms
6-15-38	42	20.0	172.0	160.0	7.0	early bloom
6-22-38	66	22.8	125.0	97.2	22.2	full bloom
6-22-38						cut

Results of Autoclaving and Subsequent Incubation

Data are presented in Table VIII to show the effect of autoclaving and subsequent incubation upon the stability of carotene in several green herbages. Less than 20 per cent of the original carotene was lost when the green herbages were autoclaved for one hour at 15 pounds pressure. When a portion of the autoclaved sample was macerated and then incubated at 37° C. for four hours only a small loss of carotene resulted. None of the autoclaved samples lost more than eight per cent of their carotene, due to this incubation. When the fresh unautoclaved samples were macerated and incubated at 37° C. for four hours they lost from 41 to 64 per cent of their carotene.

In the case of alfalfa and brome grass the unautocalved samples lost 60 to 64 per cent of their carotene when incubated while the autoclaved samples lost only two to three per cent. Somewhat similar results are shown for the soybean plant, sudan grass, and corn leaves. The sudan grass lost 41 to 56 per cent of the carotene when the fresh unautoclaved samples were incubated, while the autoclaved samples lost more than any other herbage studied, which was only 5 to 7.5 per cent.

Table VIII. Effect of Autoclaving and Subsequent Incubation Upon the Stability of Carotene

Date sampled	Micrograms of carotene per gram of dry matter							
	Fresh sample	Fresh sample macerated and incubated 37° C. for 4 hours		Autoclaved, 15 lbs. 1 hour. Immediate analysis			Macerated and incubated 37°C for 4 hours	
	Micrograms	Micrograms	Pct. loss	Micrograms	Pct. loss		Micrograms	Pct. loss
				Alfalfa				
7-25-38	222	89	59.9	197	11.3		193	2.0
8-2-38	213	79	62.9	178	16.4		174	2.3
				Brome				
7-25-38	413	158	61.7	347	16.0		338	2.6
8-2-38	362	130	64.1	321	11.3		311	3.1
				Soybean plant				
7-26-38	432	189	56.3	378	12.5		369	2.4
8-2-38	407	168	58.7	336	17.4		332	1.2
				Sudan grass				
7-26-38	198	88	55.6	160	19.2		148	7.5
8-9-38	362	213	41.2	306	14.4		291	4.9
				Corn leaves				
8-9-38	482	260	46.1	444	7.9		428	3.6

Results of Incubation in an Atmosphere of Carbon Dioxide

Data are presented in Table IX to show the effect of exclusion of oxygen during the incubation of macerated herbages, upon the stability of carotene. In all cases the samples that were incubated in an atmosphere of carbon dioxide lost much less carotene than the similar samples incubated while being exposed to air. The sample of brome grass incubated in an atmosphere of carbon dioxide lost 34.2 per cent of its carotene while the control lost 62.9 per cent. The other herbages studied lost much less carotene when incubated under carbon dioxide. These losses ranged from 11.1 per cent in the case of sudan grass, to 17.3 per cent for the corn leaves.

Table IX. Effect of Exclusion of Oxygen During Incubation at 37° C. for Four Hours Upon the Stability of Carotene

Sample and treatment	Carotene per gram of dry matter	
	Micrograms	Per cent loss
Alfalfa		
Fresh	132.8	
Incubated as control	62.5	53.0
Incubated under CO_2	115.4	13.1
Brome grass		
Fresh	317.1	
Incubated as control	117.5	62.9
Incubated under CO_2	208.8	34.2
Soybean plant		
Fresh	248.7	
Incubated as control	91.6	63.2
Incubated under CO_2	213.4	14.2
Corn leaves		
Fresh	615.7	
Incubated as control	322.8	47.6
Incubated under CO_2	508.9	17.3
Sudan grass		
Fresh	403.8	
Incubated as control	216.4	46.4
Incubated under CO_2	359.0	11.1

Buffer Curves for Alfalfa

The buffer curves shown in Figure 1 were set up in order to calculate the amount of acid needed to obtain a desired pH with 50 grams of alfalfa. The normality of the acids used was calculated on the basis of their titration value with 0.098 normal NaOH, using phenolphthalin as the indicator. This normality was then used to compute the milliequivalents of each acid added and the resulting values plotted against the determined pH of the samples. It should be noted that more acid was required to obtain a pH of 3.5 or lower, in the case of lactic and phosphoric acid than was needed when using sulphuric or hydrochloric. These curves are presented here as reference curves which were used in the following study.

Effect of Acids Upon the Stability of Carotene in Alfalfa

The results given in Tables X and XI show the effect of adding lactic, hydrochloric, sulfuric and phosphoric acid to macerated alfalfa, upon the loss of carotene during incubation. It should be noted that the stability of carotene of alfalfa was not seriously affected by reducing the normal pH of 5.5 to pH 4.0 or slightly less, using any of the four acids. The control samples lost 53.7 and 55.8 per cent carotene while the slightly acidified samples lost 50 to 70 per cent. When the pH was lowered further the percentage loss of carotene decreased regardless of the acid used. By using lactic acid to lower the pH to approximately 2.0, the loss of carotene was 9.4 per cent for one sample and 39.1 for another. When using the other three acids to lower the pH to about 2.0 the loss of carotene varied from 21.2 per cent to 37.2. The results for all the acids show that lactic and hydrochloric stabilized the carotene slightly better than did sulfuric and phosphoric, however, the differences were not very great.

