

## Colour Quenching by Carotenoids

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### INTRODUCTION

The carotenoids are a group of lipid-soluble pigments which occur in all plants and algae and in many fungi, bacteria and animals. Their basic structural feature (see Fig. 1), on which all their properties and functions depend, is the polyene chain, the sequence of conjugated double bonds, which makes up the backbone of the molecule. The number of conjugated double bonds may be as high as 15 or as low as 3, so that the carotenoids can be purple, red, orange, yellow or even colourless. The light absorption properties of the polyene chromophore are modified by the nature of the end groups; some 40 different end groups have been detected in the 350 or so naturally occurring carotenoids that are currently recognised.

Studies on the biosynthesis of these compounds, particularly in micro-organisms, have been in progress in this laboratory for a number of years. An important feature of the experimental approach is, of course, the use of suitable radioactive substrates (labelled with Carbon-14 and/or Hydrogen-3) as precursors of the carotenoids in whole organisms or in enzyme systems. Although their solubility characteristics are such that the carotenoids dissolve without difficulty in the simplest and most efficient of scintillation fluids, the very nature of these pigments dictates that such samples interfere with the basic liquid scintillation counting process by causing colour quenching. The use of liquid scintillation counting for the assay of radioactivity in numbers of carotenoid samples which are at different concentrations and which have different absorption spectra obviously requires a versatile, accurate and preferably rapid procedure for colour quench correction.

### METHODS OF CORRECTING FOR COLOUR QUENCH BY CAROTENOIDS

The ease with which carotenoids are photodegraded by ultraviolet radiation enables

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this procedure to be used prior to counting for the elimination of colour quenching.<sup>1</sup> This method has certain disadvantages in that it is destructive and is time-consuming because of the luminescence which results from the ultraviolet bleaching and which has to be allowed to decay before counting can commence. Much the same criticisms can be made of other methods (e.g. bleaching with benzoyl peroxide and light<sup>2</sup>) which have been used in the carotenoid field. The availability of liquid scintillation counters equipped with automatic external standards has enabled the exploitation of the external standard channels ratio (ESCR) method in correcting both for chemical and for colour quenching. While separate calibration procedures are necessary for the respective types of quenching, it has been shown in studies using a limited number of widely differing coloured samples (e.g.  $\beta$ -carotene, scarlet red, oracet blue, chlorophyll, road tar, etc.) that the same relationship between counting efficiency and ESCR applies to samples of all colours.<sup>3,4</sup>

In establishing quench correction curves in order to use this method for counting Carbon-14 and Hydrogen-3 in carotenoid samples, we chose ten different carotenoids (Fig. 1) which, between them, have a wide variety of absorption maxima covering the entire wavelength range of phosphor emission and photomultiplier sensitivity (Fig. 2). Each part of the spectral range of the instrument can be covered by selecting the appropriate carotenoid. In spite of their differences in chromophore, all ten carotenoids are based on the same 40-C (tetraterpenoid) skeleton; seven are hydrocarbons (carotenes) and, of these, three ( $\beta$ -carotene,  $\gamma$ -carotene and lycopene) are isomers. Because they are structurally so similar and are basically hydrocarbon in nature and are therefore not chemical quenchers, these ten pigments provide the opportunity for making an extremely detailed and controlled study not only of the validity of the ESCR method of colour quench correction, but also of the quenching phenomenon itself.

