Carr and Price (1) in 1926 introduced a reagent for the quantitative estimation of vitamin A which was destined to play an important rôle in the study of the physiology of the vitamin. This reagent, a saturated solution of antimony trichloride in chloroform, still occupies a position of first importance in most laboratories where vitamin A is estimated by chemical means. The sensitivity of the reagent and the simplicity of its application have made its use in many cases the method of choice. However, its non-specificity and the transient character of the blue coloration produced with vitamin A have at times cast doubts on its general reliability.

Although numerous non-vitamin A substances are known to be chromogenic with antimony trichloride (2), difficulty is usually encountered only in the case of the carotenoid pigments which are closely related to vitamin A and which frequently accompany the vitamin in animal tissue. The colors produced by these pigments with the reagent display distinctly different absorption spectra and a much greater apparent stability than that due to vitamin A. The effect of increased temperature and of intense illumination on the instability of the Carr-Price colors is much more pronounced in the case of vitamin A than in the case of the carotenoids. The variation in stability has been made the basis for the differentiation of the vitamin A color from that of the other chromogens by several investigators (3–5).

Several workers have reported constants relating to the absorption spectra of the Carr-Price reaction products of vitamin A and of several of the other chromogenic substances. Gillam (6) has reported the wavelength of the absorption maxima and the corresponding $E_{1\%}$ values for β-carotene, lycopene, lutein, zeaxanthin, and vitamin A. Goldhammer and Kuen (7) have given corresponding values for carotene, xanthophyll, and for several of the sterols. Von Euler et al. (8) have published curves show-
ing absorption characteristics for β-carotene immediately after adding the reagent and also 30 minutes later. Lamb, Mueller, and Beach (9) have published curves showing the antimony trichloride colors with ergosterol, cholesterol, and 7-dehydrocholesterol. Gibson and Taylor (10) recently introduced a new technique for observing the rapidly changing spectra of the antimony trichloride-chromogen systems. Their "dynamic method" involves the use of a "flowing cell" (Fig. 1) in which a steady state of flowing reagent and chromogen is maintained during the spectral measurement. These workers have presented curves for liver oil concentrates, their oxidized products, and for β-carotene, which show the rapid changes as the solution ages.

No comprehensive investigation has been found in the literature relating to the changing spectra of the antimony trichloride reaction products with vitamin A in its various forms and with the carotenoid pigments most likely

Fig. 1. Modified form of the flowing cell apparatus of Gibson and Taylor used in determining the absorption spectra by the "dynamic method."
to interfere with the Carr-Price determination of vitamin A. It is the purpose of this paper to present these fundamental data which should prove of value in the further development of the Carr-Price method.

**Procedure**

**Materials**—The Carr-Price reagent was prepared by the general method of Koehn and Sherman (11). The antimony trichloride was dissolved in the purified chloroform at the rate of 22.5 gm. per 100 ml. of chloroform. The reagent was stored at room temperature in darkness or subdued light and filtered before use. Different batches of reagent exhibited great uniformity and stability.

Vitamin A as the crystalline alcohol, crystalline acetate, and the liquid concentrate of the natural esters was available for the investigation. These preparations were preserved in the dark at -20° until ready for use. Stock solutions in U. S. P. chloroform were prepared and from these suitable dilutions ranging from 5 to 50 γ per ml. were made for use in the Carr-Price study.

The seven most common carotenoid pigments listed by Zechmeister (12) were available for these studies. These were α-carotene, β-carotene, γ-carotene, lycopene, cryptoxanthin, lutein, and zeaxanthin. Although the quantity of γ-carotene available was insufficient for the study of its Carr-Price absorption spectrum, it was used in the other phases of the work to be reported. These pigments were also stored in the dark at -20° until used. Solutions in U. S. P. chloroform ranging in concentration from 100 to 400 γ per ml. were prepared, and the concentration and purity established in all cases by reference to the spectral data to be found in the literature (12, 13). The absorption data were obtained by the use of the Beckman spectrophotometer.

**Apparatus and Methods**—Most of the data here reported were obtained by use of the Beckman spectrophotometer. Before the spectral measurements were made the wave-length dial was set at the desired point and the instrument adjusted to zero optical density with a “blank” mixture of 1 ml. of chloroform and 9 ml. of the reagent. 1 ml. of the solution under test was placed in a lipped tube and 9 ml. of the reagent added. At this instant a stop-watch was started. The reaction mixture was transferred to the

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1 The vitamin A preparations were generously supplied by Dr. P. L. Harris of the Distillation Products, Inc.