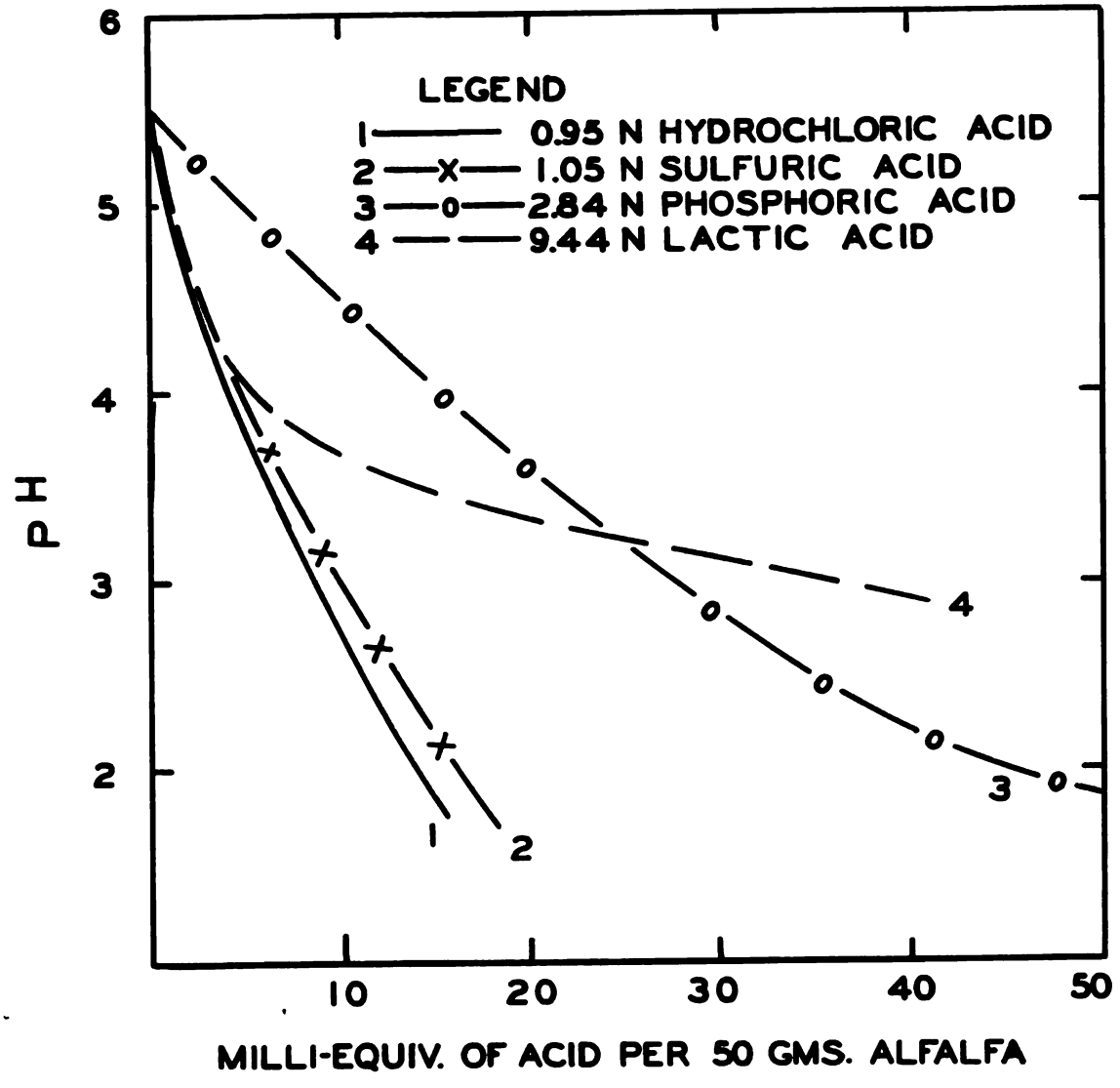


FIG. 1 BUFFER CURVE FOR ALFALFA

Table X. Effect of Acids Upon the Stability of Carotene in Macerated Alfalfa Tissue During Incubation at 37° C. for Four Hours*

Amount of acid added to 50 grams alfalfa		pH of tissue sap	Carotene per gram of dry matter	
ml.	milli-equiv.	pH	micrograms	Per cent loss
Fresh			366.7	
Check		5.48	169.8	53.7
	9.44 N - lactic acid			
0.8	7.55	3.75	121.5	66.9
1.8	16.99	3.45	164.5	55.1
3.6	33.98	3.09	226.5	38.2
20.6	194.46	2.23	223.5	39.1
	0.95 N - hydrochloric acid			
4.8	4.56	3.94	158.1	66.9
7.0	6.65	3.43	225.8	56.9
9.4	8.93	2.99	281.0	38.4
15.1	14.35	1.96	165.8	23.4
	1.05 N - Sulfuric acid			
5.0	5.25	3.88	165.8	54.8
7.1	7.46	3.43	220.4	39.9
10.0	10.50	2.96	238.5	35.0
16.7	17.54	1.83	271.0	26.1
	2.84 N - phosphoric acid			
5.0	14.20	4.15	121.5	66.9
7.1	20.16	3.53	165.8	54.8
10.0	28.40	2.96	215.4	41.3
16.7	47.43	1.88	230.6	37.1

* This sample was collected 8-23-38

Table XI. Effect of Acids Upon the Stability of Carotene in Macerated Alfalfa Tissue During Incubation at 37° C. for Four Hours*.

Amount of acid added to 50 grams alfalfa		pH of tissue sap	Carotene per gram of dry matter	
ml.	milli-equiv.	pH	micrograms	per cent loss
Fresh Check		5.50	358.2 158.2	55.8
		9.44 N - lactic acid		
0.4	3.74	4.19	150.6	58.0
1.6	15.10	3.45	226.4	26.7
4.0	37.76	2.93	282.7	21.1
20.0	188.8	2.15	424.7	9.4
		0.95 N - hydrochloric acid		
4.0	4.56	3.99	181.3	49.4
6.8	6.46	3.43	257.5	28.1
9.2	8.74	3.12	179.1	22.1
14.9	14.15	2.05	282.1	21.2
		1.05 N - sulfuric acid		
4.6	4.83	4.02	126.2	64.8
6.8	7.14	3.62	218.1	39.1
9.6	10.08	3.03	215.0	40.0
15.6	16.38	2.05	225.0	37.2
		2.84 N - phosphoric acid		
5.0	14.20	4.12	165.7	50.9
7.0	19.88	3.52	208.1	41.9
9.4	26.70	3.02	237.6	33.7
17.4	49.42	1.98	257.5	28.1

* This sample was collected 8-29-38

Destruction of Carotene by Enzymes Contained in Potato Juice

The results shown in Table XII are based on the fact that potato juice contains oxidizing enzymes (37). Results for samples 1 to 7 inclusive are given to show the relative amount of carotene extracted from each sample. The percentage loss values for samples 8 to 31 inclusive are based on an initial carotene content of 37.8 micrograms.

The results show that the amount of destruction of carotene depended upon the amount of potato juice added and the incubation time when the temperature remained constant. Potato juice, in amounts of from 0.1 to 5.0 ml., was added to sample 10 to 16 inclusive and also to samples 22 to 28 inclusive. The former series of samples was incubated at 37° C. for one hour, while the latter series was incubated for two hours. The loss of carotene from the samples incubated for two hours was 37.6 to 65.1 per cent, while the samples incubated for only one hour lost 23.3 to 62.4 per cent. In each case the loss of carotene closely paralleled the amount of potato juice added, although the smaller amounts of potato juice when incubated for two hours, were more destructive to carotene than when the incubation time was only one hour.

Inactivated potato juice was added to samples 17, 18 and 19, which were incubated for one hour, and also to samples 29, 30 and 31, which were incubated for two hours. The loss of carotene from the first three samples was negligible while the latter three lost only 2.4 to 4.0 per cent. No potato juice was added to samples 8 and 9 which were incubated for one hour, or to samples 20 and 21 which were incubated for two hours. In the former case the loss of carotene was negligible while the later two samples lost 5.8 and 9.5 per cent.

