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Received for review July 27, 1953. Accepted September 25, 1953. Presented before the Division of Agricultural and Food Chemistry at the 123rd Meeting of the AMERICAN CHEMICAL SOCIETY, *Los Angeles, Calif.*

Factors in Destruction of Alfalfa Carotene Evaluated

CAROTENE

Factors Affecting Destruction in Alfalfa

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The rapid destruction of carotene in alfalfa during field curing has been attributed to the summation of the losses by enzymatic and photochemical processes. Accurate evaluation of the extent of destruction under field conditions is confused by continual changes in temperature, moisture, light intensity, and physical state of the tissue. By incubating aqueous suspensions of macerated alfalfa leaves under controlled conditions it was possible to measure separately the effects of temperature, pH, heat treatments, and cyanide upon the enzymatic, photochemical, and autoxidative losses of carotene. The enzyme system was found to have a temperature optimum of about 43° C., to be more active at pH 4 to 5 than at higher pH values, to be heat labile, and to be partially inhibited by cyanide. Photochemical destruction was not markedly affected by pH changes between 4 and 8, by temperature changes between 10° and 45° C., or by prolonged heat treatments after enzyme inactivation. The loss of carotene in the absence of enzymatic and photochemical destruction was attributed to autoxidation. This mechanism appears to be of minor importance below 40° C., and is little affected by pH changes between 4 and 8. It is difficult to conclude that one mechanism of carotene destruction predominates over the other during field curing. Both contribute to carotene loss and both must be controlled to reduce the total loss.

ALFALFA IS POTENTIALLY A RICH SOURCE OF CAROTENE for livestock, but 45 to 90% of the carotene is lost during the field curing process (*3, 9, 13, 14*). This loss is attributed chiefly to the rapid destruction of carotene by oxidative processes which are catalyzed by enzymes (*5, 6*) and light (*5*), and to a slower destruction, probably by autoxidation in the absence of light and enzyme activity.

Attempts have been made to determine the relative importance of enzymatic and photochemical destruction of carotene during field curing by comparing the losses of carotene in samples incubated at similar temperatures in the light and dark (*4, 12*). However, it is difficult

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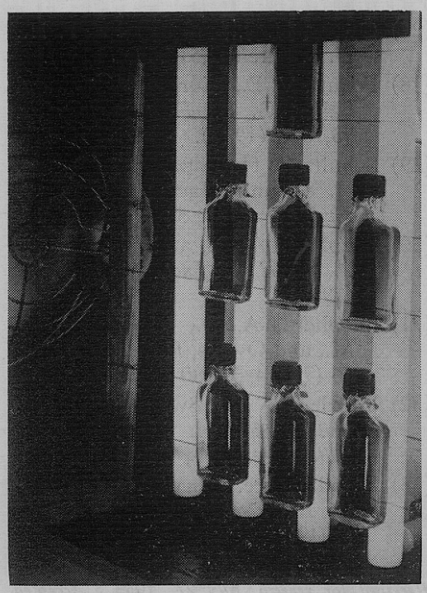

Figure 1. Apparatus used for controlling environmental conditions during incubation of suspension samples in light

to maintain comparable conditions of temperature and drying rates in the light and dark. Bernstein and Thompson (*1*) attempted to study the reactions under more precisely controlled environmental conditions, using partially dried bean leaves. They found that the relative importance of the two mechanisms of carotene destruction varies with the moisture content. To make a precise comparison of the relative importance of enzymatic and photochemical destruction during field curing requires that all environmental conditions be rigidly controlled.

The enzymatic destruction of carotene can be inhibited by heat treatments, such as blanching, autoclaving, conduction heating, and direct flaming (*7, 11*). In attempts to adapt an open flaming technique to field operations (*2*), it was found difficult to obtain complete enzyme inactivation; furthermore, the preservation of carotene was not greatly improved, possibly because of increased photochemical losses during subsequent drying in the field.