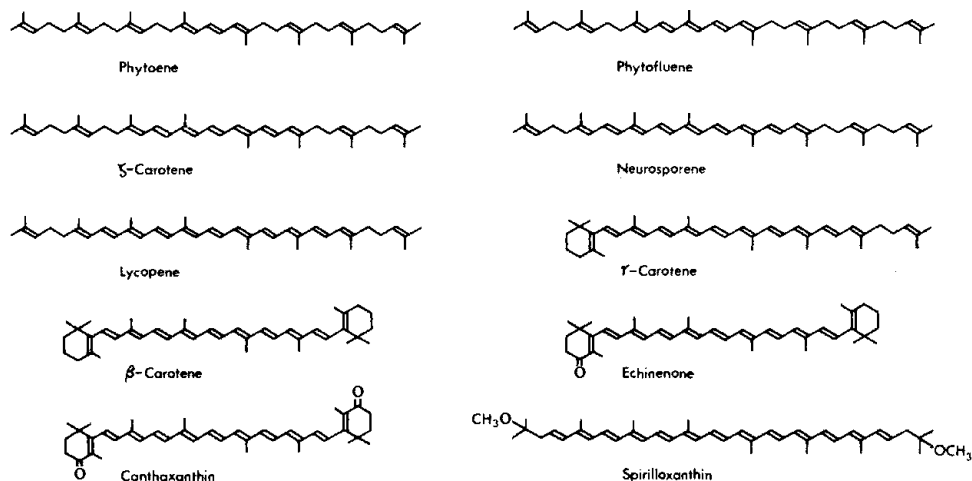


Fig. 1. Structures of the carotenoids used in these studies.

## MATERIALS AND METHODS

### Instrument

Measurements of radioactivity were made with a Nuclear Enterprises 8310

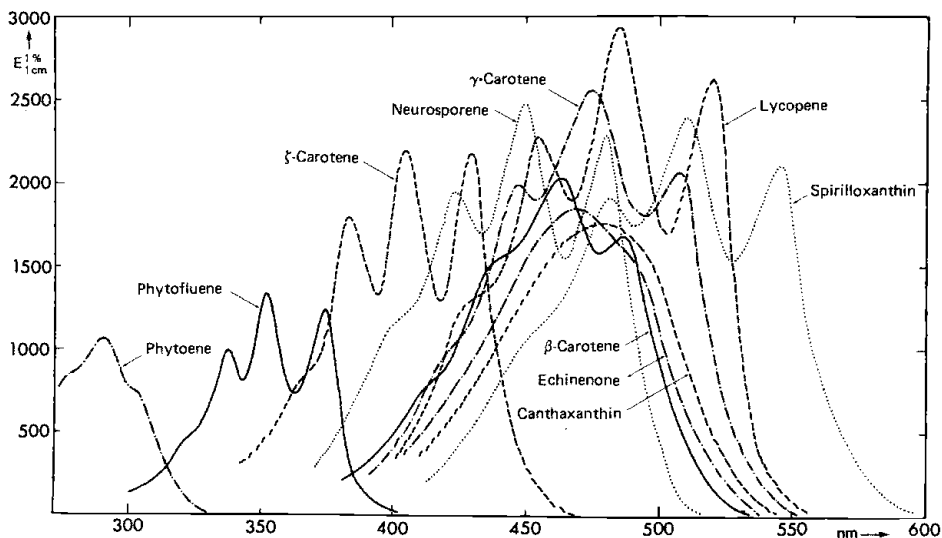


Fig. 2. Absorption spectra of carotenoids in toluene.

Automatic Liquid Scintillation Spectrometer<sup>a</sup> equipped with a Caesium-137 50  $\mu$ Ci external standard. The discriminator settings were as follows: counting channel 1,  $E = 0.6$  V,  $\Delta E = 4.4$  V; counting channel 2,  $E = 5.0$  V,  $\Delta E = 5.0$  V; counting channel 3,  $E = 0.6$  V,  $\Delta E = 9.4$  V; external standard channel 2,  $E = 9.80$  V,  $\Delta E = 10.02$  V; external standard channel 3,  $E = 8.00$  V,  $\Delta E = 4.32$  V. The counting efficiencies in the counting channels, as determined using the manufacturer's standards, were: Carbon-14, 17.4% (1), 72.7% (2), 90.7% (3); Hydrogen-3, 44.9% (1). Each experimental vial was counted in triplicate for 10 min, the counting modes used being  $\beta$ ESOL and  $\beta$ ES for Carbon-14 and Hydrogen-3 respectively. External standard channels ratios and apparent counting efficiencies were calculated with an on-line Olivetti Programma 101 computer.

