phates. Injection of vitamin A acetate or acid restored this changed pattern to normal.

2. The enzyme activities of the liver and intestinal mucosa for the sulphurylation of phenols were significantly reduced in the deficient animals and the lost activity could be restored by the addition of vitamin A alcohol or acid to the reaction mixture *in vitro*.

3. The arylsulphatase C showed no change throughout the deficiency; the arylsulphatases A and B did not change at the plateau stage, but were markedly increased at the acute deficiency stage.

4. It is suggested that the reduced excretion of ethereal sulphates and increased amounts of inorganic sulphate in the urine of vitamin-A-deficient rats are due to the marked loss of the sulphurylating activity caused by the deficiency.

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Studies in Carotenogenesis

29. ATTEMPTS TO DETECT LYCOPERSENE IN HIGHER PLANTS*

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The biosynthesis of carotenoids proceeds from isopent-3-enyl pyrophosphate (Varma & Chichester, 1962) through farnesyl pyrophosphate (Yamamoto, Yokoyama, Simpson, Nakayama & Chichester, 1961). Since squalene, a triterpene, is formed from the condensation of two C_{15} terpenyl pyrophosphate molecules (see Popják & Cornforth, 1960), it is probable that the tetraterpene carotenoids are formed by an analogous condensation of two C_{20} units. Tomato plastids and a soluble carrot-enzyme

system have been shown to form C_{20} terpenyl pyrophosphates from isopentenyl pyrophosphate and farnesyl pyrophosphate (Beeler, Nandi & Porter, 1962). If the condensation of two such units to form carotenoids is completely analogous to triterpene biosynthesis, the first C_{40} compound formed would be lycopersene (I), the C_{40} analogue of squalene. Preliminary investigations (Davies, Goodwin & Mercer, 1961) failed to detect lycopersene in carotenogenic systems, and the suggestion was made that phytoene (15,15'-dehydrolycopersene, II) and not lycopersene is the first

* Part 28: Goodwin & Phagpolngarm (1960).

(I) Lycopersene

(II) Phytoene

C₄₀ compound formed, and that the similarity between sterol and carotenoid biosynthesis terminates at the terpenyl pyrophosphate stage (Davies, 1961; Goodwin, 1961). Phytoene has been recognized for many years as a carotenoid precursor (see Goodwin, 1960*a*) and the conversion of phytoene into phytofluene by a tomato-plastid system has been demonstrated (Beeler & Porter, 1962).

The present study was carried out to assess the role, if any, of lycopersene in carotenoid biosynthesis. This work necessitated the development of a method for detecting lycopersene in biological systems. The method and some of the results were described briefly by Davies *et al.* (1961). The lower limit of the detection of lycopersene by this technique is 0.05 μ g. By exposing the plant tissues to likely isoprenoid precursors heavily labelled with ¹⁴C, the sensitivity of the method as a means of not only detecting but also assessing the importance of lycopersene has been significantly increased.

EXPERIMENTAL

Solvents. The solvents used were light petroleum (A.R., b.p. 40–60°), diethyl ether (A.R.), acetone (A.R.) and chloroform (A.R.), all obtained from British Drug Houses Ltd., Poole, Dorset. The light petroleum was redistilled and the diethyl ether was dried over sodium wire and redistilled over reduced-iron powder immediately before use. Light petroleum (A.R., b.p. 40–60°, free from aromatic hydrocarbons, Hopkin and Williams Ltd., Chadwell Heath, Essex) was used for spectrophotometric analysis.

Chromatographic adsorbents. The adsorbents used were aluminium oxide (acid-washed, M. Woelm) and silica gel G (according to Stahl) for thin-layer chromatography, obtained from E. Merck, Darmstadt, Germany.

Radioactive chemicals. DL-[2-¹⁴C]mevalonic acid lactone (specific activity 9.2 μ C/mg.) and Ba¹⁴CO₃ (specific activity 119 μ C/mg.) were obtained from The Radiochemical Centre, Amersham, Bucks.

Other chemicals. Squalene (from whale oil) was bought from L. Light and Co. Ltd., Colnbrook, Bucks. Phytoene (from carrot oil) was a gift from Professor B. C. L. Weedon, Queen Mary College, University of London, and lycopersene (synthetic) was a gift from Dr O. Isler, F. Hoffmann-La Roche and Co. Ltd., Basle, Switzerland. Neatan, an aqueous suspension of plastic for the preservation of thin-

layer chromatograms, was obtained from E. Merck, Darmstadt, Germany.

