Studies in Carotenogenesis

25. THE INCORPORATION OF $^{14}\text{CO}_2$, $[2\text{-}^{14}\text{C}]\text{ACETATE}$ AND $[2\text{-}^{14}\text{C}]\text{MEVALONATE}$ INTO β -CAROTENE BY ILLUMINATED ETIOLATED MAIZE SEEDLINGS*

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It has been known for some time that etiolated seedlings of many plants (e.g. maize, wheat, barley and sunflower) synthesize carotenoids when germinated under the most stringent conditions of light-exclusion (Beck, 1937*a*; Strain, 1938). Xanthophylls generally predominate and the individual pigments are usually the same as those xanthophylls found in green leaves (Beck, 1937*b*; Strain, 1938); β -carotene is normally present only in traces (Blaauw-Jansen, Komen & Thomas, 1950; Kay & Phinney, 1956).

According to Kay & Phinney (1956) illumination of etiolated maize seedlings results within the first 24–48 hr. in a rapid synthesis of β -carotene, with no commensurate increase in the xanthophyll fraction. Because of this observation it was considered that illuminated etiolated maize seedlings should provide good experimental material for examining the biosynthesis of β -carotene in photosynthetic tissues of higher plants. The present paper is a report of an investigation of the incorporation of labelled materials known to be carotenoid precursors in other organisms (Braithwaite & Goodwin, 1957), into β -carotene synthesized by illuminated etiolated maize seedlings.

Part of this work has already been briefly reported (Goodwin, 1958*b*).

EXPERIMENTAL

Culturing of seedlings. Maize (South African Horse Tooth) seeds were soaked overnight in water, then planted in trays containing a 3–5 cm. layer of damp, acid-washed silver sand and kept in the dark in a warm room at 28°. The seedlings were watered each morning, during which time (1–2 min.) they were exposed to a weak red light, just sufficiently intense to see to carry out the watering. When required the seedlings were removed from the sand, washed with water, placed in distilled water in small beakers and exposed in the warm room to the light from a 100 w tungsten lamp at a distance of 50 cm. Excised seedlings were prepared simply by cutting off the roots at the node connecting root and stem. The excised stems were then placed in water and illuminated in the same way as whole seedlings.

Uptake of carbon dioxide by seedlings. The seedlings, either intact or excised, were placed in water in small beakers which were then placed in a 3 l. glass vessel (vacuum desiccator). The requisite amount of $\text{Ba}^{14}\text{CO}_3$ was weighed into a small ignition tube and then sufficient unlabelled BaCO_3 added to give an atmosphere containing 0.3% (v/v) of CO_2 . The tube was then lowered by a piece of thread through the neck of the vessel into a beaker containing lactic acid, which slowly liberated the CO_2 . The bung of the vessel was replaced immediately after tipping the BaCO_3 into the acid, and the vessel was illuminated for 24 hr. under the conditions described above.

Extraction of pigments. About 6–10 seedlings (after removal of their roots) or excised stems were cut into small pieces which were immediately dropped into a mortar containing acid-washed silver sand and acetone (about 20 ml.). The tissues were thoroughly ground up and the mixture was transferred to a G4 sintered-glass filter and filtered. The vacuum was removed, further acetone (10–15 ml.) added, the mixture extracted *in situ* by stirring and grinding with a glass rod with a flattened end and then filtered. This process was repeated (usually 3–4 times) until the filtrate was colourless. An equal volume of ether was added to the combined acetone extracts in a separating funnel, followed by water dropwise until two layers formed. The aqueous layer was discarded and the ether layer twice washed with small volumes of water to free it from acetone. The ethereal solution was dried by standing for 0.5 hr. over anhydrous Na_2SO_4 , filtered through a G4 sinter, and either evaporated to dryness at 30° in a stream of N_2 or, if the total chlorophyll content was required, first made up to a suitable volume for spectrophotometric examination before evaporation to dryness.

