β -Carotene in the Flowers of *Narcissus*

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Single-flowered plants of the genus *Narcissus* may be divided into three groups. Those in the one group have flowers with yellow perianths and large yellow trumpets; and they come into bloom in Britain in March or April. They are popularly called daffodils, or systematically described as *Narcissus pseudo-narcissus*. In another group, popularly called narcissi, of which *Narcissus poeticus recurvus*, or pheasant's eye, is a common example, the flowers have small white perianths and very small pale-yellow cups or coronas fringed with red. These come into flower in May. The middle group of *Narcissus* contains many intermediate types, nearly all of which come into flower in April: they have large white or yellow perianths, and coronas less than half the length of the perianth segments. The coronas may be red, or orange, or white fringed with red. This paper describes an investigation into the nature and the concentration of the red pigment in the coronas of the narcissi in these groups.

EXPERIMENTAL

Material. Several cultivated varieties of *Narcissus* were examined, including some with deep-red coronas grown by Mr J. M. de Navarro. The yellow-trumpet specimens were from commercial varieties of daffodil. The *N. poeticus* flowers were also commercial varieties.

Taxonomy. Attempts were made to use an official taxonomic classification of *Narcissus*, but no advantage was apparent for present purposes. The specimens having red coronas are hybrids derived from various types, predominantly 'large crowned' and ultimately probably *N. poeticus*. Size and shape are dominant criteria for taxonomic classification, whereas deeply pigmented and less-pigmented varieties appear together in more than one class. Therefore it is expedient to use popular names and variety names. The yellow forms of daffodil were derived from *N. pseudo-narcissus*.

Methods

Extraction of pigments and spectrophotometry. The pigments were extracted from weighed samples of the coronas into a cold mixture of equal volumes of light petroleum (80–100°) and acetone, saturated with quinol. The acetone was washed from the extract with water, and the light petroleum solution was chromatographed on a mixture of aluminium oxide and sodium sulphate (Booth, 1957). The

carotene in solution was measured in the Unicam spectrophotometer with glass prism, model SP. 600. The extinction coefficient ($E_{1\%}^{1\text{cm}}$, at 450 m μ .) for β -carotene in light petroleum was taken as 2550. The total carotenoids, when determined, were measured in the same way before chromatography. Although carotenoids other than carotene mostly have lower E values and are thereby underestimated, this is unimportant in the present application.

RESULTS

Principal pigment from red coronas

Chromatographic behaviour. All the pigments in an extract of the red coronas of *Narcissus* flowers from the intermediate group were adsorbed from light petroleum near the top of the column of alumina. Most of the pigment was eluted by 2% acetone in light petroleum. The colour and behaviour of the most prominent chromatographic band during its elution suggested that the pigment might be β -carotene. The eluate was collected from several extracts treated in this way, acetone was washed from the solution, and the following confirmatory tests were done. Full descriptions of chromatographic technique, animal diet, saponification and other details are described by Booth (1957).

Mixed chromatogram. A portion of the light petroleum solution was mixed with a solution containing a similar quantity of β -carotene prepared in the same way from fresh lucerne (*Medicago sativa*) leaves. The mixed pigment was adsorbed on a long column of alumina and eluted with 2% acetone in light petroleum. Only one chromatographic band was seen, and this exactly resembled a band of β -carotene.

Another portion of this last solution of mixed pigments was chromatographed on magnesia. Again the only visible band resembled that of β -carotene from carrots. No band of α -carotene was seen.

Saponification. A portion of the solution was saponified by shaking with half its volume of methanol plus a half volume of *n*-propanol and 2 vol. of saturated aqueous potassium hydroxide. The pigment was epiphasic (between light petroleum and aqueous diacetone alcohol) both after and before saponification.