Maize and carrot-root preparations. Maize seedlings (South African Horse Tooth) were germinated for 5 days in the dark. They were excised above their roots, collected into bundles and the cut ends immersed either in an aqueous solution of DL-[2-¹⁴C]mevalonic acid or in a beaker of water placed within a vacuum desiccator containing ¹⁴CO₂, liberated from Ba¹⁴CO₃ with lactic acid. Thin (1 mm.) transverse slices of young carrots were freshly cut and immersed for 24 hr. in a solution containing DL-[2-¹⁴C]-mevalonic acid (40 μ C) (Braithwaite & Goodwin, 1960).

Small-scale extraction of unsaponifiable materials. The tissues (about 100 g. wet wt.) were thoroughly washed with water, cut into small pieces with scissors, placed in a blender together with 3 vol. of acetone and macerated for 3–4 min. The resulting suspension was then filtered by suction through a sintered funnel. The residue was returned to the blender, extracted again with a similar volume of acetone and filtered. This extraction process was repeated until the filtrate was colourless. The acetone extracts were bulked, diluted with 4 vol. of water and extracted several times with diethyl ether; the extraction was continued until the ether phase remained colourless. The bulked ethereal extracts were then washed several times with water, dried over anhydrous Na₂SO₄ for 30 min. and evaporated to dryness under N₂. The resulting lipid was then saponified by dissolving it in a known volume of ethanol, adding aq. 60% (w/v) KOH solution (1 ml./10 ml. of ethanolic solution) and boiling gently under a slow stream of N₂ for 15 min. The saponified mixture was then cooled, diluted with 3 vol. of water and extracted four times with ether. The bulked ethereal extracts, containing the unsaponifiable material, were then washed several times with water to remove alkali, dried over anhydrous Na₂SO₄ and taken to dryness under N₂.

Large-scale extraction of unsaponifiable material. Lipid (2 kg.) from the leaves of the common vetch (*Vicia sativa*) was saponified by refluxing for 3½ hr. with 8 l. of industrial ethanol and 600 ml. of aq. 60% (w/v) KOH solution. The saponified mixture was cooled, diluted to 40 l. with water and extracted six times with ether by using 7½ l. each time. The combined ether extracts were washed thoroughly with water, dried over anhydrous Na₂SO₄ and evaporated to dryness in a rotary evaporator.

Small-scale removal of sterols. The sterols were removed from the unsaponifiable materials (30–200 mg.) by precipitation as their digitonides. The unsaponifiable extract was dissolved in aq. 95% (v/v) ethanol (30 ml./100 mg. of

unsaponifiable material) and heated to boiling. To this solution a hot 2% (w/v) solution of digitonin in aq. 90% (v/v) ethanol (25 ml./100 mg. of unsaponifiable material) was added, and the mixture was boiled until a white precipitate appeared. Precipitation was completed at 0° overnight. The precipitate was centrifuged and the supernatant solution decanted. The sterol digitonides were washed several times with ethanol and ether and the washings were added to the supernatant solution. The supernatant solution contained a considerable quantity of free digitonin. This was removed by the addition of ether, which caused the free digitonin to be thrown out of solution, enabling it to be removed by centrifuging. Any residual digitonin in the supernatant solution was removed by washing several times with water. The supernatant solution was then dried over anhydrous Na_2SO_4 and taken to dryness under N_2 . The resulting material is referred to in this paper as the 'sterol-free unsaponifiable material'. The sterols were regenerated from their digitonides by the method of Schoenheimer & Dam (1933).

Large-scale removal of sterols. The unsaponifiable material (251.5 g.) from the tare lipid was dissolved in light petroleum and stored at -20° for 2 days, during which time a considerable quantity of sterol crystallized. The sterol was filtered off and the filtrate partitioned against an equal volume (5 l.) of aq. 95% (v/v) methanol. The light-petroleum phase (epiphase), containing the less polar hydrocarbons, was then evaporated to dryness in a rotary evaporator.

Chromatography of the 'sterol-free unsaponifiable material'. The sterol-free unsaponifiable material was chromatographed on 10 g. columns of alumina (acid-washed, weakened with water to Brockmann grade III). The columns were developed with 100 ml. each of light petroleum, 2% ether in light petroleum, 4% ether in light petroleum and 6% ether in light petroleum.

Chromatography of the epiphasic material of the tare lipid. The epiphasic material was chromatographed on a column of alumina (1200 g., acid-washed, weakened with water to Brockmann grade II). The development of this chromatogram is shown in Table 5.

