column chromatography. A portion of solution E was lyophilized, and 100 mg. of the dried substance was placed on a silicic acid column (1 × 9 cm.). The column was developed with benzene–ethyl ether–ethyl alcohol, 50:15:2. The eluted fractions were collected and weighed. Progress in the fractionation was followed by means of glass fiber paper chromatograms, using the equipment and procedures described by Dieckert and Reiser (*4*) and modified by Dieckert and Morris (*2*). The glass fiber paper (Reeve Angel, No. x-934-AH) was impregnated with silicic acid (*1*). The developing solvent for the paper chromatograms was the same as that used to develop the silicic column. After the solvent had evaporated from the paper, the chromatogram was sprayed with concentrated sulfuric acid and heated, first mildly, over a hot plate for the development of color, and finally more vigorously to char the organic substances to produce black spots on the white background (*5*).

About the first 8% of the dried eluate from the column contained substances that moved with the solvent front on the glass paper. The next 15% showed a spot well separated from both the front and the origin. This spot showed a pink to violet color when the chromatogram was sprayed with concentrated sulfuric acid and heated mildly. The R_f for the spot corresponded to that produced by an authentic specimen of β-sitosterol-D-glucoside. In Figure 2, *A* is a chromatogram of solution E, *B* one of authentic β-sitosterol-D-glucoside, *C* one of the glucoside separated from the silicic acid column, and *D* one of the glucoside crystallized from solution E.

The glucoside was hydrolyzed according to the method of Power and Salway (*6*). Five milligrams was added to a mixture composed of 3 ml. of amyl alcohol, 5 ml. of 15% aqueous HCl, and 25 ml. of methanol. The resulting solution was refluxed for 6 hours and the amyl alcohol removed by steam distillation.

The aglucone was removed from the aqueous solution by extraction with diethyl ether. It was then crystallized from methanol (m.p. 136–38° C.), with no depression of the melting point on admixture with authentic β-sitosterol. Analysis: C, 83.0; H, 12.3. Calculated for β-sitosterol: C, 83.99; H, 12.15.

The aqueous solution was treated with an excess of silver carbonate and filtered. The filtrate was chromatographed on glass fiber paper which had been impregnated with monopotassium phosphate in accordance with the method of Dieckert and Morris (*3*). The R_f value for the sugar obtained from the hydrolyzate was the same (0.78) as that obtained with D-glucose when the chromatogram was developed with acetone–*n*-propyl alcohol–water, 50:40:10.

The infrared spectra (potassium bromide disk method) of the glucoside isolated from peanut flour and of authentic β-sitosterol-D-glucoside showed the two compounds to be identical (Figure 3). The infrared spectra of the aglycone of the glucoside from peanut flour and of authentic β-sitosterol showed these compounds to be identical also (Figure 4).

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CAROTENOID EPOXIDE DETECTION

An Improved Test for Carotenoid Epoxides

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WHEN ETHER solutions of certain carotenoids are shaken with concentrated hydrochloric acid, the acid layer turns blue (*7*). Most of these carotenoids are epoxides (a number of which occur in nature) or their furanoid transformation products, which are referred to here as 5,6-epoxides and 5,8-epoxides, respectively, following the nomenclature of Goodwin (*5*). In general, a much deeper blue color forms with diepoxides than with monoepoxides (*7*); hydroxyl groups increase the stability and intensity of the blue color (*6*). An improved test has been developed for detection of carotenoid epoxides and for differentiation of diepoxides and monoepoxides; a modification of this test can be used to distinguish 5,6-epoxides from 5,8-epoxides.

Experimental

Materials. The carotenoid fractions used were obtained from known sources, especially oranges (*4*), cling peaches (*1*), and leaves (*3*); capsanthin and capsorubin were obtained from red garden peppers, and cryptoxanthin mono- and diepoxides from Meyer lemons (*2*). The identifications were based on spectral absorption curves, and on behavior in countercurrent distribution, chromatography, and acid treatment. A sample of β-apo-2-carotenal was kindly provided by the late G. F. Siemers, Hoffman-La Roche Inc., Nutley, N. J.

