[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF AGRICULTURE, UNIVERSITY OF WISCONSIN]

The Effect of Acids on Carotenoids[1]

BY FORREST WARD QUACKENBUSH, HARRY STEENBOCK AND WILLIAM HAROLD PETERSON

The action of acids on carotenoids has become a matter of practical importance as well as scientific interest in recent years. The ensiling of legumes with mineral acids, *e. g.*, mixtures of hydrochloric–sulfuric (the A. I. V. process) and phosphoric, has produced a silage which often appears to have not only all the carotene of the green alfalfa but in some cases more carotene than is found in the original herbage.[2-4] Such results naturally have raised doubts regarding the validity of the data and have indicated the need of more information regarding the character of the pigments contained in the so-called carotene fraction.

The work described in this paper shows that several carotene-like pigments are produced by the action of acids on green forage, that these pigments have no vitamin A value, and that they may to some extent be secreted into milk.

Experimental

Effect of Mineral Acid on the Apparent Carotene Content of Alfalfa.—The samples (forage or silage) were extracted with hot alcohol, the extract was saponified with alcoholic potassium hydroxide, and the carotenoids were obtained by shaking with several portions of benzine (Skellysolve B, b. p. 65–75°). The united extracts were washed with water and the non-carotene pigments (xanthophyll, etc.) removed by extracting with an equal volume of 85% ethanol in four successive portions. The residual carotene and the extracted xanthophyll were determined spectrophotometrically. The results agreed satisfactorily with the values obtained by the method of Willstätter and Stoll.[5]

Typical analytical data (Table I) showed increases in the carotene values in alfalfa which had been treated with mineral acids. It was observed that while the carotenoid pigments from fresh green alfalfa separated sharply between benzine and 85% ethanol, those from acidified alfalfa did not. With fresh alfalfa the alcohol was practically colorless after three extractions of the benzine solution but with silage or acidified fresh alfalfa even the fourth extract was highly colored. Continued extraction did not result in a sharper separation, and the use of higher concentrations of alcohol gave only slightly better results. The immediate cause of these analytical difficulties became clear when the carotene fractions were subjected to chromatographic analysis.

TABLE I

INCREASE IN "CAROTENE" FRACTION FROM ALFALFA AFTER TREATMENT WITH MINERAL ACIDS

	Sample and treatment	Carotenoids (gamma/g. dry tissue) Carotene	Xanthophyll
1	Green alfalfa	126	225
2	A. I. V. silage from above alfalfa	184	190
3	Green alfalfa	193	368
4	Green alfalfa acidified before extraction	261	134
5	A. I. V. silage from above alfalfa	302	298

Chromatographic Separation of the Carotene-like Pigments.—In the first experiments magnesium oxide (Merck U. S. P. X, heavy) was used to make the chromatographic column. The benzine solution was evaporated to dryness in a vacuum and the residue taken up with a small volume of benzine. The concentrated solution was poured on the adsorbent and the column washed with benzine to a maximum separation of the bands.

Three sharp bands made their appearance from the carotene fraction of green alfalfa. These were identified by their relative positions in the chromatogram, by their spectral absorption, and by their phasic distribution between benzine and 85% ethanol as due to α-carotene, β-carotene and lutein, respectively. β-Carotene preponderated in quantity. Acidified forage gave a fourth band almost as wide as that of β-carotene. It occupied a position in the column so close to lutein that, even after long washing, the two almost merged. The upper portion of the new band was dull red and the lower portion yellow-orange. To effect their separation the column was removed, and divided; the pigments were eluted with ether, transferred to benzine, and readsorbed on fresh magnesium oxide. By repeating this fractionation several times, small quantities of red and yellow pigments, which appeared to be free from each other, were obtained. In benzine the yellow pigment exhibited sharp maximal spectral absorption at 448 and 477 mμ. The red pigment showed no distinct maxima, but a general absorption throughout the range between these two regions.