The dry residue was then saponified, the unsaponifiable matter extracted by our standard procedures (Goodwin, 1955) and dissolved in light petroleum (the fraction with b.p. 40–60° was used throughout this investigation). The pigments of the unsaponifiable extract were first separated by chromatography on icing sugar (Goodwin, 1958*a*); β -carotene and most of the sterols are not held by this adsorbent and run straight into the filtrate. The β -carotene was then further purified by chromatography on a column of alumina weakened with methanol (Goodwin, 1955). The β -carotene zone was eluted with light petroleum containing 1% (v/v) of ethyl ether and diluted with light petroleum to a volume appropriate for spectrophotometric assay. After assay the solution was concentrated at 30° under N_2 to 3–5 ml. and quantitatively transferred to a 15 ml. tapered centrifuge tube ready for crystallization.

Crystallization of the β -carotene. As only about 100 μg . of β -carotene was usually isolated from the seedlings, it was

* Part 24: Goodwin (1958*a*).

crystallized after addition of unlabelled carrier material. Crystalline β -carotene (synthetic; Hoffman-La Roche and Co., Basel, Switzerland) (3–5 mg.) dissolved in ethyl ether (5–10 ml.) was added to the labelled β -carotene extract in the centrifuge tube. After thorough mixing of the contents, the centrifuge tube was placed in a warm-water bath (30°) and gently rotated as a stream of N_2 was directed into the tube. When the volume of solvent had been reduced by approximately half, the tube was tightly bunged and placed in a cold room (–25°) overnight. The following day the resulting crystals were centrifuged down (low-speed M.S.E. Minor centrifuge; 5 min.) at 0° and the supernatant was decanted. The crystals were then washed twice with cold (0°) methanol (5 ml.), being again centrifuged after each washing. The crystals were finally dissolved in ethyl ether or $CHCl_3$ (5–25 ml. according to activity) for ^{14}C -assay, and a small volume (0.1–0.2 ml.) was diluted appropriately for spectrophotometric assay. After the sample had been counted, the solvent was removed at 30° under N_2 from the residual solution and the β -carotene recrystallized in the manner just described. This process was repeated until a constant specific activity was attained.

Preparation and extraction of chloroplasts. Chloroplasts were prepared as described previously (Goodwin, 1958a). The chloroplast suspension was centrifuged down, the supernatant removed, and the chloroplast pellet extracted according to the procedure just described for whole tissues. The residual pigments, if any, present in the supernatant were extracted by adding an equal volume of ethanol, heating on a boiling-water bath for 2 min., cooling and shaking twice with light petroleum, each time with a volume equal to that of the original supernatant. The combined extracts were then reduced at 30° under N_2 to a volume suitable for spectrophotometric assay. The residue remaining after extracting the chloroplasts was extracted in the same way as whole tissues.

Spectrophotometric assay. The standard methods for chlorophylls (Smith & Benitez, 1955) and carotenoids (Goodwin, 1955) were used throughout, with a Unicam SP. 600 photo-electric spectrophotometer.

Labelled materials. [$2-^{14}C$]Acetate and $Ba^{14}CO_3$ were purchased from A.E.R.E., Harwell, Bucks, and DL-[$2-^{14}C$]mevalonate was very kindly synthesized by Dr R. Y. Cornforth.

^{14}C Assays. The materials were plated on planchets by using small disks of lens paper (Glover, 1956) and counted by means of a proportional counter (Nuclear Measurements Corp., Indianapolis, U.S.A.) (50% efficiency). The counts

were continued to give an accuracy of $\pm 5\%$. The amounts of material on the planchets were so small that errors due to self-absorption were insignificant (less than 10%) and corrections were never made.

RESULTS

Effect of illuminating etiolated seedlings

Experiments were usually carried out on etiolated seedlings between 6 and 9 days old; preliminary investigations with seedlings of various ages showed that such seedlings had very similar xanthophyll levels and that the pattern of β -carotene synthesis on illumination was also similar. Table 1 records some typical figures for the carotenoid content of 7-day etiolated seedlings before and after illumination for 24 hr. The pigments from the etiolated plants are almost entirely xanthophylls and their concentration is much less than that in normal green leaves [total carotenoids about 30 mg./100 g. fresh wt. (Goodwin, 1952)]. Illumination of the seedlings results in the synthesis of both β -carotene and xanthophylls, and the ratio of light-synthesized β -carotene to light-synthesized xanthophylls is typical of normal green leaves. Even after 24 hr. illumination the total concentration of carotenoids is much less than that in mature leaves. The observations of Kay & Phinney (1956) that, during the first 24 hr. of illuminating maize seedlings, only β -carotene is synthesized was not confirmed. This may be due to varietal differences; they used a single cross-strain (L 289 $\times$ L 205).