Thin-layer chromatography. Suitable samples of the sterol-free unsaponifiable material or tare epiphasic material, obtained by column chromatography on alumina, were examined by thin-layer chromatography (see the Results section), the saturation-chamber technique as described by Davies (1963) being used. The chromatograms were exposed to iodine vapour and the resulting brown zones noted. Larger samples were then diluted with authentic, non-radioactive lycopersene and run on a chromatoplate as a long strip, rather than a single spot, to prevent overloading. A narrow vertical strip of adsorbent was removed from the plate by means of transparent adhesive tape. Exposure of this strip to iodine vapour revealed on it the positions of the various hydrocarbons. By returning this stained strip to its original place on the chromatoplate, the location of these hydrocarbons on the unstained part could be marked. The squalene, lycopersene and other staining zones were carefully scraped off the plate, transferred to small centrifuge tubes and extracted with 2×3 ml. portions of chloroform and once with 3 ml. of ethanol. The bulked extracts from each zone were evaporated to dryness under N_2 and assayed for radioactivity. The lycopersene, after radioassay, was run again on another

chromatoplate, eluted in the same fashion and again assayed for radioactivity.

In later experiments it became possible to remove the whole layer of adsorbent intact from the supporting glass plate. This was achieved by spraying the chromatoplate with Neatan, which, on drying, binds the adsorbent together. The layer can then be removed easily by immersing the plate in water. After removal, the adsorbent layer was dried between filter papers and a vertical section ($1\frac{1}{2}$ in. wide) scanned for radioactivity.

Measurement of radioactivity. Measurements of radioactivity were made with a Nuclear Measurements Corp. (Chicago, Ill., U.S.A.) PCC-10A proportional counter coupled to a Panax (Redhill, Surrey) 100C E.H.T. and fast scaler unit. The geometrical efficiency was 35%. The lipid fractions were assayed by applying 0.2 ml. of a suitable solution of the lipid in CHCl_3 to a stainless-steel disk, previously prepared with a 2 cm. diam. disk of lens tissue (Greens no. 105). The lens tissue ensures an even and reproducible spread of lipid (Glover, Leat & Morton, 1957). The solvent was evaporated by irradiation with infrared lamps (250 w). The usual corrections for background, self-absorption and coincidence were made.

The strips of adsorbent removed from chromatoplates were scanned for radioactivity with a Nuclear-Chicago Corp. model C100B Actigraph II radiochromatogram scanner.

RESULTS

Detection of lycopersene

A sample of pure synthetic lycopersene was used to develop a technique for the isolation of lycopersene from carotenogenic systems. Lycopersene was found to be quite stable to the saponification procedure. The chromatographic behaviour of lycopersene on columns of alumina of different adsorbing power (according to the Brockmann scale) was investigated. Lycopersene is eluted quantitatively with light petroleum from Brockmann grade II and III alumina. Thus if a tissue unsaponifiable-lipid fraction were chromatographed on alumina, lycopersene, if present, would be eluted in the light-petroleum (hydrocarbon) fraction, a major component of which would be squalene. Squalene, the C_{30} analogue of lycopersene, would be expected to have similar physical properties, rendering the chromatographic separation of these substances from one another difficult. However, this separation was accomplished by thin-layer chromatography with silica gel G supported on glass plates (20 cm. $\times$ 20 cm.). The thin-layer chromatogram was developed with light petroleum (b.p. 40 – 60°) and the hydrocarbon zones were visualized by exposure to iodine vapour, when they became brown against the white background of the adsorbent. The R_f values of squalene, lycopersene, phytoene and phytofluene varied to some extent depending upon the age of the chromatoplate but a clear separation of these compounds was always obtained. Average R_f values for squalene,

lycopersene, phytoene and phytofluene on freshly prepared (and therefore most strongly adsorbing) chromatoplates are 0.4, 0.3, 0.2 and 0.1 respectively. As the chromatoplates age and become less strongly adsorbing these R_F values tend to increase; the separation of the hydrocarbons, however, improves. There was a slight variation in R_F values, although a saturation chamber was used, but authentic samples of phytoene, lycopersene and squalene were always chromatographed on the same plate. Since it was likely that lycopersene would be only a very minor component of the light-petroleum fraction, the ability of the thin-layer-chromatography technique to detect very small quantities of lycopersene in the presence of relatively large amounts of squalene and phytoene was investigated. Lycopersene could be separated from a mixture containing 0.05 $\mu\text{g.}$ of lycopersene, 5 $\mu\text{g.}$ of phytoene and 10 $\mu\text{g.}$ of squalene, and be detected by exposure to iodine vapour. The lower limit of lycopersene detection with this technique is 0.05 $\mu\text{g.}$ Various impurities arising from the solvents and alumina could, on subsequent thin-layer chromatography, produce zones, stainable with iodine, that could be mistaken for squalene or lycopersene. The solvents, light petroleum and diethyl ether, both of analytical grade, contained a non-volatile residue. When this residue was subjected to thin-layer chromatography it separated into several diffuse zones, which fluoresced under ultraviolet light and stained with iodine. Some of these zones had the same R_F values as squalene, lycopersene, phytoene and phytofluene. Therefore

unless these materials were removed from the solvents before use the results of the thin-layer chromatography of the light-petroleum (hydrocarbon) fractions were impossible to interpret. This difficulty was overcome by careful redistillation of the solvents.