A search was made for a more satisfactory method for separating the pigments and calcium carbonate, magnesium carbonate and aluminum oxide were found to be better than magnesium oxide. Benzene, ether, and carbon disulfide gave no better results than benzine. However, good separation was obtained in a magnesium oxide column by washing with benzine containing a small quantity of absolute alcohol. The pigments in the carotene

(1) Published with the approval of the director of the Wisconsin Agricultural Experiment Station.

(2) Virtanen, *Biochem. Z.*, **258**, 251 (1933).

(3) Peterson, Bohstedt, Bird and Beeson, *J. Dairy Science*, **18**, 63 (1935).

(4) Peterson, Bird and Beeson, *ibid.*, **20**, 611 (1937); Shinn, Kane, Wiseman and Cary, *J. Biol. Chem.*, **119**, lxxxix (1937).

(5) Willstätter and Stoll, "Untersuchungen über Chlorophyll. Methoden und Ergebnisse," Verlag von Julius Springer, Berlin; Wiseman and Kane, *J. Biol. Chem.*, **114**, cviii (1936).

fraction from silage separated into six major bands in addition to those of γ- and β-carotene. There were also some minor bands but these contained insufficient pigment for characterization. For convenience the six major bands were given the alphabetical designations, A to F.

The method finally adopted for separating the pigments was as follows. The benzine solution was forced into a uniformly packed column of magnesium oxide by means of air or nitrogen under a pressure of 1 to 1.5 atmospheres. The chromatogram was washed with benzine until the carotene bands had separated from the other pigments. Washing was then continued with a mixture of absolute ethanol (1% by volume) in benzine until bands A, B, and C had clearly separated and the carotene bands had passed out of the column. By increasing the alcohol to 2%, bands, D, E, and F were separated and bands, A, B, and C were eluted consecutively. Bands D, E, and F were eluted by increasing the concentration of alcohol, first to 3% and then to 5%. When the several eluates were collected and their extinction coefficients measured (475 mμ), it was found that less than 10% of the total pigment had been lost. A diagram of a typical chromatogram obtained with the carotene fraction from A. I. V. alfalfa silage is shown in Fig. 1.

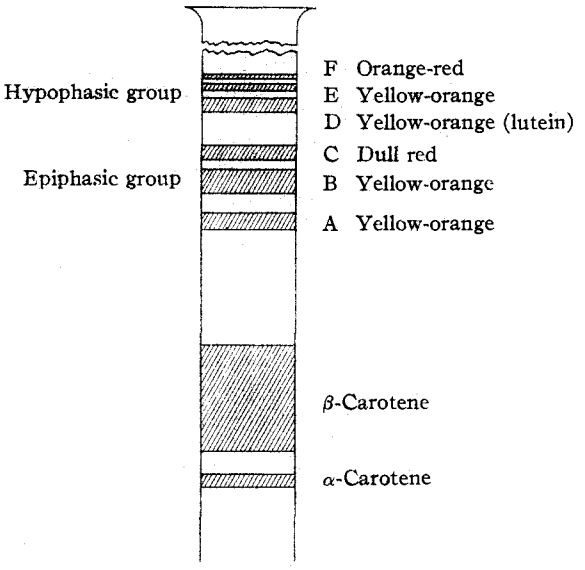

Fig. 1.—Diagram of a magnesium oxide chromatogram showing the chief carotenoid bands obtained from A. I. V. alfalfa silage.

Properties of the Pigments.—Besides occupying different positions in the chromatogram, the six pigments exhibited dissimilarities in color, spectral absorption, and phasic distribution. In the magnesium oxide column, pigments A, B, D, and E were a deep yellow, pigment C a dull brick-red, and pigment F an orange-red. In dilute benzene solution pigments A, B, D, and E were greenish-yellow and pigments C and F were reddish.

Pigments A, B, D, and E gave absorption bands which were essentially identical in form and in position of their maxima; all conformed to published data for lutein. Pigment C gave an abnormally wide absorption band which was highest in the region between 448 and 476 mμ (in benzine) but without any distinct maximum. On the other hand pigment F exhibited two distinct, though not sharp, maximal absorption bands. The absorption values of the pigments in three different solvents are given in Table II.