Heat treatments wilt the plant and cause accompanying alterations of the physical characteristics which increase carotene loss (*12*), and make it difficult to determine the effect of heat treatments upon the actual chemical mechanisms involved in carotene destruction.

This investigation was conducted to distinguish between the chemical and physical effects of heat treatments and to study some factors which affect the relative losses of carotene by enzymatic, photochemical, and autoxidative reactions.

Procedures and Results

It was found that fresh and heated alfalfa leaves could be reduced to a uniform, reproducible physical state by grinding them with water to form aqueous suspensions of macerated leaves.

Each suspension was made by freezing a 20-gram sample of autoclaved, blanched, or untreated leaves, then grinding the frozen sample with 50 ml. of ice water in a porcelain ball mill at 3° C. for 3 hours. The resultant paste was washed from the ball mill with water and diluted to 100 ml. The suspensions were stored at 3° C. in the dark and used within a week. Microscopic examination of the suspensions showed that the tissues were well macerated, almost no cells being present.

Suspension samples were incubated in the light or dark under precisely controlled environmental conditions and the carotene losses measured. The small losses of carotene occurring in suspensions of autoclaved leaves in the dark were assumed to be by autoxidation. The losses of carotene in similar suspensions incubated in the light are referred to as photochemical losses, since the losses in the absence of light are very small. The losses of carotene in suspensions of fresh leaves in the dark are referred to as enzymatic losses, although small autoxidative losses undoubtedly occur. The losses occurring in fresh suspensions in the light represent the total destruction by all mechanisms.

Incubation Conditions

Light. Five-gram suspension samples were weighed into 2-ounce prescription bottles sealed with foil-lined screw caps and placed flat side down about $^3/_4$ inch above 15-watt fluorescent lights (Figure 1). The incident light intensity was measured to be approximately 500 foot-candles. The apparatus was kept in a light-tight room maintained at a constant temperature. A fan circulated air over the bottles to keep the system from warming. A copper-constantan thermocouple was inserted in a suspension in a bottle and the suspension temperature measured. Suspensions were incubated for 6 hours at 38° C. except when the effects of temperature were being studied.

Dark. A constant temperature bath, sided with asbestos and covered with wood, was adjusted to 38° C. Five-gram suspension samples were weighed into black 2-ounce prescription bottles and immersed in the water bath, flat side down, for 6 hours.

Analysis Of Carotene

One milliliter of 1% sodium cyanide and 15 ml. of 95% ethyl alcohol were added to a 5-gram suspension sample in a prescription bottle. The bottle was almost filled with heptane (petroleum ether, 90° to 100° C. fraction), tightly covered with a foil-lined screw cap, and shaken mechanically overnight in darkness at room temperature.

The contents of the bottle were filtered through a sintered-glass funnel and the residue was washed with about 35 ml. of heptane. The filtrate, after being chilled for about an hour in the refrigerator to avoid the formation of an emulsion, was transferred to a separatory funnel. The alcohol-water layer was allowed to separate and drawn off to another separatory funnel, where it was extracted once with heptane. The heptane extracts were combined and washed five times with water to remove alcohol.

A magnesia–Super-Cel chromatographic column was prepared as described by Zscheile and Whitmore (*16*). The heptane extract was drawn through the column under vacuum until about