Excised etiolated seedlings also synthesize carotenoids on illumination and the results obtained (Table 1) were very similar to those found with whole seedlings.

Incorporation of [$2-^{14}C$]acetate and DL-[$2-^{14}C$]mevalonate into etiolated seedlings

The labelled acetate or mevalonate was added without dilution with inert material to the water in which the seedlings were placed for exposure to light. Preliminary experiments showed that

Table 1. Carotenoid synthesis by illuminated etiolated maize seedlings

7-day seedlings were illuminated for 24 hr. under a 100 w tungsten lamp at 50 cm. distance. Temp. 28°.

	Concn. of pigment (mg./100 g. fresh wt.)		β -Carotene as % of total pigments
	β -Carotene	Xanthophylls	
Intact seedlings			
Before illumination	0.07	1.19	5.9
After illumination	0.94	3.13	23.1
Increase in concn. after illumination	0.87	1.94	31.0
Excised seedlings			
Before illumination	Trace	1.19	0
After illumination	0.74	2.56	22.4
Increase in concn. after illumination	0.74	1.37	35.1

Table 2. *Incorporation of [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate into the unsaponifiable matter of illuminated, excised etiolated maize seedlings*

6-day excised seedlings were illuminated for 24 hr. in the presence of labelled substrate under conditions given in Table 1. Counts added: [2-¹⁴C]acetate, 2×10^6 /min.; DL-[2-¹⁴C]mevalonate, 1.8×10^6 /min.

Fraction	Total activity (counts/min.)	
	[2- ¹⁴ C]- Acetate	DL-[2- ¹⁴ C]- Mevalonate
Total unsaponifiable matter	405 000	506 000
β -Carotene		
1st cryst.	500	920
2nd cryst.	0	350
3rd cryst.	—	320
4th cryst.	—	410
Counts expected assuming β -carotene is 0.5% of total unsaponifiable matter	2 000	2 500

no significant uptake of either compound was observed on illuminating intact etiolated seedlings, i.e. they were unable to penetrate the intact root system. Marked uptake was, however, obtained with excised seedlings and these were used in all further experiments. Table 2 gives the results of typical experiments with both substrates. Although in both cases there is considerable incorporation into the unsaponifiable material of the seedlings, the incorporation into β -carotene is not detectable with the labelled acetate and, although, definite, only very slight with mevalonate. The incorporation from mevalonate is much less than would be expected even on the basis of the percentage of the total unsaponifiable matter represented by β -carotene, even though on illumination β -carotene is being rapidly synthesized, whereas synthesis of the remaining unsaponifiable material, mainly steroids, is negligible. Many experiments show that in 24 hr. illuminated seedlings β -carotene represents between 0.3 and 0.5% of the total unsaponifiable matter. The same results are obtained if excised seedlings are placed in contact with labelled acetate or mevalonate for 24 hr. in the dark, before being brought into the light. It

was considered that if there were a pool of 'dark' precursors of β -carotene present, the acetate or mevalonate would equilibrate with them during the 24 hr. contact in the dark, but again there was no significant incorporation into the β -carotene.

The distribution of activity in the unsaponifiable matter is different with the two labelled substrates. Table 3 shows that much more activity (about 30% of the total) appears in the weakly adsorbed 'pre- β -carotene fraction' when labelled mevalonate is used than when labelled acetate is used (about 5%). This type of distribution was confirmed in other experiments and also on paper chromatography with the solvent system of Loeffler (1955). It was also demonstrated that none of this labelling was due to trace contamination with mevalonate itself. This aspect of the problem was not pursued further but an infrared spectrum of the crude 'pre- β -carotene fraction' indicated that it was an oxygen-free, long-chain, paraffin-like compound.

Incorporation of ¹⁴CO₂ into etiolated seedlings

When ¹⁴CO₂ is used as a substrate with either intact or excised seedlings, there is a marked

Table 4. *Incorporation of ¹⁴CO₂ into the unsaponifiable matter of illuminated, intact and excised maize seedlings*

6-day seedlings were illuminated for 24 hr. under the conditions given in Table 1, and exposed in glass vessels to an atmosphere containing 0.3% (v/v) of ¹⁴CO₂. Counts added, 6×10^6 /min.