The sample of alumina that we used also contained an impurity, which could be washed off with light petroleum and on thin-layer chromatograms co-chromatographed with squalene. This material was removed from the alumina by exhaustive washing with redistilled light petroleum (see also Davies, Jones & Goodwin, 1963).

Examination of carotenogenic systems for lycopersene

Etiolated excised maize seedlings exposed to DL-[2- ^{14}C]mevalonic acid in the light. Seedlings from maize germinated for 5 days in the dark were excised from their roots and placed in water containing DL-[2- ^{14}C]mevalonic acid and illuminated for 24 hr. This system was thus absorbing labelled mevalonate and actively synthesizing carotenoids at the same time. The results of two experiments are recorded in Table 1. Expt. 1 shows that no activity could be detected in the lycopersene zone eluted from the chromatoplate. The thin-layer chromatogram of the light-petroleum fraction from Expt. 2 was directly scanned and again no activity was recorded in the lycopersene zone (Fig. 1).

Etiolated excised maize seedlings exposed to DL-[2- ^{14}C]mevalonic acid in the dark. As no lycopersene could be detected in a system known to be

Table 1. *Incorporation of radioactivity into the unsaponifiable material of etiolated excised maize seedlings exposed to DL-[2- ^{14}C]mevalonic acid in the light*

Seedlings germinated in the dark for 5 days, excised from their roots (wet wt.: Expt. 1, 208 g.; Expt. 2, 30.2 g.), were placed in an aqueous solution of DL-[2- ^{14}C]mevalonic acid and illuminated for 24 hr. Labelled substrate: 10 μC (0.67 $\mu\text{C/ml.}$) in Expt. 1 and 60 μC (10 $\mu\text{C/ml.}$) in Expt. 2.

	Wt. (mg.)		Total counts/min.		Specific activity (counts/min./mg.)	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Unsaponifiable material	147.2	23.0	—	2 598 000	—	113 000
Sterol	—	5.2	—	349 600	—	67 200
Sterol-free unsaponifiable material	101.3	17.8	567 700	2 249 000	5 600	126 300
Fraction from chromatography on alumina (Brockmann grade III)						
Light-petroleum fraction	16.8	4.5	35 900	157 400	2 140	34 980
2% E/P* fraction	15.6	0.3	122 200	17 780	7 840	59 300
4% E/P* fraction	17.9	0.5	323 100	160 200	18 900	320 400
6% E/P* fraction	7.0	1.0	50 020	1134 000	7 150	1 134 00
Thin-layer chromatography of light-petroleum fraction (one-fifth of fraction used in Expt. 1)						
Squalene zone	—	—	4 698	[see Fig. 1]	—	—
Lycopersene zone	—	—	96		—	—
Origin zone	—	—	2 390		—	—
Rechromatography of lycopersene zone by thin-layer system						
Squalene zone	—	—	Negligible		—	—
Lycopersene zone	—	—	0		—	—

* Diethyl ether in light petroleum.

Table 2. Incorporation of radioactivity into the unsaponifiable material of etiolated excised maize seedlings exposed to DL-[2-¹⁴C]mevalonic acid in the dark

Seedlings germinated in the dark for 5 days, excised from their roots (wet wt.: Expt. 3, 205 g.; Expt. 4, 145 g.), were placed in aqueous solutions of DL-[2-¹⁴C]mevalonic acid in the dark. In each Expt. 50 μc (5 μc/ml.) of labelled substrate was used. The incubation period was 6 hr. in Expt. 3 and 24 hr. in Expt. 4.