TABLE II

REGION OF MAXIMAL SPECTRAL ABSORPTION OF THE SIX PIGMENTS FROM ALFALFA SILAGE

Pigment	Solvent: CS_2	$CHCl_3$	Benzine
A	475–476	457	447
	506	486–487	477
B	475–476	457	447–448
	506	486–487	477
C	480–504	457–486	448–476
D	475–476	456–457	447–448
	506	486–487	477
E	476	457	447–448
	506	487	476–477
F	477–484	457–464	449–453
	502–509	486–491	478–481

The phasic distribution of the six pigments was especially significant. Pigments A, B, and C were distinctly epiphasic, while D, E, and F were hypophasic to the same degree. Their percentage distribution as shown in Table III was obtained by shaking the benzine solutions of the individual pigments, in similar concentrations (having equal extinction coefficients), with equal volumes of 85% ethanol.

TABLE III

PHASIC DISTRIBUTION OF THE PIGMENTS BETWEEN BENZINE AND 85% ETHANOL

(Expressed in per cent. of total)

Pigment	Isolated from silage: Benzine	Isolated from silage: 85% EtOH	Obtained from lutein by acid treatment: Benzine	Obtained from lutein by acid treatment: 85% EtOH
A	81	19	79	21
B	84	16	79	21
C	81	19	[a]	[a]
D	22	78	21	79
E	23	77	22	78
F	22	78	..	..

[a] Only a trace; insufficient for measurements.

In the hope of obtaining the epiphasic pigments in crystalline condition, the carotene fraction from 10 kg. of acidified alfalfa was fractionated chromatographically. The resulting benzine–alcohol eluates of pigments A, B, and C were concentrated to small volumes and allowed to stand in a refrigerator for several weeks. Although these solutions were many times more concentrated than a saturated solution of β-carotene, the pigments failed to crystallize. Attempts to crystallize them from ethanol, benzene plus methanol, and carbon disulfide were likewise unsuccessful.

Origin of the Pigments.—The apparent increase in the carotene content of alfalfa silage prepared with mineral acids was accompanied by a decrease in xanthophyll (Table I). This suggested that the new pigments were formed from the xanthophyll pigments by the action of acids. When the crude xanthophyll fraction from un-

treated alfalfa was refluxed with acid as is done in extracting silage, the solution was almost completely decolorized. When an acidified solution of the crude xanthophyll, 0.1 *N* hydrochloric acid in 85% ethanol, was held at 45–50° overnight, its extinction coefficient decreased 25%. When this solution was made slightly alkaline with potassium hydroxide (in 85% ethanol) and shaken with benzine 65% of the pigment passed into the benzine phase. A control sample of crude xanthophyll lost none of its color when subjected to the same treatment except for the addition of acid, and only 17% of the pigment passed into the benzine phase.

To determine the effect of acid on lutein, pure lutein was prepared from fresh green alfalfa by two successive adsorptions on magnesium oxide from alcohol–benzine mixtures. The second eluate produced typical lutein crystals. When these were dissolved in 85% ethanol, the resulting solution showed a value $E^{1\ cm.}_{475} = 4.0$. This is equivalent approximately to 0.20 mg. of lutein per cc. Ten-cc. portions of this solution were set aside at 45–50° in the dark, for fourteen hours, after the addition of various acids. The solutions were then made slightly alkaline with potassium hydroxide, and each was adjusted to a volume of 20 cc. with 85% ethanol, and shaken with 20 cc. of benzine. When equilibrium was attained, the percentage distribution between the two solvents was determined spectrophotometrically. That the stronger acids produced marked changes in the solubility of the pigment was shown by the values obtained for the benzine phase (Table IV). The values for the 85% ethanol phase represent the difference between these figures and 100.