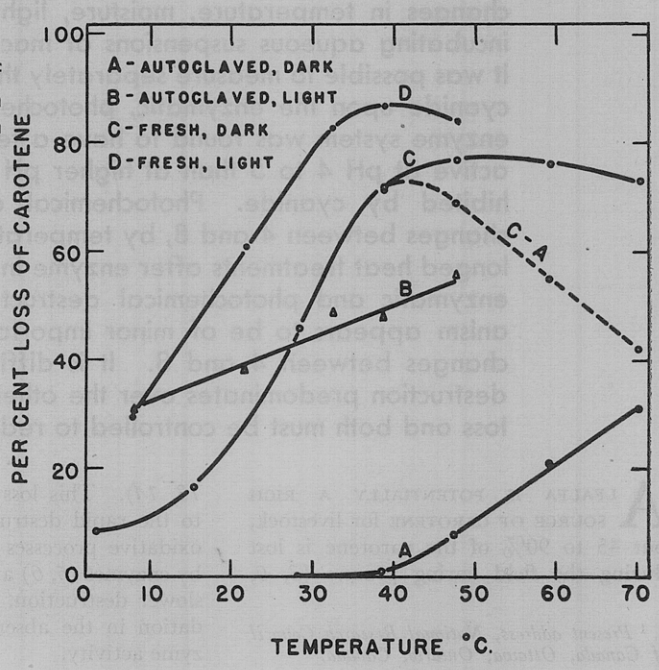

Figure 2. Effect of temperature upon loss of carotene in suspensions

30 ml. remained above the column. The carotene-free eluate was discarded at this point to reduce the final volume. Pigment adsorption was continued until almost all of the heptane extract was in the adsorbing column. The carotene was then eluted with 50 ml. of 10% acetone in heptane. The eluate was made to a final volume of 100 ml. and the absorbance (optical density) of the solution was measured in a Beckman DU spectrophotometer at 436 mμ. The carotene content was calculated using the specific absorption coefficient of 196 (*16*).

Carotene losses during incubation were expressed as per cent loss, as compared to controls which were weighed out at the same time as the experimental samples. During incubation of the experimental samples, losses were inhibited in the controls by adding 10 mg. of sodium cyanide, 15 ml. of 95% ethyl alcohol, and 35 ml. of heptane to each, and storing them at 2° C. in the dark.

Triplicate control samples and duplicate experimental samples were analyzed. All results are averages of the replicates. The differences between replicates were extremely small.

Effect of Temperature Two suspensions were prepared, one of fresh, frozen leaves and one of leaves which had been autoclaved for 1 hour at a steam pressure of 15 pounds per square inch. Each suspension was adjusted to pH 6.0 with 1 *N* sodium hydroxide or 1 *N* hydrochloric acid. No buffers were added because it had been found that the natural plant buffers would maintain the pH satisfactorily during incubation.

A series of samples of each suspension was incubated for 6 hours in the light or dark at measured temperatures ranging from 3° to 70° C. The losses of carotene were measured (Figure 2).

The increased autoxidation at temperatures above 40° C. (curve *A*) prevented a clear graphical demonstration of a sharp temperature optimum for the enzyme (curve *C*). If losses by autoxidation are subtracted from total dark losses (curve *C-A*), the temperature optimum of the enzyme system appears to be approximately 43° C.

Table II. Effect of Autoclaving and Steam-Blanching upon Subsequent Carotene Loss in Suspensions

Heat Treatment	Time of Heating, Sec.	Loss of Carotene, % In Light, No NaCN	In Light, With NaCN	In Dark, No NaCN	In Dark, With NaCN
None	...	87	74	72	54
Steam-blanched	1	88	79	77	51
	5	78	64	64	47
	10	75	63	63	..
	20	72	53	57	37
	30	68	44	46	29
	60	50	42	28	21
	120	60	46	7	6
Autoclaved	5 min.	64	43	18	3
	15 min.	62	55	6	6
	1 hour	54	55	1	3

Curve *B* indicates that a rise in temperature increased the photochemical loss. However, the intensity of light from the fluorescent lamps varied slightly with the temperature (*10*). As carotene destruction in alfalfa is proportional to light intensity around 500 foot-candles (*4*), the losses at different temperatures should be corrected for light intensity changes. The high loss at 47.5° C. should also be corrected for autoxidation. With these two corrections, the measured photochemical loss would vary only about 10% between 7.5° and 47.5° C. The small variation indicates that the photochemical loss may actually proceed independently of temperature.