	Wt. (mg.)		Total counts/min.		Specific activity (counts/min./mg.)	
	Expt. 3	Expt. 4	Expt. 3	Expt. 4	Expt. 3	Expt. 4
Unsaponifiable material	135	145	273 000	1 252 000	1 760	8 630
Sterol	45.3	24.7	125 800	472 500	2 780	19 100
Sterol-free unsaponifiable material	89.8	116.6	147 000	779 000	1 640	6 680
Fractions from chromatography on alumina (Brockmann grade III)						
Light-petroleum fraction	11.4	24.5	19 900	158 900	1 750	6 480
2 % E/P* fraction	1.0	4.7	6 170	58 500	6 170	12 450
4 % E/P* fraction	9.9	16.1	89 400	407 500	9 030	25 300
6 % E/P* fraction	2.9	5.1	18 050	88 150	6 220	17 280
Thin-layer chromatography of light-petroleum fraction						
Squalene zone	—	—	18 500	76 200	—	—
Lycopersene zone	—	—	95	5 200	—	—
Origin	—	—	240	77 500	—	—
Rechromatography of lycopersene zone by thin-layer system						
Squalene zone	—	—	Negligible	5 180	—	—
Lycopersene zone	—	—	0	0	—	—

* Diethyl ether in light petroleum.

Fig. 1. Thin-layer chromatogram of the light-petroleum fraction of the sterol-free unsaponifiable material of Expt. 2 scanned for radioactivity. Scanning conditions: scanning speed $\frac{3}{4}$ in./hr.; collimator slit $\frac{1}{8}$ in.; time-constant 40 sec.; count-rate range 1000 counts/min. A, Position of lycopersene on the chromatogram; B, position of squalene on the chromatogram.

actively synthesizing carotenoid, it was thought possible that if lycopersene were a precursor of carotenoids it might be converted into carotenoids so rapidly that its endogenous concentration would be too small to be detected even with labelled substrates. If this were the case then lycopersene

might be present at detectable concentrations in systems where the synthesis of carotenoids was inhibited to such an extent that precursors would accumulate. Such a system is the etiolated maize plant kept in the dark, when there is only a very slow synthesis of xanthophylls (Kay & Phinney, 1956; Goodwin, 1958*a, b*). To test this possibility, DL-[2-¹⁴C]mevalonic acid was administered to excised, etiolated maize seedlings kept in the dark for 6 and 24 hr. Even under these conditions no label can be found in the lycopersene zones (Table 2). After exposure to DL-[2-¹⁴C]mevalonic acid for 6 hr. almost all the radioactivity of the light-petroleum fraction was in squalene, whereas after exposure to DL-[2-¹⁴C]mevalonate for 24 hr. the radioactivity was almost equally divided between squalene and the origin zone.

Etiolated excised maize seedlings exposed to ¹⁴CO₂ in the light. Although [2-¹⁴C]mevalonic acid is well established as a precursor of isoprenoid compounds, it is not significantly incorporated into carotenoids in illuminated, excised, etiolated maize seedlings (Goodwin, 1958*a, b*), most of the label appearing in the sterols. On the other hand, ¹⁴CO₂ is rapidly and preferentially incorporated into carotenoids in both intact and excised etiolated maize seedlings when illuminated (Goodwin, 1958*a, b*). Therefore it was possible that lycopersene, if present, would become labelled to a greater extent from ¹⁴CO₂ than from [2-¹⁴C]mevalonic acid. Accordingly, in two experiments, excised etiolated maize seedlings, their cut ends immersed in water, were exposed to

an atmosphere containing $^{14}\text{CO}_2$ (derived in each case from $\text{Ba}^{14}\text{CO}_3$, 1 mc) in the light (Table 3). Again no label could be found in the lycopersene zone either on elution (Expt. 5) or after a direct scan of the thin-layer chromatogram. In Expt. 5 about 60% of the radioactivity is in the squalene, 37% in the waxy materials running with the solvent front, 2% in the phytoene and the remainder in the origin spot. The thin-layer chromatogram of the light-petroleum fraction from Expt. 6 was directly scanned for radioactivity and showed essentially the same picture.

Incubation of carrot slices with DL-[2- ^{14}C]mevalonic acid. As the amounts of the known carotenoid precursors, phytoene and phytofluene, present in green tissues are extremely small (see Goodwin, 1960b, and next section), a final experiment was carried out with isolated carrot slices. Carrots contain relatively large amounts of phytoene and phytofluene (see Goodwin, 1952) and isolated disks rapidly and efficiently incorporated DL-[2- ^{14}C]mevalonic acid into β -carotene (Braithwaite & Goodwin, 1960). Thin (1 mm.) transverse slices were cut from fresh, young carrots and immersed in a solution containing DL-[2- ^{14}C]mevalonic acid (40 μC) for 24 hr. On examination of the light-petroleum fraction of the unsaponifiable material by thin-layer chromatography it was found that the large β -carotene zone overlapped the phytoene zone. The light-petroleum fraction was chromatographed again on an acid-washed alumina (10 g.; Brockmann grade II) column, when the colourless hydrocarbons were eluted with the first 100 ml. of light petroleum whereas the β -carotene remained adsorbed at the top of the column. The colourless hydrocarbon fraction was

then chromatographed on a silica-gel-G plate in the usual manner. Again no radioactivity could be found in the lycopersene zone, although phytoene, a known precursor, contained a significant number of counts (Table 4). Squalene was again highly labelled.