Table XII. Destruction of Carotene by Enzyme Contained in Potato Juice.

Sample number	Carotene solution	Water added	Potato juice added	Total carotene	Loss of carotene *
	ml.	ml.	ml.	micrograms	per cent
			no incubation		
1	10	5	0	38.1	
2	10	5	0	37.5	
3	10	4.9	0.1	36.5	
4	10	4.5	0.5	38.4	
5	10	4.0	1.0	38.4	
6	10	2.0	3.0	37.8	
7	10	0	5.0	37.5	
			incubated at 37° C. for one hour		
8	10	5	0	37.5	0
9	10	5	0	38.1	0
10	10	4.9	0.1	29.0	23.3
11	10	4.7	0.3	25.9	31.5
12	10	4.5	0.5	23.4	38.1
13	10	4.0	1.0	23.6	37.6
14	10	3.0	2.0	18.0	52.4
15	10	2.0	3.0	14.2	62.4
16	10	0	5.0	14.4	61.9
17	10	4.5	0.5 **	37.5	0
18	10	4.0	1.0 **	38.4	0
19	10	0	5.0 **	38.1	0
			incubated at 37° C. for two hours		
20	10	5	0	34.2	9.5
21	10	5	0	35.6	5.8
22	10	4.9	0.1	23.6	37.6
23	10	4.7	0.3	23.2	38.6
24	10	4.5	0.5	19.3	48.9
25	10	4.0	1.0	19.4	48.7
26	10	3.0	2.0	17.2	54.5
27	10	2.0	3.0	16.1	57.4
28	10	0	5.0	13.2	65.1
29	10	4.5	0.5 **	36.3	4.0
30	10	4.0	1.0 **	36.5	3.4
31	10	0	5.0 **	36.9	2.4

* Percentage loss was calculated on basis of 37.8 micrograms of carotene contained in 10 ml. of original solution.

** Potato juice inactivated by boiling for 10 minutes.

DISCUSSION OF RESULTS

The results for the herbages studied clearly show that the carotene content per gram of dry matter is much higher when the plants are in the earlier stages of growth than after they reach the stage of maturity at which they are usually harvested. In this investigation the carotene analyses were started during the stage of early growth, usually several weeks before the time of blooming. The determinations were made at weekly intervals. The early growth alfalfa contained more than 300 micrograms of carotene per gram of dry matter, but when cut for hay, the first crop contained only 171 micrograms and the second crop 133 micrograms. The carotene content of the third crop was considerably higher, however, the samples were taken from a different field so that the results may not be directly comparable. Soybeans, on the other hand, when 10 to 15 inches high, contained slightly less than 300 micrograms of carotene per gram of dry matter but this value increased to 432 micrograms when the plants were 22 inches high, and by the time the beans were developing, at which stage they are usually cut for hay, this value was approximately 200 micrograms.

First crop brome grass contained 141 micrograms of carotene per gram of dry matter when cut for hay, while the second crop contained 317 micrograms. This difference was probably due to the fact that the first crop developed to a more mature stage than did the second. The carotene content of sudan grass, 18 to 20 inches high, was slightly more than 400 micrograms, but after heading this value dropped to about 200 micrograms. Both crops followed the same rate of decrease. The oat plant when 6 to 10 inches high contained more than 400 micrograms but after heading this value dropped to below 150 micrograms. The sweet clover used in this investigation was lower

in carotene than the other plants studied. The maximum of 244 micrograms per gram of dry matter was noted at 18 inches, which value decreased to 125 micrograms when in full bloom. On the other hand, corn leaves reached a maximum of 646 micrograms when the ears were in early milk stage, which value decreased to 517 micrograms in the dough stage, although as the leaves became yellow the carotene decreased very rapidly to a low value of 25 micrograms.

The decrease in carotene content as the plants matured was not progressive in all cases. During the latter part of May, alfalfa, brome grass, oats and sweet clover did not show the normal decrease, as a matter of fact in some cases a slight increase in the trend was noted. This was probably due to an increase in the rate of growth of the plants caused by the additional rain fall during the latter part of May. A report by the U. S. Weather Bureau revealed that the total precipitation for the month was 5.73 inches, most of which fell during the latter half of the month. This additional rain fall apparently affected the carotene content in the same manner as growth was affected. Carotene content and maximum growth go hand in hand (128).

Some of the variations in carotene content during the growing season cannot be explained on the basis of an increase in the rate of growth. Attention should be called to the fact that the carotene analyses were made on different plants in successive weeks, thus some of the variations from week to week may have been due entirely to sampling, since it is almost impossible to select random samples under normal growing conditions that are exactly comparable. Most of the carotene of herbages is contained in the leaves (36), thus in the event of selecting a sample for analysis which contained more stems, the carotene content would be lower while a plant with more leaves would contain more carotene. This, and also the fact

that plants are biological tissue in which changes are continually taking place, might help explain some of the variations from the normal trends.

The differences in trends for the carotene content of different plants were interesting. In the case of alfalfa, brome grass, oats, and sudan grass, the carotene content decreased from the earliest samples until maturity. The second growth of brome grass did show an increase during the first week, but thereafter the regular decrease was noted. In the case of sweet clover there was a gradual increase during the first two weeks, after which the trend was downward. In contrast to these plants the soybeans showed a progressive increase in carotene up to and including three weeks growth after the first blossoms appeared, but thereafter a definite downward trend was noted. In the case of corn leaves, sampling was not begun until the tassels were out, at which time the carotene content was increasing. The increase continued for two weeks, after which a definite and progressive decrease was noted. These variations in trends may be due entirely to plant species differences. It should be mentioned, however, that the blooming process, in the case of soybeans is slow and gradual. In the case of the corn, leaves rather than the entire plant were used in this investigation.

In order to study the stability of carotene in green herbages as affected by the possible presence of oxidizing enzymes, each of the herbages was incubated subsequent to various laboratory treatments. The results show that when plants were macerated and incubation at 37° C. for four hours they usually lost more carotene than when a portion of the same plant was cut in half inch lengths and incubated for 44 hours. These percentage loss values for the various herbages show variations in both cases, however, more

emphasis is placed on the losses from the macerated tissue since the results were more consistent. Also since the macerated samples usually lost more carotene, it seemed evident that the losses were due quite largely to enzymatic destruction while the latter might be due in part to other factors such as bacteria. The question of the importance of enzymes in the destruction of carotene will be discussed later.