TABLE IV

EFFECT OF VARIOUS ACIDS UPON LUTEIN

Sample	Normality and kind of acid	% of pigment remaining after treatment	% of remaining pigment found in the benzine phase	Chromatographic distribution of pigments after treatment (Total pigment remaining = 100% in each case) A	B	D	E
1	None	100	20			100	
2	*N*/40 acetic	80	24	10	Trace	90	
3	*N*/40 lactic	98	38	28	5	62	5
4	*N*/40 oxalic	98	71	69	17	7	7
5	*N*/40 H_2SO_4	75	70	56	36	6[a]	
6	*N*/80 HCl	57	65	60	18	16[a]	
7	*N*/40 HCl	65	69	60	25	11[a]	
8	*N*/20 HCl	65	71	51	32	12[a]	
9	*N*/5 HCl	31	72	6	74	Trace	Trace

[a] Pigments from bands D and E combined.

The chromatogram yielded not less than three and in some cases five well-defined bands which in their position and appearance resembled strikingly the bands obtained from silage. Treatment with acetic acid produced bands A, B, and D. All other acids produced one additional band, E, and in two cases a fifth band, C. Lutein subjected to all the manipulations except acidification retained its chromatographic behavior unchanged.

The individual pigments were collected quantitatively as they were eluted from the chromatogram. The extinction coefficient and solution volume were then measured for each fraction. From these data the percentage of each pigment in the sample was calculated (Table IV). Care was taken to maintain a fairly constant volume of eluate, and to make the spectrophotometric readings with solution depths which would give log I/I_0 values very close to 0.50. Close agreement was then obtained between the values for total pigment before and after separation.

The phasic distribution and spectral absorption of pigments A, B, D, and E obtained from lutein were found to be identical with those of the corresponding pigments found in silage (Tables II and III).

Pigment D proved to be residual lutein. When the various D-fractions from all of the samples were combined and analyzed chromatographically, the pigment remained almost quantitatively in a single band; other pigments appeared only in traces. When a sample of lutein was mixed with the purified pigment D, and a chromatogram prepared, again a single band appeared. However, when pure lutein was mixed with any of the other three purified pigments, *viz.*, A, B, and E, two bands appeared in the chromatogram. Analyzed separately each produced a single band.

From the various data which have been presented it is concluded that pigments A, B, and E are derived from lutein by the action of acids. The origin of pigments C and F has not as yet been clearly ascertained.

Occurrence and Distribution of the Acid-derived Pigments.—When it was found that the acid-derived pigments could be extracted from freshly acidified alfalfa, the question arose as to whether they were products of ensiling or artifacts formed during extraction under acid conditions. To determine this a sample of alfalfa silage was made slightly alkaline with potassium hydroxide before extraction and its carotenoids were compared with those extracted from silage in the usual way, *i. e.*, without alkalization. The epiphasic fractions from these two extracts were found to contain equal quantities of total pigment, and the chromatograms from both fractions contained the eight bands previously described (Fig. 1).

It is evident from the results presented in Table IV that mineral acids are more effective than lactic or acetic acid in converting lutein to pigments A and B. It might be predicted that a carotene fraction from silage produced by a natural lactic acid fermentation would contain a relatively higher percentage of true carotene than that from silage prepared with mineral acids. This was found to be the case (Table V). Only 57% of the pigment in the carotene fraction from the A. I. V. silage was found in the carotene

TABLE V

RELATIVE PROPORTIONS OF THE CAROTENOID PIGMENTS IN ALFALFA FORAGE FOLLOWING VARIOUS TREATMENTS OF IDENTICAL MATERIAL

Sample	Percentage distribution Epiphasic pigments Carotene	A	B	C	Hypophasic D + E + F	Recovery after chromatographic separation, %
Oven-dried alfalfa	85	..	..	..	15 (D only)	...
A. I. V. alfalfa silage—total pigments	38	7	14	4	37	90
A. I. V. alfalfa silage—carotene fraction	57	9	19	5	10	100
Molasses alfalfa silage—total pigments	50	4	7[a]		39	96
Molasses alfalfa silage—carotene fraction	75	5	9[a]		11	101

[a] Pigment from bands B and C combined.

bands of the chromatogram as compared with 75% for the naturally fermented silage.