#### Experimental samples

The counting efficiencies of Carbon-14 were assayed by preparing, for each of the ten carotenoids, a series of ten counting vials, each containing a different accurately measured amount of the carotenoid (0 to 10  $\mu$ g or, in the case of phytoene, 0 to 25  $\mu$ g) and 10 ml of a scintillation fluid containing 3.0 g PPO<sup>b</sup> and 0.5 g dimethyl-POPOP<sup>c</sup> per litre of toluene. The scintillation fluid also contained a concentration of (1-Carbon-14)n-hexadecane<sup>d</sup> equivalent to 18731 d.p.m. per vial. The counting efficiencies of Hydrogen-3 were assayed by an identical procedure except that each vial contained an equal amount (equivalent to 34056 d.p.m.) of (1, 2 Hydrogen-3)n-hexadecane.<sup>e</sup>

<sup>a</sup> Nuclear Enterprises Ltd., Sighthill, Edinburgh 11, Scotland, U.K.

<sup>b</sup> 2,5-Diphenyloxazole; Packard Instrument Co. Inc., La Grange, Illinois, U.S.A.

<sup>c</sup> 1,4-Bis(4-methyl-5-phenyloxazol-2-yl)benzene; Packard Instrument Co. Inc., La Grange, Illinois, U.S.A.

<sup>d</sup> Specific activity 1.10  $\mu$ Ci/g; Radiochemical Centre, Amersham, Bucks., U.K.

<sup>e</sup> Specific activity 2.00  $\mu$ Ci/g; Radiochemical Centre, Amersham, Bucks., U.K.

### Carotenoids

The carotenoids were obtained as follows: phytoene, phytofluene,  $\zeta$ -carotene, neurosporene,  $\gamma$ -carotene, lycopene and  $\beta$ -carotene were isolated from normal and inhibited cultures of a number of mutants of the fungus Phycomyces blakesleeanus,<sup>5,6</sup> spirilloxanthin was obtained from cultures of the purple, non-sulphur, photosynthetic bacterium Rhodospirillum rubrum,<sup>7</sup> while samples of echinenone and canthaxanthin were a generous gift from F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland. All the carotenoids were purified to spectral purity by standard methods of column and thin-layer chromatography.<sup>8</sup>

### Absorption spectra

Absorption spectra (Fig. 2 and Table 1) were determined in toluene using a Unicam SP800 Recording Spectrophotometer, the wavelength scale of which was calibrated using a holmium oxide filter. Quantitative measurements were made in one of a matched pair of 1 cm path-length silica cuvettes and specific extinction coefficients ( $E_{1\text{cm}}^{1\%}$ ) were calculated (Table 1) from those quoted for solutions in other solvents after quantitative comparisons of carotenoid spectra in toluene and in the appropriate solvent. Samples were dispensed into the counting vials as accurately pipetted aliquots from standard solutions and were evaporated to dryness prior to dissolving the residue in 10 ml scintillation fluid containing the appropriate labelled species of n-hexadecane.

Table 1. Absorption characteristics of carotenoids in toluene.

Carotenoid	Absorption maxima (nm)			$E_{1\text{cm}}^{1\%}$ at $\lambda_{\text{max}}$ *
Phytoene	(277)	290.5*	302	1040
Phytofluene	337	354 *	374	1140
$\zeta$ -Carotene	383	406 *	431	2200
Neurosporene	425	451 *	480	2480
Lycopene	457	484 *	519	2950
$\gamma$ -Carotene	450	474 *	507	2580
$\beta$ -Carotene	(434)	463 *	490	1960
Echinenone		468 *	(498)	1830
Canthaxanthin		479 *		1910
Spirilloxanthin	478	508 *	542	2400

### QUENCH CORRECTION CURVE

After recording in triplicate the counting efficiencies of some 200 standard samples and the corresponding values of ESCR, it was possible to plot four curves representing the relationships between the efficiency of isotope counting in the different channels and the ESCR (Fig. 3). The results of previous workers<sup>3,4</sup> are confirmed in so far as the nature of the carotenoid is irrelevant, for points for all the pigments fitted the same lines. Any slight deviations of the individual points (all <2%) could satisfactorily be accounted for on the basis of pipetting errors.

These calibration curves have been used for the routine counting of Carbon-14 in carotene samples as part of our biosynthetic studies. They have consistently given results comparable with those obtained following a bleaching procedure; the latter has now been abandoned in this laboratory.