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Crystal form. A portion of the solution prepared from *Narcissus coronas* was concentrated by evaporation under reduced pressure. A few drops of the concentrated solution were placed on a microscope slide and, after the light petroleum had evaporated, the residue was examined under a microscope (480 diameters). The largest crystals were reddish-brown, and the more regular among them were roughly rhombic in shape. They resembled crystals prepared in the same manner from lucerne leaves, and those shown for synthetic all-*trans* β -carotene by Isler, Lindlar, Montavon, Rüegg & Zeller (1956).

Spectral-absorption curve. The pigment from red coronas was adsorbed from light petroleum on to alumina and eluted with 2% acetone in *n*-hexane. The spectral-absorption curve resembled that of β -carotene from other sources: it had maxima at 451 and 479 m μ . The variety of *Narcissus* used was 'Tantrum'. Another variety, 'Carrowmore', was extracted, and the major pigment gave a similar spectral curve. Both varieties were from the intermediate group.

Rat growth. The carotene-like pigment was extracted from coronas of 'Carrowmore', purified chromatographically, and dissolved in arachis oil. A batch of eight rats, 3½ weeks old, was given a diet free from vitamin A for 3 weeks. Three rats (group C) were given the solution, corresponding in amount to 2.1 μ g. as β -carotene, on alternate days. Three other rats (group R) were each given a similar amount of a solution prepared from another variety, 'R419'. Two rats (group O) received none. Both these control rats developed xerophthalmia, became listless and, after 2 weeks, lost weight. All the dosed rats thrived. The average gains in weight were: group R, 44 g. in 24 days; group C, 40 g. in 24 days; group O, -5 g. in 20 days.

Another eight rats were similarly maintained on the diet free from vitamin A. When they had almost ceased gaining weight, four rats (group RR) were given the second solution, corresponding to 2.1 μ g. of β -carotene, on alternate days, and the other four (group OO) were given none. In 12 days group RR gained an average of 28 ± 2.3 g. and group OO lost 17 g.

Liver storage. Two of the RR rats were then given all that remained of the two solutions, corresponding to 53 μ g. of β -carotene/rat. Their livers were removed, digested with potassium hydroxide, and extracted with diethyl ether (Davies, 1933). The ether was evaporated, and the residue dissolved in chloroform and treated with antimony trichloride reagent. From the extinction of the blue colour, determined in an absorptiometer, it was calculated that the livers had 29 and 38 i.u. of vitamin A. No vitamin A was found in the livers of two undosed rats from group OO.

Carotenoid contents of coronas

Carotene. The major pigment in the coronas of narcissi in the intermediate group was estimated on the assumption that it was β -carotene. Varieties examined included 'Brodie 132/F/30', 'Carrowmore', 'Firecracker', 'Firemaster', 'Limerick', 'Mahmoud', 'Matapan', 'Scarlet Elegance' and 'Tantrum'. The β -carotene contents of the specimens of coronas ranged from 1.1 to 3.1 mg./g. based on fresh weight. Dry weights, determined by weighing before and after heating at 100° for 4 hr., of these and other varieties ranged from 11.8 to 16.5%, average 13.9. The sample having the highest carotene content had dry matter 15.2%; hence its β -carotene content on a dry-weight basis was 20.4 mg./g.

The flowers of the varieties named above had red coronas. Samples of other varieties having orange or deep-yellow coronas were also analysed. These had β -carotene contents of from 0.15 to 0.9 mg./g. based on fresh weight; they included 'Flamenco', 'Gala', 'Kilworth', 'Monique' and others whose names were not known.

The carotene values are not listed because it is doubtful whether the specimens examined were representative, for the following reasons. (1) The pigment in *Narcissus* coronas is bleached by sunlight, particularly in richer varieties; (2) the carotene content may vary with the maturity and growing conditions as it does in the carrot (Booth & Dark, 1949); (3) only small samples were examined.

Physical state of carotene. Sections of *Narcissus* coronas from the intermediate group were mounted in water and examined under a microscope (1000 diameters). Large numbers of long, roughly rectangular, yellow crystals were visible.