Effect Of pH Two suspensions were prepared; one of fresh, frozen leaves and one of autoclaved leaves (10 minutes at 15 pounds per square inch). Each suspension was divided into two equal parts. Sodium cyanide was added to one part of each suspension to a final concentration of 0.04 *M* (10 mg. of sodium cyanide per 5 grams of suspension sample).

The pH of the freshly prepared suspensions, without cyanide, was approximately 6. A series of aliquots of each of the four suspensions was adjusted with 1 *N* sodium hydroxide or 1 *N* hydrochloric acid to pH values ranging from 4 to 10. One series of samples of each aliquot was incubated in the dark, another in the light. Losses of carotene during incubation are recorded in Table I.

The enzymatic destruction of carotene was greatly affected by changes in pH over the range of 4 to 10. Increasing the acidity from pH 6 to 4 accelerated the loss of carotene, while increasing the pH decreased the loss. The photochemical loss was not affected markedly by pH changes, although there appears to be a significant rise at pH 10. A good correlation between autoxidation and pH was not observed. However, as in the case of photochemical loss, the destruction was highest at pH 10. The total loss in the fresh leaf suspensions was observed to vary inversely with pH in the range from 4 to 10.

Cyanide partially, but not completely, inhibited the enzymatic destruction of carotene. No significant effect of cyanide was apparent in the suspension of autoclaved leaves.

Effect of Heat Treatments Twenty-gram samples of frozen alfalfa leaves were defrosted by immersion in water for 1 minute, then blanched with live steam or autoclaved at 15 pounds per square inch, for definite periods of time (Table II), and refrozen again as quickly as possible.

An aqueous suspension was made of each sample, adjusted to pH 6.0, and incubated in the light or dark. A parallel series contained 10 mg. of sodium cyanide per sample at a pH of 6.0.

Blanching or autoclaving inhibited the enzymatic destruction of carotene, but the photochemical destruction was not significantly affected by an extended period of autoclaving after enzyme inactivation. Cyanide did not reduce the total loss of carotene in the light in a sample autoclaved for an hour. As the enzyme system was completely inactivated in this sample, it appears that the inhibitory action of sodium cyanide must be upon the enzymatic destruction.

Effect of Cyanide Various amounts of sodium cyanide in 1 ml. of water at pH 6.0 were added to 5-gram aliquots of a suspension. One ml.

Table I. Effect of pH upon Losses of Carotene in Suspensions of Alfalfa Leaves, with and Without Cyanide

Suspension	Incubation	pH 4	5	6	7	8	10
		Loss of Carotene, %					
Fresh	Light	92	86		79	76	60
	Dark	81	82	66	55	42	36
Fresh with NaCN	Light	79		65		58	56
	Dark	64		36		25	20
Autoclaved	Light	50		51		48	73
	Dark	11		8		3	22
Autoclaved with NaCN	Light	48		42		57	59
	Dark	1		4		17	30

of water was added to a control sample. Samples were incubated in the dark.

Addition of 0.001 mg. of sodium cyanide did not significantly inhibit carotene destruction (Table II). A range of cyanide from 0.1 to 137 mg. per sample partially inhibited the enzymatic destruction of carotene. As there was only about 300 mg. of alfalfa leaf dry matter per sample, it can readily be seen that the amount of cyanide required for marked enzyme inhibition was very large. The results indicate that this is not a simple noncompetitive enzyme inhibition.

Discussion

When suspensions were first used, it was hoped that sodium cyanide could be used to inhibit the enzyme system completely (*11*) and thus make possible the measurement of photochemical losses directly during incubation in the light. Although preliminary work indicated that this might be a satisfactory method, further study showed that the inhibitory effect of sodium cyanide was due largely to its strongly basic properties, which shifted the suspension pH from 6 to 9 or 10, thus retarding the enzyme activity. It seems likely that the complete enzyme inhibition by sodium cyanide observed by Mitchell and Hauge (*11*) in aqueous extracts of the enzymes of alfalfa also was due partially to a decided shift in the pH of their system towards basicity.