Since silages constitute a considerable part of the ration of the dairy cow, it is important to know whether the acid-derived pigments are present in butter fat produced on such rations. Four samples of butter fat which have been analyzed contained such pigments. Gillam and Heilbron[6] have reported that the chief carotenoids in butter fat are carotene, kryptoxanthin and lycopene. The presence of kryptoxanthin was confirmed, but lycopene was not detected in our samples.

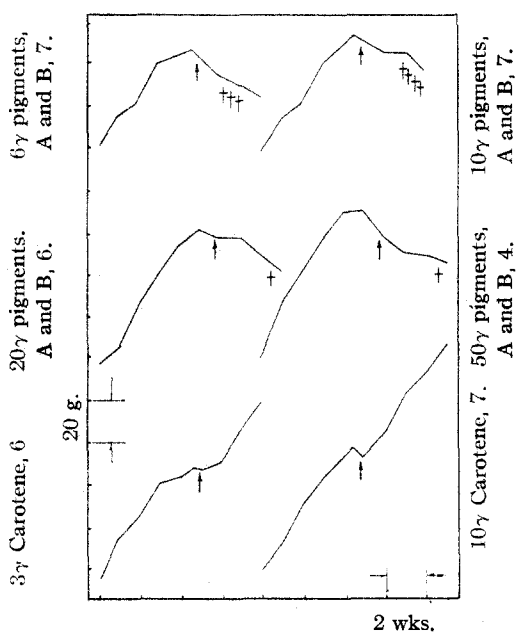

Fig. 2.—Growth curves of rats which received β-carotene and pigments A and B.

Biological Assay of the Epiphasic Pigments, A and B.—The epiphasic pigments, A and B, were tested for vitamin A activity before our technique for chromatographic fractionation had been fully developed. The tests were made on a mixture of the two pigments in which pigment A was present in somewhat larger amounts than pigment B. The assays were executed essentially according to the technique of Baumann and Steenbock.[7] The control rats were fed pure β-carotene separated chromatographically from extracts of fresh green alfalfa. To facilitate convenient dosage the pigments were incorporated in Wesson oil by evaporating their concentrated benzine solutions with the oil under reduced pressure. Resumption of growth, cure of ophthalmia, and the production of normal vaginal smears were employed as criteria of vitamin A activity. The β-carotene was fed at levels of 3 and 10 gamma daily. The mixture of pigments A and B was fed at levels equivalent to 6, 10, 20 and 50 gamma of β-carotene based on the extinction coefficients of their solutions. While the feeding experiments were in progress the oil solutions were checked for deterioration from time to time by spectrophotometric methods.

The data show clearly that the epiphasic pigments, A and B, could not function as precursors of vitamin A (Fig. 2). While the daily administration of 3 gamma of β-carotene restored growth and normal oestrus and cured the ophthalmia completely, even the highest dosage with pigments A and B, equivalent to 50 gamma of β-carotene, failed to restore even normal oestrus.

Discussion

When the separation of the non-carotene pigments into six distinct bands was first observed, its significance was viewed with some suspicion. It was well known that certain extraneous substances can affect the distribution of pigments in a chromatogram. Furthermore, it has been reported[8] that carotenes may undergo profound changes during chromatographic adsorption. However, the possibility that the pigments were of chromatographic origin is not supported by the experimental evidence. The non-carotene portion of the chromatogram from silage was unlike that from fresh alfalfa. When the chromatogram from alfalfa was washed with benzine–alcohol mixtures, bands A, B, and C failed to make their appearance; practically all of the pigment remained in band D. Also, after the initial separation, each of the new pigments gave a single characteristic band when chromatographed by itself. And, finally, besides differing in their chromatographic behavior, the individual pigments showed other differences in physical properties, *e. g.*, solubility and in some cases spectral absorption.

It is probable that Kuhn, *et al.*,[9] dealt with a mixture of pigments A, B, D, and E when they treated lutein with oxalic acid. The severity of their treatment was not markedly different from ours, for while they used a lower concentration of acid they employed a higher temperature. Although they did not differentiate their resultant pigment from lutein in its chromatographic behavior or spectral absorption, it was distinctly more epiphasic than lutein. We likewise did not succeed at once in separating our pigments from lutein chromatographically although we used a number of adsorbents and solvents. It was only later through a fortunate choice of mixtures of solvents that we were able to effect a good separation.