The variations in the losses seem to follow trends somewhat in accord with the stage of maturity, although in many cases the differences may not be great enough to be significant. It should be mentioned that the maceration and incubation procedure was begun in early July, thus no data on this relationship are presented for sweet clover. The data are also incomplete in some of the other crops. Where the data are more complete the general trend in the loss of carotene seems to be an increase during early growth up to a certain point and then a slight decrease at the more mature stages. This trend was followed by the second crop of alfalfa, but in the third crop, for three successive weeks during early growth the losses remained almost constant at 54 to 56 per cent, while for the next three weeks during the blooming stage the losses varied from 36 to 39 per cent.

The percentage loss of carotene in the second crop of brome grass, due to maceration and incubation, increased from 38 to 63 per cent during the growing season. The trend seemed definite for an increased loss as the plants matured. A similar trend was noted in the case of the first crop sudan grass, however, the results for the second crop are more complete and the increase progressed only to the stage of growth at which seeds were developing, after which a definite downward trend was noted. The few data

obtained with the oat plants in late maturity showed an increase in loss as the plants matured.

The data for soybeans are more complete than for most other plants studied and the results are very interesting. Only 8.4 per cent of the carotene was lost in the macerated tissue during the four hour incubation. This value gradually increased to a loss of slightly more than 60 per cent at the time the beans began to develop. During the time the beans were developing the loss showed a definite decrease. In this respect the results are comparable with those of other workers (42) in which it was pointed out that young soybeans 12 to 15 inches in height lost less "vitamin A" during hay drying after the beans had formed in the pods.

The maceration and incubation procedure for oats was begun only four weeks before the crop was harvested. The limited data show that the loss of carotene increased as the grain developed from the milk stage to maturity, with the losses varying from 49 to 69 per cent. In the case of the corn leaves the data are complete from the time the tassels were out until the corn was ripe. The loss of carotene varied from 34 to 48 per cent, with the highest value during the earlier growth, which was followed by a decrease at maturity up to the glaze stage. Thereafter a slight increase was noted.

Several investigators (36) (38) have indicated that the loss of carotene, under somewhat comparable incubation conditions to those used in this study, was due largely to enzymatic destruction. Other investigators (18) (34), commenting on this subject stressed the importance of factors such as light, oxygen, bacteria, and molds in the destruction of carotene, especially under hay making conditions. The fact that autoclaved and macerated samples retained more than 90 per cent of their carotene during

incubation at 37° C. for four hours, while similar unautoclaved samples lost from 40 to 60 per cent, indicates that some heat labile factor or factors were largely responsible for the loss during incubation of the macerated tissue. It may be argued that the losses from the unmacerated samples when incubated for 44 hours were due in part to bacteria, however, in the case of the macerated samples it seems very evident that some factor or factors within the plant cells must be largely responsible for the loss. The loss in the latter case could hardly be due to bacteria or molds because of the short incubation period. These factors are most probably oxidizing enzymes.

Oxidizing enzymes are probably not the only cause for loss of carotene in the macerated plant tissue, although they evidently play an important role. The destruction of carotene seems to be an oxidative process, since by incubating macerated samples in an atmosphere of carbon dioxide the usual high losses were greatly reduced. The results in Table IX illustrate the oxidative destruction of carotene since in all cases, except brome grass, the loss of carotene was less than 20 per cent while the corresponding samples incubated in an atmosphere of air lost from 46 to 63 per cent. These differences are quite notable and it seems probable that the loss of carotene from the samples incubated under carbon dioxide might have been even less if the air could have been removed immediately. The procedure required about 30 minutes to get the samples completely under carbon dioxide gas during which time considerable oxidative destruction could have taken place.

The addition of acids to macerated alfalfa during the four hour incubation procedure did not greatly lessen the destruction of carotene

until the pH was decreased to 3.5. The results in Tables X and XI show that as the pH of the macerated and incubated samples was lowered from 3.5 to 2.0 the loss of carotene was greatly reduced thus indicating that whatever the factor responsible for the destruction of carotene it is less active at the lower pH.

Lactic, hydrochloric, sulfuric, and phosphoric acid did not give values sufficiently different to warrant any definite statement as to their relative efficiency in preserving carotene. A comparison between the losses of carotene from acidified silage (39) (58) (125) and those from the macerated and acidified samples incubated at 37° C. for four hours indicate that the higher losses in the latter case must be due to maceration and exposure of the tissue to oxygen. Most of the air is excluded during ensiling which was not true under the laboratory conditions.

It seems evident that carotene in macerated plants is destroyed by heat labile factor or factors which are more active in the presence of oxygen than when oxygen is excluded, and also that, an optimum pH is needed for greatest destruction. By macerating plant tissue the destruction of carotene is hastened, partly because of exposure to air and also because there exists within the plant cells certain heat labile factors, most probably oxidizing enzymes. Other unknown factors may cause destruction of carotene, however, the process is probably one of oxidation.

The stage of maturity may effect the loss of carotene from macerated tissue during incubation, in that more plant enzymes may be present to increase destruction, or antioxidents may be formed to decrease the loss, and also, as the plants mature the cells may become more resistant to break down

in the grinding procedure, which would not allow as much carotene to be exposed to enzymes and oxygen as would be for the plants in early growth.

Samples of the various herbages incubated for 44 hours did not lose carotene nearly as rapidly as did the macerated samples. By comparing the losses for the unmacerated samples with the losses for samples cured in the field (3) (17) (59), a more rapid loss of carotene from the field cured samples is noted. Therefore, enzymes may not be so important in field curing conditions unless the enzymatic effect is photochemical. However, in order to demonstrate this, autoclaved samples should be placed in the field to dry which was not done. The results of the macerated samples compared with the unmacerated samples, would indicate that in silage making a cutter that cuts the plants cleanly and does not tear, bruise or crush them, would be helpful in preventing some of the losses in this process.

In order to present more evidence for the idea that oxidizing enzymes are destructive to carotene, a solution of carotene was prepared to which potato juice was added and the loss of carotene observed. Potato juice is known to contain oxidizing enzymes (37). The results shown in Table XII demonstrate that at least some heat labile factor present in potato juice is destructive to carotene. Samples of the carotene solution which contained inactivated potato juice or no potato juice at all, lost little or no carotene during incubation at 37° C. for one or two hours. When similar samples contained potato juice, as high as 65 per cent of the carotene was lost during incubation for two hours. The loss of carotene from the samples varied with the amount of potato juice added.

This procedure might be used to study the presence and concentration of oxidizing enzymes in various plant saps. This could be done by using the various plant saps instead of potato juice in this procedure.

SUMMARY AND CONCLUSIONS

1. Variations in the carotene content of seven herbages were studied during their growing season. These herbages included alfalfa, brome grass, corn leaves, the oat plant, the soybean plant, sudan grass, and sweet clover.

2. The carotene content of these herbages is much greater during the earlier stages of growth than after they reach the stage of maturity when they are usually harvested.