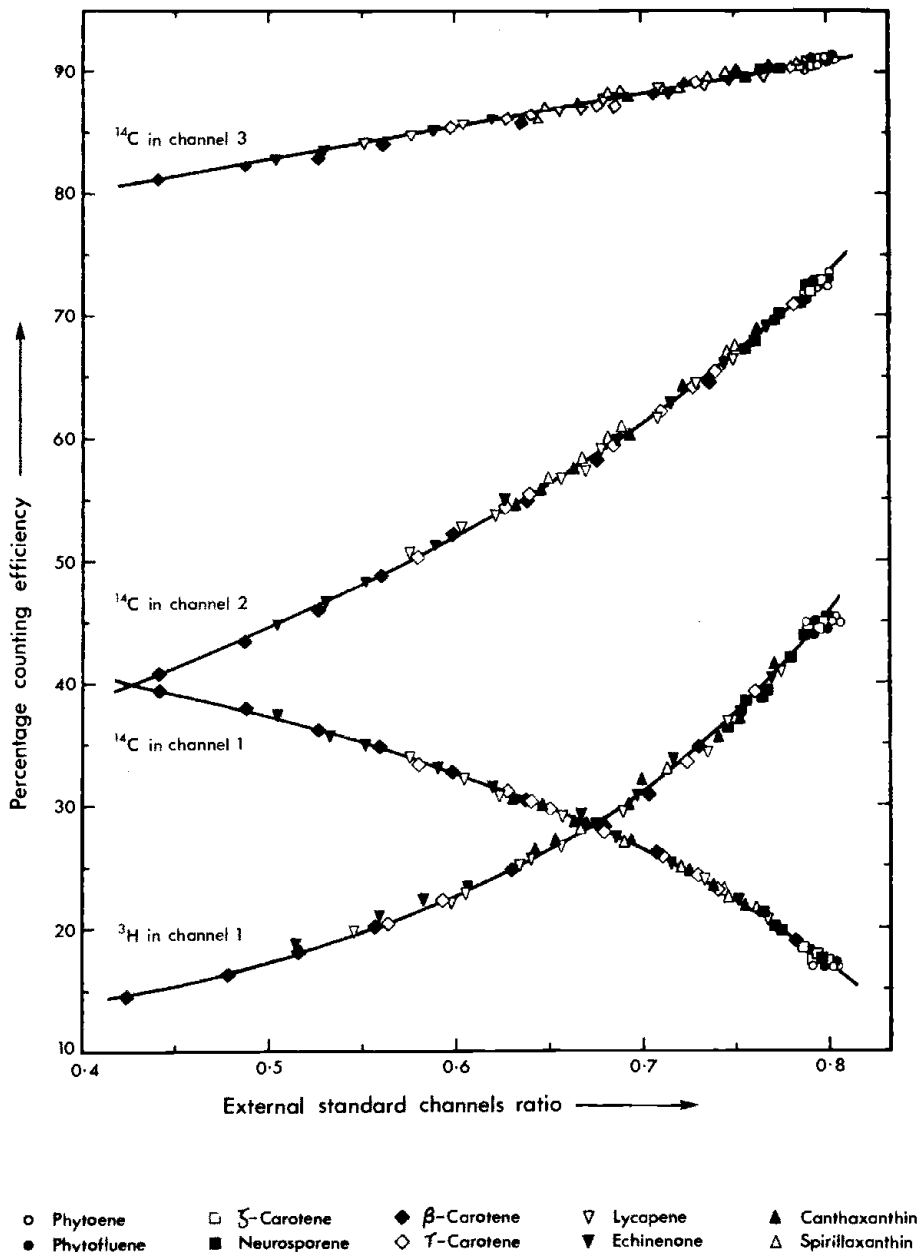


Fig. 3. Relationship between external standard channels ratio and counting efficiencies for Carbon-14 and Hydrogen-3 colour quenched by various carotenoids (see text for conditions)