It is evident that the pigments derived from lutein are the chief cause of the abnormal caro-

(6) Gillam and Heilbron, *Biochem. J.*, **29**, 834 (1935).

(7) Baumann and Steenbock, *Science*, **76**, 417 (1932).

(8) Gillam and El Ridi, *Biochem. J.*, **30**, 1735 (1936).

(9) Kuhn, Winterstein and Lederer, *Z. physiol. Chem.*, **197**, 141 (1931).

tene values reported for A. I. V. silage. The extent to which the apparent carotene values differ from the true values is dependent on such factors as strength of acid used, lutein content of the forage, temperature of the silage and period of ensiling. While the usual method of analysis may give carotene figures that are nearly twice the true values for A. I. V. silage, too high results are also obtained in the analysis of silages made by normal fermentation processes. The need for a more reliable method of determining carotene in these silages is obvious.

Chromatographic data on butter indicate the presence of the same pigments as those found in silage. Although the amount of these new pigments has not been determined, their presence points to the need of more information regarding the value of acid-preserved silages for increasing the vitamin A potency of milk.

Summary

Five new carotenoids, designated as pigments A, B, C, E, and F, have been obtained from alfalfa silage and from acidified fresh alfalfa. They were not present in untreated forage. Dilute solutions of A, B, and E in benzine were greenish-yellow and those of C and F were reddish.

The pigments were partially fractionated by their phasic distribution between benzine and 85% ethanol; A, B, and C were epiphasic, E and F were hypophasic. Quantitative separation was effected by use of the magnesium oxide chromatogram and elution with benzine–alcohol mixtures. The bands of A, B, and C formed below that of lutein; those of E and F above it. The pigments were eluted from the column in the alphabetical order given by gradually increasing the percentage of alcohol in the solvent. Spectral absorption of the pigments was markedly less valuable for differentiation than chromatographic behavior. Absorption curves of A, B, and E were essentially the same as those of lutein; those of C and F showed no well-defined maxima.

A high order of solubility in the usual carotenoid solvents was shown by A, B, and C. Attempts to effect their crystallization were not successful.

A, B, and E were produced from lutein in large amounts by treatment with either 0.025 hydrochloric or sulfuric acid, and in smaller amounts with 0.025 *N* lactic and acetic acids. The stronger acids favored the production of B; weaker acids the production of A. The origin of C and F was not clearly established.

Inasmuch as the usual methods of carotene analysis fail to differentiate between carotene and pigments A, B, and C, values obtained by these methods on silages, especially those prepared with mineral acids, are obviously too high.

When fed to vitamin A deficient rats, A and B exhibited no biological activity. Both pigments were found in butters produced by cows on A. I. V. silage.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

Researches on Pyrimidines. CLIX. Synthesis of 6-Benzyl- and 5-Benzyluracils[1]

BY TREAT B. JOHNSON AND JOSEPH C. AMBELANG[2]

One conclusion predictable from an examination of the skeleton structure of the pyrimidine cycle as represented by formula II, is the existence of two interdependent zones of chemical activity which envelop the pyrimidine molecule. Each of these zones is characterized by its encompassment of a specific organic structure. Zone "A" includes the unsaturated amidine portion of the pyrimidine cycle, while zone "B" incloses the cyclic allyl structure of the molecule. Any alteration or tautomeric change in the specific, unsaturated structure embraced by one zone necessarily leads to a corresponding change in the constitution of the grouping in the other, and also its chemical reactivity. In other words, we are dealing with a dual system of reactions in our study and development of pyrimidine chemistry and if tautomeric structures be accepted for these unsaturated cyclic groupings in the different zones, as theory provides, positions 1 and 3 included in zone "A" and positions 4 and 6 in zone "B," respectively, are identical.

(1) Constructed from a portion of a dissertation presented by Joseph C. Ambelang, in June, 1938, to the Graduate Faculty of Yale University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(2) Sterling Professorship of Chemistry Research Assistant 1938–1939.