Carotenols. Other yellow fat-soluble pigments, presumably hydroxycarotenoids, that could be eluted from alumina by polar solvents, beginning with 5% acetone in light petroleum, were also present in the extracts of *Narcissus* coronas. They were colorimetrically equivalent to almost half the β -carotene content. About half of this carotenol fraction was readily eluted in a manner strongly suggesting that it was in the esterified form. The higher concentrations of acetone that were necessary to elute them, and the complexity of their chromatographic bands, suggested that the other pigments comprised a variety of carotenoids. When a diethyl ether extract of coronas was treated with concentrated hydrochloric acid, no green colour was produced. Evidently furanoid epoxides were not present in appreciable amounts.

Pigments in perianth. The perianths of the flowers having deepest red coronas were usually white; but some of the varieties having red coronas had yellow perianths. The bulk of the pigments extracted from

yellow perianths were held on alumina in the manner of carotenols, and only a trace of carotene was present. No green colour was seen when a solution of the pigments in diethyl ether was treated with concentrated hydrochloric acid.

Pheasant's eye narcissus

The coronas or cups of flowers of the pheasant's eye type have deep-red fringes less than 1 mm. wide. The fringes from several flowers of *N. poeticus recurvus* were cut off and extracted. Most of the pigment behaved chromatographically like β -carotene. The mean yield from three samples of different origin was 51.5 mg./g. Since the dry weight was 31.1 %, the major pigment formed 16.6 % of the dry weight.

The pigment from *N. poeticus* was not studied in such detail as that from the coronas of flowers in the intermediate group. However, the chromatographic behaviour, the colour of the chromatographic band, the colour of the solution, the resistance to saponification, the shape and colour of the crystals and the position of the spectral bands in the visible region resembled those of β -carotene. The shape of the spectral absorption curve between 280 and 380 m μ . closely resembled that for all-*trans* β -carotene shown by Zechmeister (1944).

Yellow daffodils

Carotenoid content. The yellow coronas or trumpets from flowers of the *N. pseudo-narcissus* type, having yellow perianths, were similarly extracted, and the light petroleum solution was chromatographed on a mixture of alumina and sodium sulphate. The greater part of the pigment was not eluted by 2 % acetone in light petroleum. The major pigment was therefore not carotene, but its behaviour was consistent with its being carotenoid in nature. The trumpets had a total carotenoid content of about 0.87 mg./g. and a β -carotene content of 0.013 mg./g. Thus in carotenol content these yellow trumpets resembled red coronas, but their carotene content was of a different order altogether. The dry matter, determined as before, was 11.8 %. Hence the β -carotene content on a dry-weight basis was 0.11 mg./g.

A sample of another variety of daffodil having very large trumpets (5 cm. long) was examined. The total carotenoid content of the trumpets was 0.22 mg./g., but the carotene content was only 0.004 mg./g.

When diethyl ether extracts of the daffodil trumpets (both 5 and 3½ cm. types) were treated with concentrated hydrochloric acid, a green colour developed slowly. Evidently the daffodil pigments included epoxides.

Leaves

Leaves of *Narcissus*, from each group taken at time of full bloom, had an average carotene content of 0.056 mg./g. based on fresh weight.

DISCUSSION

Courchet (1888), Bidgood (1905) and van Wisselingh (1914) reported the presence of carotenoids in flowers of *Narcissus*, and Coward (1923) found that the flowers supported growth in vitamin A-deficient rats. Hence it could be inferred from this early qualitative work that *Narcissus* blooms contain a carotenoid having vitamin A activity, but no estimates of its concentration have been found in the literature.

The identity tests described in this paper (colour in solution, chromatographic appearance, mixed chromatogram, resistance to saponification, crystal form, spectral-absorption curve and biological response) taken together provide good evidence for the hypothesis that the major component of the red pigment in *Narcissus* coronas is all-*trans* β -carotene. Although sixteen rats are too few for assaying biological activity, it is improbable that any pigment other than β -carotene would give so high a response; for, while ten rats thrived on 1.05 μ g./day, all the undosed rats declined.