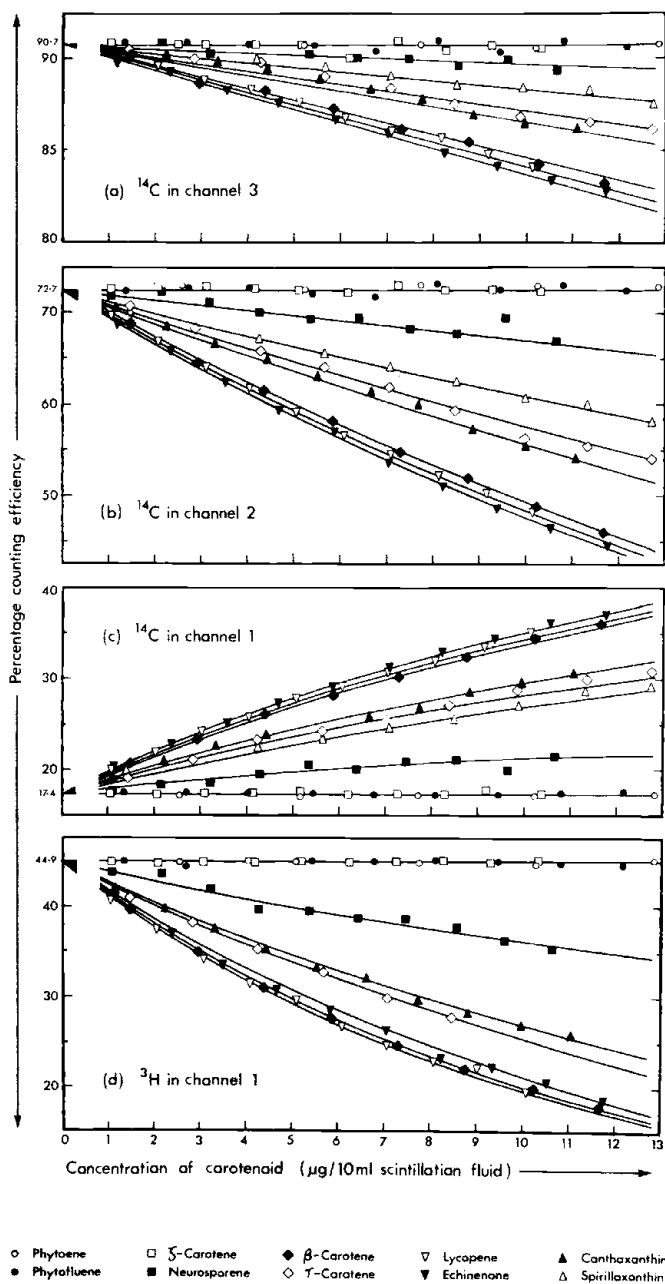


Fig. 4. Variations in counting efficiency with carotenoid concentration (see text for conditions).

### COLOUR QUENCHING BY DIFFERENT CAROTENOIDS

The results upon which the quench correction curves are based were studied in more detail. When the percentage counting efficiencies were plotted against the concentration of carotenoid in each vial, the values corresponding to each individual pigment gave rise to a different line (Fig. 4). This confirms that, as would be expected, some carotenoids are more efficient quenching agents than others.

It has often been assumed<sup>9</sup> that the counting of phytoene and phytofluene, which are both colourless but absorb ultraviolet radiation (Table 1), would require some correction procedure, especially phytofluene, whose strong fluorescence (maximum excitation 369 nm, maximum emission 490 nm, both values in ethanol<sup>10</sup>) might be expected to lead to some spurious results. The presence of neither of these polyenes showed any effect on the recorded count rates of standard samples. As the sensitivity of the EMI 9635QB Photomultipliers<sup>a</sup> (with which the NE 8310 scintillation counter is fitted) is maximal at about 380 nm<sup>11</sup> and the fluorescence emission spectrum of the scintillator mixture (in toluene) has maxima at 410 (infl.), 430 and 455 nm,<sup>12</sup> it would be expected that the conjugated heptaene,  $\zeta$ -carotene ( $\lambda_{\max}$  in toluene 383, 406 and 431 nm), and the conjugated nonaene, neurosporene ( $\lambda_{\max}$  in toluene 425, 451 and 480 nm), would show the highest degree of colour quenching per unit concentration. This was not the case. Of the coloured carotenoids, these two polyenes showed the least quenching, although neurosporene was a stronger quenching agent than  $\zeta$ -carotene. Rather, the most efficient quenchers were lycopene ( $\lambda_{\max}$  in toluene 457, 484 and 519 nm) and echinenone ( $\lambda_{\max}$  in toluene 468 nm), both of which absorbed maximally in a region where both photomultiplier sensitivity and phosphor emission are at some 60% of their highest values.