3. In making hay or silage in order to obtain the greatest carotene content the plants should be cut at an early stage of maturity.

4. By macerating the plant tissue and incubating at 37° C. for four hours higher losses of carotene resulted than when a portion of the plants was cut in half inch lengths and incubated for 44 hours.

5. Autoclaving for one hour at 15 pounds pressure destroyed 8 to 19 per cent of the carotene present in the original plants. Only small additional losses resulted when the autoclaved tissue was macerated and incubated at 37° C. for four hours.

6. Incubation of the macerated tissue in an atmosphere of carbon dioxide resulted in much lower losses of carotene than when incubated in air.

7. The addition of lactic, hydrochloric, sulfuric, and phosphoric acids to alfalfa was effective in preserving carotene during incubation only when the pH was reduced to below 3.5. A still smaller loss of carotene occurred when the pH was lowered to about 2.0.

8. The evidence presented indicates that carotene is destroyed by some heat labile factor or factors present in plants which are probably oxidizing enzymes.

9. The evidence showed that potato juice is destructive to carotene in solution. The factors responsible are heat labile and are probably oxidizing enzymes.

LITERATURE CITED

(1) Atkeson, F. W., Peterson, W. J. and Aldous, A. E.
1937 Observations on the carotene content of some typical pasture plants. J. Dairy Sci. 20: 557-562.

(2) Barnes, W. C.
1936 Effects of some environmental factors for growth and color of carrots. Cornell Uni. Agri. Expt. Sta. Memo. 186: 36 pp.

(3) Bartlett, S., Henry, K. M., Kon, S. K., Osborne, L. W., Thompson, S. Y. and Tinsley, J.
1938 The effect of different methods of drying on the biological value and digestibility of the proteins and on the carotene content of grass. Biochem. J. 32: 2024-2030.

(4) Bauman, C. A. and Steenbock, H.
1933 Fat-soluble vitamins. XXXVII. The stability of carotene solution. J. Biol. Chem. 101: 561-572.

(5) Beck, W. A.
1937 Development of plant pigments in seedlings grown in the dark. Studies Inst. Divi. Thomas 1: 109-116. (Abstr.) Chem. Abstr. 31: 7078.

(6) Beck, W. A.
1937 Pigments formed in etiolated sunflower seedlings; Protoplasma 28: 273-282. (Abstr.) Chem. Abstr. 31: 7470.

(7) Bender, C. B., Bartlett, J. W., Tucker, H. H. and Mixner, J.
1937 Molasses grass silage as the sole roughage diet for milk production and growth of dairy animals. J. Dairy Sci. 20: 424-425.

(8) Bethke, R. M. and Kick, C. H.
1929 Vitamin A content of alfalfa hay. Ohio Agri. Expt. Sta. Bul. 431. (47th Ann. Rept) 117-118.

(9) Bethke, R. M., Record, P. R. and Wilder, O.H.M.
1938 The stability of carotene and vitamin A in mixed rations. Progress of Agri. Res. in Ohio; Ohio Agri. Expt. Sta. Bul. 592: 99.

(10) Bogert, M. T.
1938 Carotenoids - The polyene pigments of plants and animals. Chapter 14 of Organic Chem. by Gilman, 2: 1138-1219. John Wiley & Sons, New York.

(11) Bolin, D. W.
1939 Stability of carotene in green grasses and alfalfa stored at five degrees Fahrenheit. J. Dairy Sci. 22: 111-113.

(12) Brooke, R. O., Tyler, S. W. and Baker, W. S.
1939 Determination of beta-carotene in alfalfa meals. Ind. Eng. Chem., Anal. Ed. 11: 104.

(13) Capper, N. S.
1930 Vitamin A and carotene. Nature 126: 685.

(14) Churchill, B. R. and Horwood, R. E.
1938 Methods of making and feeding alfalfa-molasses silage. J. Dairy Sci. 21: Abstr. 105.

(15) De, N. K.
1936 Factors affecting the carotene content of certain vegetable food stuffs. Indian J. Med. Research 24: 201-212.

(16) Deleano, N. T. and Dick, J.
1933 Studies on carotene 1. New methods for the preparation, demonstration and determination. Bichem. Z. 259: 110-133 (Abstr.) Chem. Abstr. 27: 2979.

(17) Dexter, S. T. and Moore, L. A.
1937 Carotene (vitamin A) in alfalfa hay. Mich. Agri. Expt. Sta. Quart. Bul. 20: 75-76.

(18) Douglass, E., Tobiska, J. W. and Vail, C. E.
1933 Studies on changes in vitamin content of alfalfa hay. Col. Expt. Sta. Tech. Bul. 4: 68.

(19) Drew, J. P., Deasy, D., O'Sulivan, G. F., Senior, B. J. and Sheeley, E.J.
1938 The recovery of the nutrient of grass herbages as A.I.V. and as untreated silage, etc. J. Dept. Agri. Ireland, 35: 1-32.

(20) Esselen, W. B., Fellers, C. R., Insgur, B.
1937 Vitamin A, C and D in maize as affected by variety and stage of growth. J. Nut. 14: 503-511.

(21) Euler, H. v., and Hellstrom, H.
1929 The formation of xanthophyll, carotin and chlorophyll in illuminated and unilluminated barley seedlings. Z. Physiol. Chem. 183: 177-183.

(22) Euler, H. v., Karrer, P. and Rydbom, M.
1929 Relation between vitamin A and carotenoids. Ber. 62-B: 2445-2451. (Abstr.) Chem. Abstr. 24: 1143.

(23) Fagan, T. W. and Ashton, W. M.
1938 The effect of partial field drying and artificial drying on the chemical composition of grass. The Welsh J. of Agri. 14: 160-1761.

(24) Ferguson, W. S.
1935 Curves for use in colorimetric estimation of carotene. Analyst 60: 680-683.

(25) Fraps, G. S.
1938 Evaluation of Vit. A. potency of feeds. Ind. Eng. Chem., Anal. Ed. 10: 525-527.

(26) Fraps, G. S. and Kemmerer, A. R.
1937 Losses of vitamin A and carotene from feeds during storage. Texas Agri. Expt. Sta. Bul. 557: 28 pp.

(27) Fraps, G. S. and Treichler, R.
1933 Vitamin A content of foods and feeds. Texas Agri. Expt. Sta. Bul. 477: 34 pp.

(28) Fraps, G. S., Treichler, R. and Kemmerer, A. R.
1936 Relation of the carotene content of certain feed materials to their vitamin A potency. J. Agri. Res. 53: 713-716.

(29) Gillam, A. E. and Ridi, M. S. El
1936 The isomerization of carotene by chromatographic adsorption. I. Pseudo-alpha-carotene. Biochem. J. 30: 1735.