	Total activity (counts/min.)	
	Intact seedlings	Excised seedlings
Total unsaponifiable matter	125 000	142 000
β -Carotene		
1st cryst.	4 500*	7 000
2nd cryst.	8 200	6 000
3rd cryst.	7 200	4 800
4th cryst.	8 500	4 600
Counts expected assuming β -carotene is 0.5% of total unsaponifiable matter	650	700

* Unexpectedly low.

Table 3. *Distribution of labelling in the unsaponifiable matter of excised etiolated maize seedlings illuminated in the presence of [2-¹⁴C]acetate and DL-[2-¹⁴C]mevalonate*

6-day excised seedlings were illuminated as in Table 1. Unsaponifiable matter was chromatographed on weakened alumina; the developer was light petroleum (b.p. 40–60°) containing varying amounts of ethyl ether.

Fractions in order of increasing adsorptive affinity	Solvent required for elution	Total activity (counts/min.)	
		[2- ¹⁴ C]Acetate	DL-[2- ¹⁴ C]Mevalonate
1. Unknown	Light petroleum	4 300	59 000
2. Unknown	Light petroleum + 1–2% (v/v) of ether	700	10 000
3. β -Carotene (crude)	Light petroleum + 2% (v/v) of ether	4 000	1 800
4. Sterols	Light petroleum + 20% (v/v) of ether	71 400	144 000

Table 5. *Intra- and extra-plastidic distribution of carotenoids in illuminated excised etiolated maize seedlings*

6-day seedlings were illuminated for 24 hr. under the conditions described in Table 1; the chloroplast was prepared as described under Experimental.

	Concn. of carotenoid (mg./100 g. fresh wt.)		
	Chloroplasts	Residue	Total
Before illumination			
Total carotenoids	—	1.19	1.19
After illumination			
Total carotenoids	1.61	0.85	2.56
β -Carotene	0.61	Trace	0.61
β -Carotene as percentage of total carotenoids	38.2	—	23.8

preferential incorporation of label into β -carotene compared with that appearing in the other unsaponifiable constituents; up to ten times that expected from simple quantitative analysis of the unsaponifiable matter is observed (Table 4).

Distribution of β -carotene in chloroplasts and whole seedlings

The results just described strongly suggested that β -carotene was being synthesized from photo-synthetically-fixed CO_2 . If this were so synthesis would most likely occur into the chloroplasts, the development of which are stimulated by light, and in which photosynthesis takes place. Further, the carotenoids of normal green leaves are entirely concentrated in the chloroplasts (Goodwin, 1952, 1958a).

Table 5 gives the results of a typical carotenoid analysis of the chloroplasts and extra-plastidic residue of illuminated etiolated maize seedlings. They show that (i) the extra-plastidic carotenoids represent quantitatively and qualitatively the pigments present in unilluminated etiolated seedlings (compare Table 1); (ii) all the β -carotene formed on illumination is in the chloroplasts; and (iii) all the xanthophylls formed on illumination are similarly situated.

It is never possible to remove all the chloroplasts from the residual extra-plastidic material, and the results given in Table 5 were obtained by using a calculation based on the fact that the chlorophylls exist only in the chloroplasts. First, the concentration of chlorophylls and carotenoids are determined in the chloroplast fraction and in the residue; secondly, the 'chloroplast carotenoids' present in the residue are calculated from the concentration of chlorophylls in the residue and the chlorophyll/carotenoid ratio for the pure chloroplasts fraction; finally, this calculated value is subtracted from the observed total carotenoid concentration and added to the chloroplast-carotenoid figure to give the gross amount of carotenoids in the chloroplasts.

Table 6. *Uptake of $^{14}\text{CO}_2$ into unsaponifiable matter by illuminated etiolated maize seedlings in the presence and absence of hydroxylamine*

7-day seedlings were illuminated under the conditions given in Table 1; chloroplasts were prepared as described under Experimental; hydroxylamine concn., 0.001 M.