Observations on the Hydrochloric Acid–Ether Test. Hydrochloric acid–ether tests were made by the procedure previously described (*4*) on many carotenoid fractions obtained from various fruits and leaves. A deep blue color was formed by violaxanthin (zeaxanthin 5,6,5′,6′-diepoxide), its 5,8-epoxide isomerization products, and some polyol monoepoxides. In other cases (such as cryptoxanthin diepoxides and neoxanthin) a definite, but weaker color was formed, and in still other cases (mutatoxanthins and cryptoxanthin monoepoxides) the blue color was pale or uncertain. Capsanthin (a diol ketone) gave a light salmon color in the hydrochloric acid layer, capsorubin (a diol

An improved hydrochloric acid color test for carotenoid 5,6- and 5,8-epoxides has been developed in which the reaction is carried out in one phase of ether-methanol. Diepoxides give greenish blue colors; monoepoxides, usually yellow-green; while two monoepoxides of unknown structure, persicaxanthin and sinensiaxanthin, give red-violet colors. A modified test, using citric acid instead of hydrochloric acid, can be used to detect the presence of 5,6-epoxides. Both of these tests can be readily followed spectrophotometrically. Contrary to earlier reports, neither β-apo-2-carotenal nor capsorubin gave a blue color in the hydrochloric acid–ether test.

diketone) a stronger salmon color, and trollein (a nonepoxide polyol) (*4*) a rather strong yellow color; some polyol monoepoxides gave green colors. β-Apo-2-carotenal gave a negative test. It was reported that the latter substance gives an intense, stable blue coloration in this test (*8*); while capsorubin which was reported earlier (*7, 9*) yields a deep blue color. It appears that for the last two, the preparations used in the earlier work had epoxide impurities.

Ether is much more soluble in concentrated hydrochloric acid than it is in water; about 2.2 volumes of ether (U.S.P.) dissolve in one volume of acid. The resulting solution has an appreciable solubility for polyoxygen carotenoids; hence these may give a color in the lower layer, although they are not epoxides.

The substance or substances responsible for the blue color are unknown. A solution of violaxanthin in ether was extracted repeatedly with concentrated hydrochloric acid until the extract was only a pale blue. The extracts were added to a mixture of ether and water, and potassium hydroxide in methanol was added until the blue color disappeared; on mixing, the ether layer became red-orange. The recovered pigment on chromatography yielded a number of bands. The strongest appeared to be a *cis*-mutatoxanthin (spectral absorption maxima in benzene, 460, 434, and 319 mμ), but the others did not resemble known carotenoids. Hence, the blue compound did not revert to the 5,8,5′,8′-diepoxide on neutralization.

The Hydrochloric Acid – Ether–Methanol Test for Carotenoid Epoxides. Formation of a blue color with hydrochloric acid and carotenoid epoxides occurs in other solvents such as methanol, ethanol, and acetone. It forms rapidly in acetone, but with some carotenoids there is a considerable increase in yellow or orange color. Not all carotenoids are readily soluble in methanol or ethanol; a 1:1 mixture of ether and methanol will dissolve both carotenoids and concentrated hydrochloric acid. The color formed is not affected by partition of the carotenoid between the two phases as in hydrochloric acid–ether. A convenient amount of carotenoid is that equivalent to 20 ml. of solution having an absorbance at the principal maximum of 1 in a 1-cm. cell. The procedure is as follows:

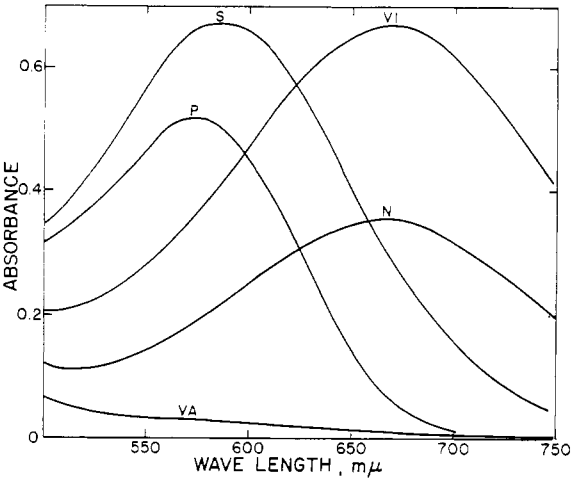

Figure 1. Absorbance at wave lengths 500 to 750 mμ of solutions of carotenoids in ethyl ether–methanol–concentrated hydrochloric acid, 10 to 9 to 1 by volume

N. Neoxanthin, 7 hours
P. Persicaxanthin, 40 minutes
S. Sinensiaxanthin, 2 hours 20 minutes
VA. Valenciaxanthin, 4 hours 35 minutes
VI. Violaxanthin, 7 hours
Initial absorbance of controls at principal maxima ca. 1.9

Evaporate two equal aliquots of carotenoid solution in vacuo, preferably in a rotary evaporator. Dissolve the residues in 10 ml. of ether (preferably U.S.P., peroxide free), and add 9 ml. of methanol. Add 1 ml. of water to one solution as a control, and 1 ml. of concentrated hydrochloric acid to the other. Compare the colors of the two solutions in similar test tubes. The acid solution will often become greener at once if an epoxide is present. At the end of 1 hour, acid-treated diepoxide solutions will be greenish blue, monoepoxides usually yellowish green. Nonepoxides are usually a little paler than the controls, but some *cis*-isomers may show an increase in the yellow or orange color, as does also valenciaxanthin (a 5,6-epoxide). A red-violet color indicates presence of epoxides such as persicaxanthin or sinensiaxanthin with a shorter conjugated double bond system.

In the above test, the blue colors formed with typical mono- and diepoxides are similar, but the residual color of the carotenoid is different. 5,6-Monoepoxides on treatment with acid are rapidly converted to 5,8-epoxides with a decrease in wave length of the spectral absorption maxima of about 20 mμ, while with 5,6,5′,6′-diepoxides the decrease is over twice as great. The color change is more pronounced with 5,6-epoxides than with 5,8-epoxides.

Test for Carotenoid 5,6-Epoxides. The following simple test for distinguishing 5,6-epoxides from 5,8-epoxides or nonepoxides is based on the shift in spectral absorption maxima of the 5,6-epoxides on contact with acid. In order to avoid formation of a blue or green color, citric acid is used instead of hydrochloric acid. The procedure is as follows:

Pipet out equal aliquots of the carotenoid solution (a convenient amount is the equivalent of 10 ml. of solution with

an absorbance at the principal maximum of 1 in a 1-cm. cell). Evaporate in vacuo and dissolve the residues in 5 ml. of ether and transfer to test tubes of the same diameter. To one, add 5 ml. of methanol and to the other, 5 ml. of a 1% solution of citric acid in methanol. Stopper and allow to stand for 1 hour. If a 5,6-epoxide is present, the solution with acid added will become definitely paler when compared crosswise. Slight decreases in color may be observed in nonepoxide carotenoids when viewed vertically, probably due to *trans–cis*-isomerization. With valenciaxanthin and persicaxanthin, which have very pale colors initially, there is a marked increase in greenish fluorescence in ultraviolet light.

This test is positive for 5,6-, 5,6,5′,6′-, and 5,6,5′,8′-epoxides, but negative for 5,8- and 5,8,5′,8′-epoxides and for nonepoxides. In this test, 5,6,5′,6′-diepoxides show a much greater color change than 5,6-epoxides or 5,6,5′,8′-diepoxides.