(30) Gillam, A. E., Ridi, M. S. El and Kon, S. K.
1937 The isomerization of carotenes by chromatographic adsorption. II. Neo-alpha-carotene. Biochem. J. 31: 1605-1610.

(31) Goldblatt, H. and Barnett, H. M.
1932 Carotene and Vitamin A. Proc. Soc. Exptl. Biol. Med. 30: 201-204.

(32) Gortner, R. A.
1938 Outline of Biochemistry, 2nd. Ed. John Wiley & Sons, New York. 875 pp. illus.

(33) Guilbert, H. R.
1934 Determinations of carotene as a means of estimating the vitamin A value of forage. Ind. Eng. Chem., Anal. Ed. 6: 452-454.

(34) Guilbert, H. R.
1935 Factors affecting the carotene content of alfalfa hay and meal. J. Nutr. 10: 45-62.

(35) Hathaway, I. L., Davies, H. P. and Brauer, J. C.
1936 The vitamin A content of A.I.V. molasses and normal silage and the effect of feeding these silages upon the vitamin A content of milk. J. Dairy Sci. 19: 452.

(36) Hauge, S. M.
1934 Vitamin A value of alfalfa cut at different stages of maturity. J. Assoc. Official Agri. Chem. 17: 304-307.

(37) Hauge, S. M.
1935 Evidence of enzymatic destruction of the vitamin A value of alfalfa during the curing process. J. Biol. Chem. 108: 331-336.

(38) Hauge, S. M. and Aitkenhead, W.
1931 The effect of artificial drying upon the vitamin A content of alfalfa. J. Biol. Chem. 93: 657-665.

(39) Hayden, C. C., Perkins, A. E., Krauss, W. E., Monroe, C. F. and Washburn, A. G.
1937 Legume silage for dairy cows. Ohio Agri. Expt. Sta. Bimonthly Bul. Vol. 22: No. 184: 21-27.

(40) Hegsted, D. M., Porter, J. W. and Peterson, W. H.
1938 An improved method for determination of carotene in silage. Paper at Milwaukee meeting Amer. Chem. Soc. September 5-9.

(41) Heilbron, I. M., Morton, R. A. and Webster, E. T.
1932 The structure of vitamin A. Biochem. J. 26: 1194-1196.

(42) Hilton, J. H., Hauge, S. M. and Wilbur, J. W.
1935 The vitamin A activity of butter produced by cows fed alfalfa hay and soybean hay cut in different stages of maturity. J. Dairy Sci. 18: 795-800.

(43) Holmes, H. N.
1936 U. S. Patent No.2051257. The Official Gaz. U. S. Patent Office 469, No. 3, 616.

(44) Holmes, H. N. and Corbet, R. E.
1939 Catalytic effects of porous powders on pure vitamin A. J. Biol. Chem. 127: 449-456.

(45) Holmes, H. N. and Leicester, H. M.
1932 Isolation of carotene. J. Am. Chem. Soc. 54: 716-20.

(46) Ijdo, J. B. H.
1935 Relation between soil condition and the carotene and vitamin C content of plants. Acta. Brevia Neerland. Physiol. Pharmacol., microbiol. 5: 167-169 (Abstr.) Chem. Abstr. 30: 3462.

(47) Ijdo, J.B.H.
1936 The influence of fertilizers on the carotene and vitamin C content of plants. Biochem. J. 30: 2307-2312.

(48) Kane, E. A., Wiseman, H. G. and Cary, C. A.
1937 The loss of carotene in hays and alfalfa meal during storage. J. Agri. Res. 55: 837-847.

(49) Kane, E. A., Wiseman, H. G., Hartman, A. H. and Cary, C. A.
1936 Spectrophotometric data bearing on the character of the pigments obtained in routine determinations of carotene in hays, silages, and freshly cut plant materials. J. Dairy Sci. 19: 466.

(50) Karrer, P., Helfenstein, A., Wehrli, H. and Wettstein, A.
1930 Plant pigments XXV. The constitution of lycopin and carotene. Helv. Chim. Acta. 13: 1084-1099. (Abstr.) Chem. Abstr. 25: 519.

(51) Karrer, P. and Morf, R.
1931 Plant pigments XXXV. The constitution of beta-carotene and beta-dihydro-carotene. Helv. Chim. Acta. 14, 1033-1036. (Abstr.) Chem. Abstract 26: 733.

(52) Karrer, P., Morf, R. and Schöpp, K.
1931 Vitamin A from fish oils. Chim. Acta. 14, 14-31-1436. (Abstr.) Chem. Abstr. 26: 4359.

(53) Karrer, P., Morf, R. and Schöpp, K.
1933 Synthesis of perhydronitamin A. Chim. Acta. 16, 557-561. (Abstr.) Chem. Abstr. 27: 3976.

(54) Karrer, P., Morf, R. and Walker, O.
1933 Constituion of alpha-carotene. Nature 132: 171.

(55) Karrer, P. and Schlientz, W.
1934 Plant pigments LV. The occurrence of alpha-, and beta-carotene in different national products. Helv. Chim. Acta. 17: 7-8. (Abstr. (Chem. Abstr. 28: 3076.

(56) Kerppola, W.
1929 Isolation, identification and occurrence of vitamin A. Acta. med. Scand. suppl. 34: 170-175. (Abstr.) Chem. Abstr. 24: 5347.

(57) Krauss, W. E., Hayden, C. C., Perkins, A. E. and Washburn, R. G.
1938 A trial with temporary silos. Ohio Agri. Expt. Sta. Bimonthly Bul. 23: 71-76.

(58) Krauss, W. E. and Washburn, R. G.
1936 Studies on A.I.V. silage. Part 3. Carotene preservation and biological properties of the milk. J. Dairy Sci. 19: 454-455.

(59) Krauss, W. E. and Washburn, R. G.
1938 Loss of carotene in hay making. Progress of Agri. Res. in Ohio; Ohio Agri. Expt. Sta. Bul. 592: 81.

(60) Kuhn, R. and Lederer, E.
1931 The separation of carotene into its components. 1. The growth vitamin. Ber. 64 B. 1349-1357. (Abstr.) Chem. Abstr. 25-5694.

(61) Lederer, E. and Moore, T.
1936 Echinenone as a provitamin A. Nature 137: 996.

(62) Mackinney, G.
1935 Leaf carotenes. J. Biol. Chem. 111: 75-84.

(63) McDonald, F. G.
1933 The stability of carotene in ethyl esters of fatty acids, and in liver and vegetable oils. J. Biol. Chem. 103: 453-460.

(64) Miller, E. S.
1935 Rapid and accurate quantitative method for the determination of the common carotenoids: Analysis of beta-carotene and leaf xanthophyll in thirteen plant tissues. J. Am. Chem. Soc. 57: 347-349.