	Activity (counts/min./g. wet wt.)	
	Without hydroxylamine	With hydroxylamine
Chloroplasts	740	120
Residue	140	150
Total	880	270

Action of hydroxylamine

According to Bandurski (1949), 0.001 M-hydroxylamine inhibits photosynthesis without inhibiting respiration. It has been found that etiolated maize seedlings, placed in 0.001 M-hydroxylamine and then illuminated, fail to produce any increase in their carotenoid levels and also fail to produce chlorophylls. In other words, development of the photosynthetic apparatus is inhibited. In an experiment in which uptake of $^{14}\text{CO}_2$ by illuminated etiolated seedlings was measured in the presence and absence of hydroxylamine, the uptake was 3–4 times as great in the absence of the inhibitor, but there was a significant uptake in the presence of the inhibitor (Table 6). As this occurs in the absence of photosynthesis, these results indicate that a carboxylation reaction can proceed which is not dependent on photosynthesis.

Distribution of total unsaponifiable matter in chloroplasts and whole seedlings

Two determinations of the distribution of unsaponifiable matter (mainly sterols) between the chloroplasts and extra-plastidic material of maize seedlings illuminated for 24 hr. indicated that 89

(86–92) % is present in the chloroplasts; this agrees with the distribution of $^{14}\text{CO}_2$ recorded in Table 6.

DISCUSSION

The present investigation demonstrates that the illumination of etiolated seedlings results in the development of chloroplasts in which chlorophyll and carotenoids are synthesized; the ratio β -carotene/xanthophylls in these chloroplasts is the same as that observed in mature leaves. As the dark carotenoids are entirely xanthophyllic in nature, illumination appears at first sight to bring about a specific synthesis of β -carotene. However, when the xanthophylls synthesized in the dark are allowed for, there is no such preferential synthesis of β -carotene; the β -carotene and xanthophylls are being synthesized in the chloroplast in about the same ratio as in normal leaves (Goodwin, 1952). These observations differ from those of Kay & Phinney (1956), who found that on illuminating etiolated seedlings of their strain of maize only β -carotene was synthesized over the first 24 hr. The reason for this difference may be due to the different strains used. The marked incorporation of $^{14}\text{CO}_2$ into the β -carotene produced on illumination indicates that the pigment is produced afresh in the newly developed chloroplast by the photosynthetically fixed CO_2 . This conclusion is confirmed by the action of 0.001M-hydroxylamine, which inhibits both photosynthesis and formation of β -carotene. There is no indication whatever from the present experiments that illumination triggers off the conversion of dark-formed precursors into β -carotene. If this were so, a significant incorporation of $^{14}\text{CO}_2$ into the molecule would not be expected, and certainly there would not be an enrichment of the isotope in the pigment compared with the remainder of the unsaponifiable material. Such a light-triggering action in carotenogenesis does, however, occur in *Neurospora* (Zalokar, 1955).

The failure of labelled acetate and mevalonate to become incorporated into β -carotene in the excised illuminated etiolated seedlings is almost certainly due to their failure to reach the site of carotenoid biosynthesis in the chloroplast rather than to the existence of an entirely novel pathway of biosynthesis. At least this is the simplifying assumption which must be made at the moment, and it is supported by the fact that both acetate and mevalonate are incorporated into the sterols of the seedlings and it is known that the basic pathways of sterol and carotenoid synthesis in other organisms are similar. The present observation that most of the sterols (80–90 %) of the illuminated seedlings are in the chloroplasts is consistent with the views of Chibnall (1939), who found a high lipid content in the chloroplast com-

pared with that of the cytoplasm, and Menke & Jacob (1942), who found that 20 % of the chloroplast lipids were sterols. These chloroplast sterols become highly active in the presence of labelled acetate and mevalonate, and the question arises why these and not the carotenoids are labelled. This cannot be answered at the moment but two possibilities exist: (a) acetate and mevalonate cannot enter the chloroplast as such, but in the cytoplasm are rapidly converted into trace amounts of labelled sterols, which are then transferred to the chloroplasts; (b) the acetate and mevalonate can penetrate the chloroplast but are very rapidly taken up by the sterol-synthesizing system before they can reach the carotene-synthesizing site.