Large-scale examination of plant lipid for lycopersene. The failure to find lycopersene in relatively small quantities of tissues, even though the detection procedure was made more sensitive by the prior incubation of these tissues with likely radioactive precursors, led us to prepare much larger quantities of plant material in an attempt to find this compound. Tare lipid (2 kg., obtained from about 150 kg. wet wt. of tare leaves) was saponified and yielded 251.5 g. of unsaponifiable material, which was dissolved in light petroleum and stored for 2 days at -20° , when 123 g. of sterol crystallized out. After the sterol crystals were filtered off, the light-petroleum solution was partitioned against 95% (v/v) methanol. The petroleum phase (epi-phase) yielded 74 g. of material, which was chromatographed on alumina. The development and results of this chromatography are given in Table 5.

A preliminary thin-layer chromatogram of small samples of fraction 1-8 from this column showed that fractions 3 and 4 contained the colourless polyenes. These two fractions were bulked and chromatographed on a column (10 g.) of silica gel G. The column was developed with light petroleum, 100 fractions (5 ml.) being taken.

Thin-layer-chromatographic analysis of these fractions showed that a waxy material was present in fractions 10-15 inclusive. This material ran with the solvent front on a thin-layer chromatogram.

Table 3. *Incorporation of radioactivity into the unsaponifiable material of etiolated excised maize seedlings exposed to $^{14}\text{CO}_2$ in the light*

Seedlings germinated in the dark for 5 days, excised from their roots (wet wt.: Expt. 5, 185 g.; Expt. 6, 40 g.), were placed in an atmosphere (vol. 5 l.) of $^{14}\text{CO}_2$ (from 1 mc of $\text{Ba}^{14}\text{CO}_3$) and illuminated for 24 hr.

	Wt. (mg.)		Total counts/min.		Specific activity (counts/min./mg.)	
	Expt. 5	Expt. 6	Expt. 5	Expt. 6	Expt. 5	Expt. 6
Unsaponifiable material	135.2	30.1	803 700	795 600	5950	26 500
Sterol	20.7	5.7	135 500	90 400	6 540	15 860
Sterol-free unsaponifiable material	114.5	24.4	668 200	705 200	5 860	28 900
Fractions from chromatography on alumina (Brockmann grade III)						
Light-petroleum fraction	19.4	6.1	50 350	81 060	2 590	13 280
2% E/P* fraction	3.0	Negligible	26 370	64 400	8 790	—
4% E/P* fraction	21.3	9.6	359 000	150 500	16 860	15 670
6% E/P* fraction	6.9	3.2	79 100	183 600	11 450	57 360
Thin-layer chromatography of light-petroleum fraction (one-half of fraction used in Expt. 5)						
Squalene zone	—	—	15 175	—	—	—
Phytoene zone	—	—	500	—	—	—
Lycopersene zone	—	—	0	—	—	—
Solvent-front zone	—	—	9 500	—	—	—

* Diethyl ether in light petroleum.

Table 4. *Incorporation of radioactivity into the unsaponifiable material of carrot slices incubated with DL-[2-¹⁴C]mevalonic acid*

Slices (wet wt. 44 g.) were immersed in 100 ml. of water containing 40 μ C of DL-[2-¹⁴C]mevalonic acid for 24 hr.

	Wt. (mg.)	Total counts/min.	Specific activity (counts/min./ mg.)
Unsaponifiable material	51.9	—	—
Sterol-free unsaponifiable material	41.9	396 600	9 460
Fractions from chromatography on alumina (Brockmann grade III)			
Light-petroleum fraction*	14.5	290 200	20 000
2% E/P† fraction	1.2	8 430	7 030
4% E/P† fraction	2.9	66 000	22 750
6% E/P† fraction	1.8	17 400	9 670
Thin-layer chromatography of light- petroleum fraction (one-quarter of fraction used)			
Squalene zone	—	66 200	—
Phytoene zone	—	1 200	—
Lycopersene zone	—	290	—
Rechromatography of lycopersene zone by thin-layer system			
Squalene zone	—	210	—
Lycopersene zone	—	0	—

* First rechromatographed on alumina (Brockmann grade II) (see text).