(65) Moon, F. E.
1938 The carotene - xanthophyll ratio in fresh and dried grass. J. Soc. Chem. Ind. 57: 457-460.

(66) Moore, L. A.
1939 Vitamin A and carotene studies in the bovine. Thesis for PhD. Michigan State College, 95 p.

(67) Moore, L. A.
Michigan State College - Unpublished data.

(68) Moore, T.
1929 Carotin and vitamin A. Lancet 1929, 1. 499-500.

(69) Moore, T.
1929 The relation of carotene to vitamin A. Lancet 1929. 2. 380-381.

(70) Moore, T.
1929 Vitamin A and carotene 1. The association of vitamin A activity with carotene in the carrot root. Biochem. J. 23: 803-811.

(71) Moore, T.
1929 Vitamin A and carotene. II The vitamin A activity of red palm oil carotene. III The absence of vitamin D from carotene. IV The effect of various dietery modifications upon the vitamin A activity of carotene. Biochem. J. 23: 1267-1272.

(72) Moore, T.
1930 Vitamin A carotene. V The absence of liveroil vitamin A from carotene. VI The conversion of carotene to vitamin A in vivo. Biochem. J. 24: 692-702.

(73) Moore, T.
1931 Vitamin A and carotene. VIII Distribution of vitamin A and carotene in the body of the rat. Biochem. J. 25: 275-286.

(74) Moore, T.
1932 Vitamin A and carotene. IX Notes on the conversion of carotene to vitamin A in the cow. Biochem. J. 26: 1-9.

(75) Moore, T.
1933 Vitamin A and carotene. X The relative minimum doses of vitamin A and carotene. Biochem. J. 27: 898-902.

(76) Munsey, V. E.
1937 Report on carotene. J. Assoc. Official Agri. Chem. 20:459-468.

(77) Murneek, A. E.
1934 Relation of carotenoid pigments to sexual reproduction in plants. Science 79: 528.

(78) Murneek, A. E.
1934 Biochemical study of the internal mechanism of photoperidism. Mo. Agri. Expt. Sta. Bul. 340 (Ann. Rpt. 1933): 63-64.

(79) Murri, I. K.
1937 A rapid method for the determination of carotene. Biokhimija. 2: 831-840. (Abstr.) Chem. Abstr. 32: 2554.

(80) Myburgh, S. J.
1935 The carotene content of some South African feeds. Onderstepoot. J. Vet. Sci. 5: 475-482.

(81) Norris, R. J.
1933 Observations on the development of chlorophyll and carotonoid pigments in etiolated plants. Cincinnati Inst. of Sci. Res. Bul. of Basic Sci. Research 5: 23-32.

(82) Ott, M.
1937 The vitamin C and carotene content of field and garden products with different fertilizers. Angew. Chem. 50: 75-77. (Abstr.) Chem. Abstr. 31: 2336.

(83) Page, H. J. and Watson, S. J.
1935 The present position of silage making. Agri. Progress 12: 99-102.

(84) Palmer, L. S.
1916 The physiological relation of plant carotinoids to the carotinoids of the cow, horse, sheep, goat, pig and hen. J. Biol. Chem. 27: 27-32.

(85) Palmer, L. S.
1922 Carotinoids, and related pigments. Am. Chem. Soc. Memo. Series. The Chem. Catalog Co., Inc. N. Y. 316 pp. Illus.

(86) Palmer, L. S.
1938 The chemistry of vitamin A and substances having a vitamin A effect. J. Amer. Med. Assoc. 110: 1748-1751.

(87) Palmer, L. S. and Eckles, C. H.
1914 Carotin - The principle natural yellow pigment of milk fat; Its relation to plant carotene and the carotin of the body fat, corpus luteum and blood serum. I The chemical and physiological relation of the pigments of milk fat to the carotin and xanthophyll of green plants. J. Biol. Chem. 17: 191-210.

(88) Peterson, W. H.
1938 Pea vine silage and quality milk. Canning Trade 60: 7-8. (Abstr.) Expt. Sta. Record 79: 678.

(89) Peterson, W. H. and Bird, H. R. and Beeson, W. M.
1937 Chemical changes in the making of A.I.V. alfalfa silage and nutritive qualities of milk produced therefrom. J. Dairy Sci. 20: 611-623.

(90) Peterson, W. H., Bohstedt, G., Bird, H. R. and Beeson, W. M.
1935 The preparation and nutritive value of A.I.V. silage for dairy cows. J. Dairy. Sci. 18: 63-78.

(91) Peterson, W. J., Hughes, J. S. and Freeman, H. F.
1937 Determination of carotene in forage. A modification of the Guilbert method. Ind. Eng. Chem. Anal. Ed. 9: 71-72.

(92) Pfaff, C. and Pfützer, G.
1937 The effect of fertilizing on the content of carotene and ascorbic acid of various vegetables and fodder plants. Angew. Chem. 50: 179-184. (Abstr.) Chem. Abstr. 31: 3620.

(93) Pfützer, G. and Pfaff, C.
1935 The carotene and vitamin C contents of vegetables and fodders. Angew. Chem. 48: 581-583. (Abstr.) Chem. Abstr. 29: 7550.

(94) Quackenbush, F. W., Steenbock, H. and Peterson, W. H.
1938 The effects of acids on carotenoids. J. Amer. Chem. Soc. 60: 2937-2941.

(95) Richardson, H. E.
1935 The handling, processing and storing of legume crops for feed. Agri. Eng. 16: 469-471.

(96) Russell, W. C.
1929 The effect of the curing process upon the vitamin A and D content of alfalfa. J. Biol. Chem. 85: 289-297.

(97) Russell, W. C., Taylor, M. W. and Chichester, D. F.
1934 Effect of the curing process upon carotene and vitamin A content of alfalfa. N. J. Agri. Expt. Sta. Bul. 560: 8 pp.

(98) Russell, W. C., Taylor, M. W. and Chichester, D. F.
1935 Colorimetric determinations of carotene in plant tissue. Plant Physiol. 10: 325-340.

(99) Schertz, F. M.
1928 The extraction and separation of chlorophyll (A and B), carotin and xanthophyll in fresh leaves, preliminary to their quantitative determination. Plant Physiol. 3: 211-216.

(100) Scheunert, A. and Schieblich, M.
1933 The stability of the international standard carotene in oil solution. Biochem. Z. 263: 454-457. (Abstr.) Chem. Abstr. 27: 5383.

(101) Scheunert, A. and Schieblich, M.
1934 The influence of artificial and natural drying on the vitamin content of alfalfa. Biedermanns. Zentr. B. Tierernähr. 6: 112-132. (Abstr.) Chem. Abstr. 28: 5102.