Table I. Absorption Maxima of Blue Colors Formed by Addition of Hydrochloric Acid to Solutions of Carotenoids in Methanol-Ether

No.	Component	Type of Epoxide	Absorbance Maxima of Control	Absorption Maxima Formed in Presence of Hydrochloric Acid: Time, hr.	Wave length, Mμ	% absorbance of principal maximum of control
1	Cryptoxanthin	5,8-epoxide	451, 426, (404)[a]	1	673	15
2	Cryptoxanthin	5,8,5′,8′ diep.	426, 401, 375	1	623	23
3	Mutatoxanthin [a]	(5, 8)	450, 424, (404)	1	670	5
4	Mutatoxanthin [b]	(5, 8)	451, 426, (406)	1	676	12
5	Violaxanthin	(5, 6, 5′, 6′)	470, 439, 416	0.25	667	13
6	Luteoxanthin [a]	(5, 6, 5′, 8′)	449, 422, 399	1	654	26
7	Luteoxanthin [b]	(5, 6, 5′, 8′)	448, 422, 398	1	674	16
8	Auroxanthins	(5, 8, 5′, 8′)	426, 401, 380	1	636	26
9	Neoxanthin	(5, 6)	465, 436, 413	1	665	10
10	Neochrome [a]	(5, 8)	448, 422, 398	1	666	7
11	Neochrome [b]	(5, 8)	448, 421, 398	0.25	667	17
12	Valenciaxanthin	(5, 6)	390, 369, 351	1	...	1.5 (at 545 mμ)
13	Persicaxanthin	(5, 6)	392, 371, 353	1	566	29
14	Sinensiaxanthin	(5, 6)	427, 402, 380[b]	0.67	586	36

[a] Values in parenthesis represent shelves or humps (not maxima) on spectral absorption curves.
[b] In benzene.

Spectrophotometric Observations. The above tests can conveniently be adapted to a spectrophotometer. The development of the blue color was studied for numerous carotenoid epoxides, using a Cary Model 14. The absorbance at the principal maximum of the control (10 ml. of U.S.P. ether, 9 ml. of methanol, and 1 ml. of water) was about 1.9 in a 1-cm. cell. A duplicate aliquot was evaporated in vacuo, the residue dissolved in 10 ml. of ether and 9 ml. of methanol, and 1 ml. of concentrated hydrochloric acid was added. Spectrophotometric curves were run as soon as possible, and then at intervals up to 24 hours, over the range 750 to 300 mμ. In the initial curves, the 5,6-epoxides showed a complete conversion to 5,8-epoxides and a single broad maximum appeared at wave lengths above 550 mμ (Figure 1). The latter increased in magnitude with time, in some cases reaching a value 50 or 60% of that of the principal maximum of the control in 24 hours or less, while the maxima of the 5,8-epoxide decreased and eventually disappeared. In most cases, the blue color was still present after several days.

In Table I are given comparative data on the effect of hydrochloric acid in methanol-ether on 14 carotenoids. The positions of the long wave length maxima were at wave lengths of 670 to 676 mμ for derivatives of β-carotene monoepoxide (numbers 1, 3, and 4) and at 665 to 667 for those of α-carotene monoepoxide (numbers 9, 10, and 11). Positions of the blue maxima were much more variable for derivatives of β-carotene diepoxide (numbers 2, 5, 6, 7, and 8), ranging from 623 to 674 mμ.

In Table I, data are included for three isomeric 5,8-epoxide pairs, numbers 3 and 4, 6 and 7, and 10 and 11. In all three cases, the blue color formed in one of each pair at about twice or more the rate of the other (right-hand column).

The positions of the long wave length maxima with sinensiaxanthin and persicaxanthin (two polyol 5,6-epoxides of incompletely known structure obtained from oranges and cling peaches, respectively) were at much shorter wave lengths. These maxima developed and then faded much more rapidly than in numbers 1 to 11.

Valenciaxanthin was unique in that after acid treatment there was a small amount of absorbance from 500 to 750 mμ with no maximum (Figure 1). In the hydrochloric acid–ether test it formed a light blue color (*4*). Persicaxanthin and valenciaxanthin can hence be differentiated by treatment with hydrochloric acid as well as by countercurrent distribution (*1*). The valenciaxanthin solution in hydrochloric acid–ether–methanol became more yellow on standing, with maxima appearing at 416 and 390 mμ, with an inflection at around 466 mμ.

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