† Diethyl ether in light petroleum.

Table 5. *Fractionation of the epiphasic unsaponifiable material from tare lipid on acid-washed alumina (Brockmann grade II)*

For preparation of epiphase material see text.

Fraction no.	Vol. (l.)	Solvent	Eluate	Residue	
				Appearance	Wt. (g.)
1	1	Light petroleum (b.p. 40–60°)	Colourless	White waxy solid	0.490
2	1		Colourless	Pale-yellow solid	6.628
3	0.5		Pale yellow	Yellow oil	0.210
4	1		Orange-red	Brown oil	0.474
5	1		Orange-red	Brown oil	0.607
6	1		Orange-red	Brown oil	0.487
7	1		Orange-red	Brown oil	0.329
8	1		Orange-red	Brown oil	0.225
9	1		Light orange	Brown oil, solidified on cooling	0.327
10	1		Light orange		0.201
11	1	0.5% E/P*	Light orange		0.215
12	1		Light orange		0.292
13	1		Light orange		0.335
14	1		Orange-red		1.020
15	1		Orange-red		1.559
16	1		Orange-red		2.798
17	1		Orange-red		4.442
18	1		Orange		4.661
19	1		Ether		14.937
20	1		Orange-red		17.835
21	1	Ether	Orange		3.395
22	2	Ethanol	Orange		8.290
Total	22.5	—	—	—	69.76

* Diethyl ether in light petroleum; recovery 96.5%.

Fractions 15–40 contained squalene, the concentration of which was maximal in fraction 25. Phytoene was present in fractions 30–70, its maximal concentration being in fraction 50. Squalene and phytoene were identified in these

fractions by co-chromatography with authentic samples of these substances. Preliminary experiments had shown that authentic, synthetic lycopersene runs between squalene and phytoene on silica gel G (Davies *et al.* 1961); therefore if lyco-

persene were present it would be expected to appear in fractions 25–50. Careful chromatography of these fractions on thin layers of silica gel G failed to reveal its presence.

The presence of phytoene in fractions 30–70 was confirmed by spectrophotometric analysis (absorption maxima at 275, 285, 296 $m\mu$ in light petroleum). Phytofluene, as indicated by its fluorescence and absorption maxima (331, 348, and 367 $m\mu$) in light petroleum, was eluted from the column subsequently to phytoene. Phytoene and phytofluene were determined spectrophotometrically ($E_{1\text{cm}}^{1\%}$ of phytoene in light petroleum at 285 $m\mu$ is 1220, and that of phytofluene at 348 $m\mu$ is 1350). The concentration of phytoene in tares is 28 $\mu\text{g./100 g.}$ fresh wt. and that of phytofluene 15 $\mu\text{g./100 g.}$ fresh wt. The latter concentration is of the same order as that reported by Zechmeister & Karmakar (1953).

DISCUSSION

Absence of lycopersene from plant tissues. Although it is difficult to prove conclusively that a substance is absent from plant tissues, the experiments reported here indicate that the incorporation of $^{14}\text{CO}_2$ or DL-[2- ^{14}C]mevalonate into lycopersene cannot be demonstrated. Under our experimental conditions an activity of 20 counts/min. could easily be detected, so that if it is assumed that the lycopersene zones in our experiments contained 10 counts/min., then, in both maize and carrot, lycopersene could exist only in a concentration of less than 0.015% of that of squalene. A similar calculation for carrot would indicate a maximum concentration of 1% that of phytoene.

In the large-scale experiment with lipid from tares no lycopersene could be detected, i.e. 0.05 $\mu\text{g.}$ could not be detected in an extract from approximately 150 kg. of starting material. Thus the concentration of lycopersene could not exceed 3.3×10^{-5} $\mu\text{g./100 g.}$ fresh wt. The usual concentration of phytoene and phytofluene in green tissues is 10 $\mu\text{g./100 g.}$ (Zechmeister & Karmakar, 1953) and of β -carotene, 4 mg./100 g.

From these figures the reasonable conclusion is that lycopersene does not exist in higher plants, and this view has also been put forward by Anderson & Porter (1962). Lycopersene was not detected in photosynthetic bacteria (Davies *et al.* 1961), in *Phycomyces blakesleeanus* (B. H. Davies, unpublished observations) and in *Neurospora crassa* (Davies *et al.* 1963). Grob & Boschetti (1962) have, however, reported the presence of lycopersene in *N. crassa*.