(102) Shepherd, J. B. and Woodward, T. E.
1938 Further investigations in chopping alfalfa hay at the time of storage. J. Dairy Sci. 21: 89-96.

(103) Shepherd, J. B. and Woodward, T. E.
1938 Experience in ensiling partially cured alfalfa, methods used, losses sustained, and feeding value. J. Dairy Sci. 21, Abstr. page 104-105.

(104) Sherman, H. C. and Munsell, H. E.
1925 The quantitative determination of vitamin A. J. Amer. Chem. Soc. 47: 1639-1646.

(105) Shinn, L. A., Wiseman, H. G., Kane, E. A. and Cary, C. A.
1938 The carotene content of market hays and corn silage as determined by a quantitative adsorption procedure. J. Dairy Sci. 21: Abstr. page 113-114.

(106) Smith, M. C.
1936 The effect of storage upon the vitamin A content of alfalfa hay. J. Agri. Research 53: 681-684.

(107) Smith, M. C. and Briggs, I. A.
1933 The vitamin A content of alfalfa as affected by exposure to sunshine in the curing process. J. Agri. Res. 46: 229-234.

(108) Smith, M. C. and Stanely, E. B.
1938 The vitamin A value of Blue Gramma Range grass at different stages of growth. J. Agri. Res. 56: 69-72.

(109) Smith, Ora
1936 Effect of light on carotenoid formation in tomato fruits. Cornell Univ. Agri. Expt. Sta. Memoir 197: 26 pp.

(110) Sprague, H. B.
1928 A convenient method of measuring quantities of chloroplast pigments. Science 67: 167-169.

(111) Sprague, H. B. and Shive, J. W.
1929 A study of the relations between chloroplast pigments and dry weights of tops in dent corn. Plant Physiol. 4: 165-192.

(112) Steenbock, H.
1919 White corn vs. yellow corn and a probable relation between the fat soluble vitamin and yellow pigments. Science 50: 352-353.

(113) Steenbock, H. and Boutwell, P. W.
1920 Fat soluble vitamin. III The comparative nutritive value of white and yellow maizes. J. Biol. Chem. 41: 81-96.

(114) Steenbock, H. and Boutwell, P. W.
1920 Fat soluble vitamin. V Thermostability of the fat-soluble vitamin in plant materials. J. Biol. Chem. 41: 163-171.

(115) Steenbock, H., Sell, M. A., Nelson, E. M. and Buell, M. V.
1921 The fat soluble vitamin. Proc. Am. Soc. Biol. Chem. J. Biol. Chem. 46: 32-33.

(116) Strain, H. H.
1934 Carotene VIII Separation of carotenes by adsorption. J. Biol. Chem. 105: 523-535.

(117) Strain, H. H.
1935 Carotene IX Carotenes from different sources and some properties of alpha and beta carotene. J. Biol. Chem. 111: 85-93.

(118) Taylor, M. W.
1934 Stability of provitamin A in alfalfa and silage. N. J. Agri. 16, 6, 3.

(119) Taylor, M. W.
1937 Carotene and vitamin A in silage. N. J. Agri. 19, No. 5: 2-3.

(120) Taylor, M. W. and Russell, W. C.
1938 The stability of carotene in plant tissues. J. Nut. 16: 1-13.

(121) Thomas, B. and Moon, F. E.
1938 A preliminary study of the effects of fertilizing treatment and of age on the carotene content of grass. Empire J. Exptl. Agri. 6: 235-245.

(122) Tswett, M.
1906 Physikalischemische studies uber das chlorophyll. Die adsorptionen. Ber. Botan. Ges. 24, 316-323 Cited by Palmer, L. S., Carotenoids and related pigments. p. 260. The Chem. Catalog Co., Inc. N. Y.

(123) Turner, R. G.
1934 The stability of carotene in olive oil. J. Biol. Chem. 105: 443-454.

(124) Vail, C. E., Tobiska, J. W. and Douglass, Earl
1936 Further studies on vitamins in alfalfa hay. Colo. Expt. Sta. Tech. Bul. 18: 19.

(125) Virtanen, A. I.
1933 Conservation of vitamins in cattle feed. Biochem. Z. 258: 251-256.

(126) Virtanen, A. I.
1936 Vitamins and plants. Nature 137: 779-780.

(127) Virtanen, A. I. and Hausen, S. v.
1932 Vitamin formation in plants. Naturwissenschaften 20: 905. (Abstr.) Chem. Abstr. 27: 1657.

(128) Virtanen, A. I., Hausen, S. v. and Saastamoinen, S.
1933 Studies on vitamin production in plants. Biochem. Z 267: 179-191.

(129) Wackenroder, H.
1826 Uber das oleum radicis dauci aetherum, das carotin, (etc.); Diss. de Anthelminiticis, Gottigen; Geige's magaz. Pharm. 33: 144-172 (1831) From Palmer, L. S., Carotenoids and related pigments. p. 25. The Chemical Catalog Co. Inc. N. Y.

(130) Watson, S. J.
1934 The conservation of grassland herbage. J. Royal Agri. Soc. Eng. 95: 103-116.

(131) Wilder, O.H.M. and Bethke, R. M.
1938 The loss of carotene in dehydrated alfalfa leaf meal stored under different conditions. Progress of Agri. Res. in Ohio; Ohio Agri. Expt. Sta. Bul. 592:100.

(132) Willstatter, R. and Stoll. A.
1913 Investigations on chlorophyll. English translation by Schertz, F. M. and Mertz, A. R. (1928) Science Press, Lancaster, Pa., 385 pp. illus.

(133) Wiseman, H. G. and Kane, E. A.
1936 The determination of carotene in fresh plant materials. Proc. Am. Soc. Biol. Chem. J. Biol. Chem. 114:CVII.

(134) Wiseman, H. G., Kane, E. A. and Cary, C. A.
1934 The losses involved in determining carotene in hays and in fresh green plants. J. Biol. Chem. 105: CI - CII.

(135) Wiseman, H. G., Kane, E. A. and Cary, C. A.
1936 Rate of decomposition of carotene in hays during storage at different seasons of the year. J. Dairy Sci. 19:466-467.

(136) Wise, H. G., Kane, E. A., Shinn, L. A. and Cary, C. A.
1938 The carotene content of market hays and corn silage. J. Agri. Res. 57: 635-669.

(137) Woodward, T. E. and Shepherd, J. B.
1936 An experiment in chopping alfalfa hay at the time of storage. J. Dairy Sci. 19: 697-706.

(138) Woodward, T. E. and Shepherd, J. B.
1938 Methods of making silage from grasses and legumes. U.S.D.A. Tech. Bul. 611: 55 pp.

ROOM USE ONLY