SUMMARY

1. The carotenoids of etiolated maize seedlings are almost entirely xanthophylls, only traces of β -carotene being present.
2. Intact or excised etiolated seedlings synthesize β -carotene and xanthophylls when illuminated. All the light-synthesized carotenoids are present in the chloroplasts which also develop on illumination.
3. $[2-^{14}\text{C}]$ Acetate and $[2-^{14}\text{C}]$ mevalonate cannot penetrate the root system of intact maize seedlings, and are thus not incorporated into the unsaponifiable matter. They are rapidly incorporated into the unsaponifiable matter of excised seedlings kept either in the dark or illuminated. There is, however, only an insignificant incorporation into β -carotene in illuminated excised seedlings.
4. $^{14}\text{CO}_2$ is rapidly incorporated into the unsaponifiable matter of illuminated intact and excised seedlings, and there is a very marked and preferential incorporation into β -carotene.
5. Hydroxylamine (0.001M) inhibits the synthesis of β -carotene, chlorophyll and, presumably, chloroplast development in illuminated excised seedlings.

6. It is concluded that the synthesis of β -carotene in illuminated seedlings occurs in the chloroplasts and utilizes photosynthetically-fixed CO_2 ; this is an integral part of the process of turning an etiolated plant into a photosynthetic plant.

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Canavanine and Related Compounds in Leguminosae

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Canavanine (α -amino- δ -guanidinoxybutyric acid) occurs in the free state in the seeds of the jack bean, *Canavalia ensiformis*, and in the seeds of *C. lineata* and *C. obtusifolia* (Kitagawa & Tomiyama, 1929; Kitagawa, 1937; Damodaran & Narayanan, 1939). Fearon (1946) showed that compounds containing the guanidoxy grouping,

react with trisodium pentacyanoammonioferrate in aqueous solution at pH 7, to give colours which range from orange-red with *N*-methoxyguanidine to magenta with canavanine itself. With this reagent a representative selection of plants were tested for the presence of canavanine, and positive results were obtained with species of *Medicago*, *Ornithopus* and *Colutea*. Canavanine was isolated from *C. arborescens* (Fearon & Bell, 1955).

The inhibition of the pentacyanoammonioferrate colour reaction by such compounds as ascorbic acid and creatinine (Fearon & Bell, 1955) makes its direct application to biological tissues or extracts of limited value. This difficulty has been overcome by using paper chromatography and paper ionophoresis to separate pentacyanoammonioferrate-reacting compounds from others which inhibit or mask the colour reaction.

The occurrence of canavanine in concentrations of 3–5% of dry weight in the seeds of *Canavalia ensiformis* and *Colutea arborescens* suggests its function as a nitrogen-storage product and hence the existence of an enzyme system associated with its utilization. Damodaran & Narayanan (1940) reported the existence in the seeds of *Canavalia ensiformis* of an enzyme which brought about the hydrolysis of canavanine to canalin and urea. They also showed that this enzyme was identical with

jack-bean arginase, though the optimum pH of hydrolysis was 9.4 for arginine and 7.5 for canavanine. It was suggested that canavanine might be the natural substrate of this arginase. In view of these facts, and of the occurrence of canavanine in plants other than *Canavalia*, it seemed likely that other known sources of arginase in the Leguminosae, such as vetch (*Vicia sativa*) and red clover (*Trifolium pratense*), might also prove to be sources of canavanine. This is now shown to be so. It is also shown that canavanine occurs in the seeds of many leguminous plants; quantitative determinations have been made on those seeds which give the strongest colour reaction with trisodium pentacyanoammonioferrate, and the amino acid has been isolated from two of them. The presence in leguminous seeds of compounds other than canavanine which also react with the pentacyanoammonioferrate at pH 7 has been demonstrated.

EXPERIMENTAL

Pentacyanoammonioferrate reagent (PCAF). A 1% (w/v) solution of PCAF (Fearon, 1946) in Cu-free distilled water was used.

Phosphate buffer (pH 7). This was prepared by mixing equal volumes of 0.066 M- NaH_2PO_4 and 0.066 M- Na_2HPO_4 .

Determination of α -amino nitrogen. This was determined as α -amino acid carboxyl by the method of Van Slyke, MacFadyen & Hamilton (1941).

Specificity of the PCAF colour reaction for canavanine

Fearon (1946) found that, of the substituted guanidines which he tested, only the guanidoxy compounds reacted with PCAF to give orange and red colours in the range pH 5–7.5, and of these only canavanine gave a vivid magenta.