The coronas of *Narcissus* represent one of the most concentrated sources of carotene in Nature. Coronas from the intermediate group had carotene contents ranging from 0.15 to over 3 mg./g., and upon microscopic examination of the coronas, crystals of carotene were clearly seen. In the richest specimens the carotene comprised 2 % of the dry weight. Some of the most deeply coloured varieties are noteworthy for fading in sunlight. Therefore it is unlikely that the carotene was determined while it was at maximal concentration, and, if more specimens and more varieties were examined, doubtless even higher values would be found. In the fringes from the coronas of *N. poeticus recurvus* the average concentration was 16.5 % of the total dry matter, and in addition other carotenoids were present with the carotene. It is noteworthy that the dry-matter content of these fringes was higher than that of coronas from other types of *Narcissus*. These very high concentrations of carotene suggest that this material might be useful in studies on carotenogenesis.

The pigments in the trumpets of the yellow daffodil, although presumably also carotenoids, only included a small percentage of β -carotene, thereby differing from the *Narcissus* type. The carotene contents of *Narcissus* leaves were only of the same order as those of other coarse leaves.

SUMMARY

1. The major pigment in the red coronas of certain cultivated varieties of *Narcissus* was separated from other pigments chromatographically on alumina.

2. The unknown pigment showed a chromatographic behaviour like that of β -carotene, and it could not be separated from β -carotene on alumina or magnesia. The spectral-absorption curve resembled that of β -carotene. In colour and shape the crystals resembled those of all-*trans* β -carotene. The pigment supported growth in rats deficient in vitamin A, and led to storage in their livers of a substance that gave a blue colour with antimony trichloride reagent. It is concluded that the pigment is β -carotene.

3. The carotene was found in the coronas in concentrations up to 3 mg./g. based on the fresh weight, or 2% of the dry weight. In red fringes only from the coronas of *N. poeticus recurvus* the carotene comprised about 16.5% of the dry weight.

4. Other pigments, probably free and esterified

carotenols, were present in amounts approximately equivalent to half the β -carotene contents.

5. Pigments from trumpets of yellow forms ('daffodil'), although presumably carotenoids, included only small proportions of carotene.

6. Tests with hydrochloric acid suggested that the trumpets of yellow forms contained epoxides but that the coronas and perianths of the red-corona forms did not.

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Steroids of Pregnant Mare's Urine

5. IDENTIFICATION OF TWO ANDROSTANE-3:16-DIOLS.
THE STRUCTURE OF 'URANOLONE'*

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Brooks, Klyne, Miller & Paterson (1952) described the fractionation of the neutral non-ketonic lipids of pregnant mare's urine and the isolation of a number of compounds, some of which were not identified. The main purpose of the present paper is to report the identification of two of these unknown compounds (*MN* and *MM*) as the 5α -androstane-3 β :16 α - and -3 β :16 β -diols. A preliminary account of this work has already been given (Brooks & Klyne, 1956).

The structure of 'uranolone' (Marker, Lawson, Wittle & Crooks, 1938b) has been confirmed.

*Identification of 5α -androstane-3 β :16 α -
 and -3 β :16 β -diols*

The compounds *MM* and *MN* were obtained as their benzoates by the methods described by Brooks *et al.* (1952). Comparison of the properties of

* Part 4. Brooks, Klyne, Miller & Paterson (1952).

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MM and its derivatives with those quoted by Fajkoš & Šorm (1955) for 5α -androstane-3 β :16 β -diol (I; cf. also Huffman & Lott, 1955) suggested that the two substances might be identical. Comparison of our materials with authentic samples supplied by Professor Šorm proved the identity conclusively (see Table 1, p. 665).

In the structural formulae of the steroids in this paper the convention followed by the Chemical Society has been used, i.e. interrupted lines represent α -configuration and bold continuous lines β -configuration.

5α -Androstane-3 β :16 β -diol