True comparisons of quenching efficiencies, however, cannot be made on the basis of concentration alone, for this takes no account of the different extinction coefficients of the carotenoids. Further comparisons were therefore made. In the first, values for percentage quench determined from the above data at carotenoid concentrations calculated to give extinctions of 0.25 at their wavelengths of maximal absorption were plotted against the wavelengths of maximal absorption (Fig. 5). This indicated quite clearly that, on the basis of matching peak wavelengths, those carotenoids which absorb maximally at about 465 nm, well away from the expected region, are the most efficient quenchers of this liquid scintillation system.

Since this anomaly may have arisen through considering peak wavelengths alone instead of spectral overlap integrals, a second approach was used. In this, values were found for the percentage quench shown by those graphically determined concentrations of the different carotenoids which absorbed a standard total amount of light over their entire absorption range, and these were plotted against the corresponding mean wavelengths of light absorption, again determined graphically for each carotenoid. There was, however, no significantly better correlation between the apparent wavelength of maximal quenching (465 to 470 nm) and the wavelengths of maximal phosphor emission and photomultiplier response.

### THEORETICAL APPROACHES TO COLOUR QUENCHING BY CAROTENOIDS

Two theoretical attempts were made to correlate the experimental data with expected behaviour. The first utilised the integral of Ross,<sup>13</sup> where the percentage

<sup>a</sup> EMI Electronics Ltd., Electron Tube Division, Hayes, Middlesex, U.K.

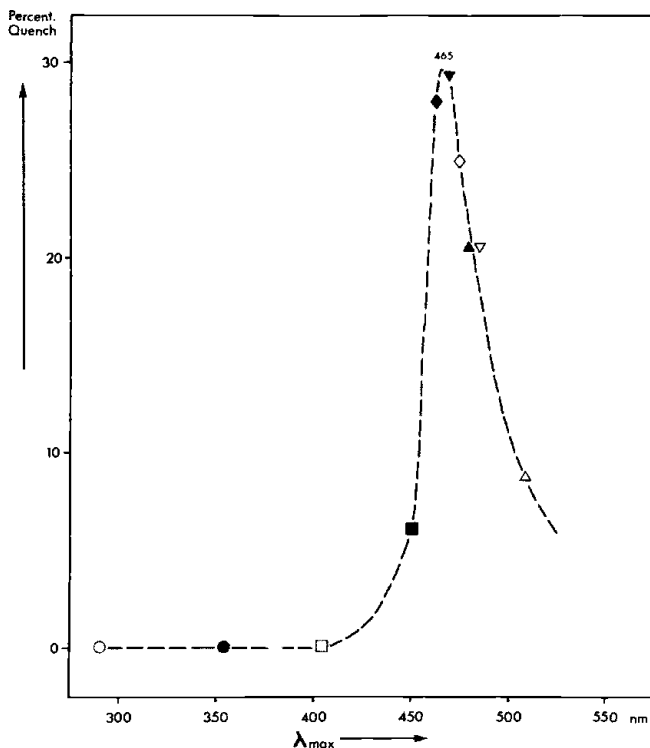


Fig. 5. Percentage quench of Carbon-14 counts in counting channel 2 by carotenoid solutions of  $E_{max}$  0.25 plotted against  $\lambda_{max}$  (carotenoid symbols as for Figs. 3 and 4).

colour quench ( $\Omega$ ) of a sample is given by:

$$\Omega = \int_{\lambda_1}^{\lambda_n} (K) (A) d\lambda$$

where  $K$  is the quenching coefficient,  $A$  is the absorbance of the solution and  $\lambda$  is the wavelength. This approximates to:

$$\Omega = (K_{\lambda_1}) (A_{\lambda_1}) + (K_{\lambda_2}) (A_{\lambda_2}) + (K_{\lambda_3}) (A_{\lambda_3}) \dots + (K_{\lambda_n}) (A_{\lambda_n})$$