The evidence further indicates the difficulty of predicting the exact relative significance of enzymatic and photochemical destruction during field curing. Changes in the temperature of drying would affect the enzymatic destruction greatly but have little effect upon the photochemical destructive process. Therefore temperature changes would change the relative significance of the two destructive mechanisms. Furthermore, the two destructive processes probably vary in their importance at the different moisture contents of the hay during drying (*1*). The theory was first expressed by Guilbert (*5*) that photochemical destruction was more important than enzymatic in rapidly drying hay, while enzymatic destruction would predominate in slowly drying hay.

Even under the controlled environmental conditions employed during incubation of the aqueous leaf suspensions, it is difficult to state exactly how much each destructive process contributed to the total carotene loss. Because two major destructive processes are competing for the same substrate, acceleration of one should decrease the rate and importance of the other by simply depriving it of part of the carotene substrate. This is illustrated in Figure 2. At 40° C., the enzymatic loss alone accounted for 73% destruction; the photochemical loss alone was 50%. The total loss which resulted from the simultaneous effects of these two mechanisms was 87%. It is difficult to say how much of this 87% loss was by enzymatic destruction and how much by photochemical destruction.

Carotene was destroyed more rapidly in the suspensions than in whole tissue, undoubtedly because more intimate contact of the reactants causes carotene loss. If one mechanism is accelerated more than the other by the suspension conditions, measurements of the relative destruction by the enzymatic and photochemical mechanisms may not depict the actual relative importance of these mechanisms in intact tissue.

It is interesting to contrast the postulated alfalfa lipoxidase (*11*) with the lipoxidase isolated from soybeans (*15*). The greatest enzyme activity in alfalfa is below a pH of 5, while soybean lipoxidase has greatest activity at about pH 10 and very little activity at pH 5 (*8*). The temperature optimum of the alfalfa enzyme is about 43° C., while the optimum for soybean lipoxidase is about 30° C. Sodium cyanide does not affect the activity of the soybean lipoxidase but the alfalfa enzyme appears to be partially inhibited by sodium cyanide. These observations indicate that the two enzymes differ greatly in their properties. Furthermore, this may indicate that alfalfa contains two enzymes which destroy carotene.

Table III. Effect of Sodium Cyanide upon Enzymatic Destruction of Carotene in Suspensions

Mg. NaCN per 5-Gram Suspension Sample	Loss of Carotene, %
0	71
0.001	72
0.1	64
1.0	62
10.0	52
34.2	37
68.5	32
137.0	26

Summary

By incubating aqueous suspensions of macerated alfalfa leaves under controlled environmental conditions, it was possible to measure separately the effects of temperature, pH, heat treatments, and cyanide upon the amount of carotene destruction by enzymatic, photochemical, and autoxidative mechanisms, thus avoiding the variations resulting from physical alterations of alfalfa tissue which accompany the usual methods of preservation.

The carotene-destroying enzyme system in alfalfa appears to have a temperature optimum of approximately 43° C. The photochemical destruction does not appear to be sensitive to temperature changes.

Enzymatic losses of carotene were progressively inhibited by increases in pH from 5 to 10.

Heat treatments inactivate the carotene-destroying enzyme system, but continued autoclaving for 1 hour did not cause any marked increase in the lability of carotene to subsequent photochemical destruction.

Cyanide partially inhibits the enzymatic losses of carotene in alfalfa.

The loss of carotene in alfalfa during preservation cannot be satisfactorily reduced unless both the enzymatic and the photochemical mechanisms can be controlled. Inhibition of one mechanism merely leaves more carotene substrate for destruction by the other mechanism.

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Received for review August 17, 1953. Accepted September 8, 1953. Journal paper No. 738, Purdue University Agricultural Experiment Station. Investigation supported in part by a grant from Indiana Farm Bureau.