2 Dr. L. Zechmeister of the California Institute of Technology contributed samples of lycopene and zeaxanthin which were used in this study. Dr. John Porter of Purdue University and Dr. G. Mackinney of the University of California kindly supplied the lutein and the cryptoxanthin, respectively. The remaining carotenoid pigments were either prepared in this laboratory or purchased.
absorption cell, which was quickly placed in position for measurement in
the Beckman spectrophotometer. At 30 seconds the first reading of
optical density was taken, and readings were taken at intervals thereafter
for a period of 10 minutes. This set of data represented the change in the
optical density at the chosen wave-length, with time. The wave-length
dial was reset, and the measurements repeated. This process was repeated
at frequent intervals in the wave-length range from 500 to 700 m\(\mu\), well on
either side of the 620 m\(\mu\) maximum of the vitamin A Carr-Price reaction
product. In plotting the data, all of the optical densities obtained at the
same time after mixing were plotted against the wave-length. A series of
absorption curves was thereby obtained, each member of which corresponds
to a specific age of solution. These curves, viewed together, present a
picture of the changing Carr-Price reaction product from 30 seconds to
10 minutes after mixing.

For time intervals less than 30 seconds recourse was had to the "dynamic
method" of Gibson and Taylor, with a modified flowing cell apparatus.
Fig. 1 shows the essential features of the flowing cell which was placed over
the aperture of a visual Bausch and Lomb spectrophotometer. The
reagent and chromogen solutions were allowed to mix at a constant rate
and flow through the absorption cell at a time after mixing controlled by
the level held in the retention chamber. When a steady state was reached,
the absorption curve of the flowing mixture was determined in a normal
fashion. The time after mixing was calculated by dividing the ml. of
solution between the point of mixing and the center of the observation
window by the rate of flow of the solution in ml. per minute. With the
apparatus used, reproducible results could be obtained with mixtures from
2 to 30 seconds of age.

Calculations—All optical densities were converted to the corresponding
\(E^1\%_{1\text{cm.}}\) values by means of the Bouger-Beer (Beer-Lambert) law. Thus

\[
E^1\%_{1\text{cm.}} = \frac{D}{c \cdot l}
\]

where \(D\) is the observed optical density, \(c\) is the concentration in per cent of
the chromogen, and \(l\) is the thickness of the absorption cell in centimeters.

DISCUSSION

The results obtained in this investigation are presented graphically in
Figs. 2, 3, and 4. In these figures the \(E^1\%_{1\text{cm.}}\) values are shown plotted
against the wave-length, which extends well on either side of the 620 m\(\mu\)
maximum of the vitamin A Carr-Price reaction mixture. The age of the
solutions measured from the instant of mixing is indicated on the various
curves by numbers as well as by the coded lines. The solid lines represent
the solution of least age, while the dotted lines represent the oldest of the solutions.

Examination of the curves reveals marked differences between those relating to vitamin A in any of its forms and the curves of the carotenoid pigments. Vitamin A is seen to have chromogenic powers 10- to 25-fold greater than the common carotenoids in the region of the vitamin A maxima. Vitamin A, as alcohol, acetate, or natural ester, is unique among the chromogens in possessing a single strong absorption band (maximum at 620 m\textmu) which rapidly decreases with time. Changes of a more complex nature are exhibited by \(\beta\)-carotene, lutein, and zeaxanthin. Here the absorption maxima shift as a wave or in steps toward the red end of the spectrum as the solution ages. The remaining chromogens, \(\alpha\)-carotene,
lycopene, and cryptoxanthin, show relatively simple absorption spectra, which gradually rise or fall with time. In no case can the Carr-Price color be termed "stable."

A study of these absorption curves makes clear the differences in the "stability" of the Carr-Price colors, as normally measured with a filter colorimeter or a diffraction instrument of equally wide wave band. In the case of vitamin A, only the decreasing absorption in the region of 620 m\(\mu\) is recorded, while in the case of the carotenoids major changes occurring in the character of the absorption spectra may be entirely overlooked, due to the width of the filter band used in making the measurement. Thus, the Carr-Price colors with the various carotenoid pigments may be recorded as stable, increasing, or decreasing, depending on the integrated area under the absorption curves between the limits imposed by the optical system involved in the measurement.

![Absorption spectra of the Carr-Price reaction products with \(\beta\)-carotene, lutein, and zeaxanthin at various times after mixing of the reactants.](http://www.jbc.org/)
SUMMARY

1. Spectral absorption curves for the Carr-Price reaction mixture with vitamin A as alcohol, acetate, and concentrate of the natural esters have been presented for reaction mixtures of ages varying from 2 seconds to 10 minutes.

![Graphs of spectral absorption curves for carotenoids and cryptoxanthin.](Image)

**Fig. 4.** Absorption spectra of the Carr-Price reaction products with α-carotene, lycopene, and cryptoxanthin at various times after mixing of the reactants.

2. Similar data have been presented for six of the common carotenoid pigments, α-carotene, β-carotene, lycopene, cryptoxanthin, lutein, and zeaxanthin.

The authors wish to thank Dr. L. Zechmeister, Dr. John Porter, Dr. G. Mackinney, and Dr. P. L. Harris for the gift of the carotenoids and the vitamin A preparations used in this study. Further, we wish to acknowledge the technical assistance of Mrs. Howard Hamlin who aided materially in this project.
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Changes in the absorption spectra due to aging of the Carr-Price reaction mixture with vitamin A and the common carotenoid pigments
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