The second method, suggested to us by Dr. J.B. Birks, likens the system at wavenumber  $\bar{\nu}$  ( $\text{cm}^{-1}$ ) to (1) an emitter (phosphor) of fluorescence  $F(\bar{\nu})$ , (2) a detector with response  $S(\bar{\nu})$  and (3) a filter of transmittance  $T(\bar{\nu})$ , equivalent to an absorbing solution of molar extinction coefficient  $\epsilon$  and molar concentration  $c$  in a scintillation vial of internal diameter  $t$ , where:

$$\log_{10} T(\bar{\nu}) = -\epsilon(\bar{\nu})ct/2$$

The factor 't/2' is an approximation, since for one of the pair of photomultipliers the length of the absorbing path varies from zero to  $t$ , while for the other it varies

from  $t$  to zero. The quenching factor ( $\Omega$ ) is given by:

$$\Omega = \frac{\int F(\bar{\nu})T(\bar{\nu})S(\bar{\nu})d\bar{\nu}}{\int F(\bar{\nu})S(\bar{\nu})d\bar{\nu}}$$

Unfortunately, neither calculation gave useful results. This is presumably due to the complex and persistent multi-peaked absorption spectra of the carotenoids, especially those of the acyclic carotenes which show pronounced fine structure. Such spectra do not lend themselves to the use of simple integrals in an experiment with only a limited number of samples.

#### SUMMARY AND COMMENTS

When viewed solely from the point of view of the carotenoid specialist, the data presented above justify the use of the ESCR method of correcting for colour quenching by a wide variety of these pigments. One set of correction curves is applicable to all the compounds of this class that we have examined, and it is unlikely that any others are sufficiently powerful chemical quenchers to introduce inaccuracies into the results and so necessitate an alternative approach. As long as the limitations of the technique, with regard to very high degrees of quench<sup>14</sup> and to doubly labelled samples,<sup>9</sup> are appreciated, the method is as accurate and is certainly more convenient than other procedures available.

Attempts to correlate the degree of colour quenching by the individual carotenoids with their light absorption characteristics led to the unexpected, if not anomalous, observation that those pigments which absorb maximally in the region 350 to 450 nm (phytofluene,  $\zeta$ -carotene and neurosporene) are not necessarily the most powerful quenchers in the system examined. Indeed, phytofluene consistently shows no quenching, while the effect of  $\zeta$ -carotene is virtually negligible. The latter observation contrasts with a report that the conjugated heptaene is a strong quencher.<sup>2</sup> Perhaps the reason for the discrepancy lies in the different instrumentation used or in the difficulty of separating  $\zeta$ -carotene from  $\beta$ -zeacarotene and  $\beta$ -carotene.<sup>15</sup>

Although losses in efficiency in counting carotenoid samples are due solely to colour quenching, the use of a series of different carotenoid pigments to investigate the relationship between colour quench and wavelength did not give as satisfactory a correlation between experimental observation and theoretical prediction as has been achieved by other workers. Iwakura and Kasida<sup>16</sup> used a series of 14 dyes, which were very different in their individual structures and which required methanol for solubilisation, in order to carry out a similar study. That a better correlation between their experimental data and theory was obtained than in our case must be attributable, at least in part, to the relative simplicity of the absorption spectra of the dyes chosen for their study.

#### ACKNOWLEDGEMENTS

Our grateful thanks are due to F. Hoffmann-La Roche & Co. Ltd., Basel, Switzerland, for the generous provision of samples of synthetic echinenone and canthaxanthin, to Dr. J. B. Birks of the University of Manchester for his suggestions on the theoretical treatment of colour quenching, and to the Science Research Council for a Research Studentship (P.M.B.) and a Research Grant for studies on carotenoid biosynthesis.

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## DISCUSSION

**E.B. Mueller:** Have you tried counting carotenoid solutions with no fluor or with only primary fluor? Are the (non-quenching) carotenoids possibly acting as fluors or wavelength shifters, and this accounts for their not quenching?

**B.H. Davies:** No, but we have seen no shift in the emission spectrum of samples of PPO + Me<sub>2</sub> POPOP in the presence of carotenoids, and they are present at such small concentrations that they would not be expected to act as fluors.