If lycopersene is not the first C_{40} compound formed in carotenoid biosynthesis, then phytoene probably fulfils this role. Circumstantial evidence for this view has been available for some time (see

Goodwin, 1961), and Beeler & Porter (1962) have demonstrated the conversion, by tomato plastids, of phytoene into phytofluene, the next compound in the series leading from phytoene to lycopene (Anderson & Porter, 1962).

A consequence of the formation of phytoene as the first C_{40} terpene, rather than lycopersene, is that the centre of the molecule becomes more rigid, and may well prevent the extensive cyclization of the tetraterpene; so that, although fused polycyclic triterpenes formed from squalene abound in Nature, the C_{40} analogues do not. If lycopersene were found in biological systems, then the existence of polycyclic tetraterpenes would not be unexpected. This problem has been discussed in detail (Goodwin, 1961).

In systems from higher plants, NADP^+ appears to be effective in stimulating the formation of phytoene, whereas NADPH is inhibitory (Anderson & Porter, 1962). In cholesterol biosynthesis in a rat-liver system, NADPH is the cofactor concerned in the formation of squalene (Goodman & Popják, 1960). Anderson & Porter (1962) considered these observations to be circumstantial evidence for the existence of different pathways of terpene biosynthesis in plants and animals, NADPH operating in animals and NADP^+ in plants. Yokoyama, Nakayama & Chichester (1962), however, have demonstrated the participation of NADP^+ in carotenogenesis by their system from *Phycomyces blakesleeanus*, and Lynen (1959) has shown that NADPH is a cofactor in squalene formation in an enzyme system from yeast. Thus it is more likely that the regulating mechanism for terpene biosynthesis involves the balance of NADP^+ and NADPH, NADPH favouring the formation of squalene and sterol, and NADP^+ the formation of carotenoids. Yokoyama *et al.* (1962) also considered the possibility of NAD^+ being a governing factor, since ergosterol synthesis in *Phycomyces* has a greater requirement for NAD^+ than has carotene synthesis.

General observations. A number of general conclusions can be drawn from Tables 1, 2 and 3 on the incorporation of DL-[2- ^{14}C]mevalonic acid and $^{14}\text{CO}_2$ into the unsaponifiable material of plants: (a) in illuminated seedlings the counts in the sterol fraction represent about 15% of the total incorporation into the unsaponifiable material irrespective of whether the substrate is mevalonate or carbon dioxide; (b) with mevalonate, seedlings kept in the dark show a much higher (40%) relative incorporation into the sterols; (c) with mevalonate, the specific activity of the sterol-free unsaponifiable material is much greater than that of the sterol fraction in illuminated seedlings and much less in seedlings kept in the dark; (d) with carbon dioxide the total counts in the sterol-free unsaponifiable

material are greater than that in the sterol fraction after 24 hr. of illumination.

The main labelled material contributing to the total counts in the sterol-free unsaponifiable material from the $^{14}\text{CO}_2$ -treated maize is phytol, derived from the chlorophyll synthesized during illumination (Mercer & Goodwin, 1962). Phytol, however, does not become significantly labelled when $[2\text{-}^{14}\text{C}]$ mevalonate is the substrate (Mercer & Goodwin, 1962). It therefore behaves towards mevalonate and carbon dioxide in the same way as does β -carotene (Goodwin, 1958*a*, *b*). The compounds that become highly labelled in the sterol-free unsaponifiable material from maize treated with $[2\text{-}^{14}\text{C}]$ mevalonate include squalene and an unidentified substance, whose specific activity is considerably greater than that of the sterols. This substance does not appear to become appreciably labelled when maize is exposed to $^{14}\text{CO}_2$ for 24 hr.

The activity in squalene of carrot slices represents some 60–70% of the total activity of the sterol-free unsaponifiable material. In all the experiments with maize it was never greater than 15%.

SUMMARY

1. A method for the detection and identification of lycopersene has been developed. The sensitivity limit is 0.05 μg .

2. Analysis of the unsaponifiable components of excised, etiolated maize seedlings exposed to $[2\text{-}^{14}\text{C}]$ mevalonate under conditions of light and darkness, and to $^{14}\text{CO}_2$, has failed to reveal the presence of lycopersene.

3. $[^{14}\text{C}]$ Carbon dioxide is incorporated into both squalene and phytoene by maize seedlings, but $[2\text{-}^{14}\text{C}]$ mevalonate is incorporated only into squalene.

4. $[2\text{-}^{14}\text{C}]$ Mevalonate is incorporated into both squalene and phytoene by carrot-root slices; no lycopersene could be detected.

5. Tare (*Vicia sativa*) lipid was also examined for the presence of lycopersene, but none was detected.

6. Lycopersene is probably not a precursor of carotenoids.

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