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Incorporation of [2-^{14}C]Mevalonic Acid into Phytoene by Isolated Chloroplasts

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1. Chloroplasts prepared by the non-aqueous technique will, after fragmentation by ultrasonic treatment, incorporate [2-^{14}C]mevalonic acid into phytoene, the first C_{40} compound formed in the biosynthetic sequence to coloured carotenoids. 2. With suspensions containing 3·5mg. of chlorophyll, the optimum amounts of cofactor required were ATP (10μmoles), magnesium chloride (20μmoles) and glutathione (20μmoles); neither NAD^+ nor $NADP^+$ was required. 3. Very small amounts of squalene are also formed and synthesis is stimulated by addition of NADH or NADPH. Phytoene synthesis was not affected by the presence of these cofactors and no lycopersene (the C_{40} homologue of squalene) was detected. 4. The phytol side chain of chlorophyll is also labelled under these conditions. 5. Preparations of developing chloroplasts are more active than preparations of mature chloroplasts.

Much evidence on the structure, function and chemical composition of the chloroplasts of higher plants suggests that the carotenoid pigments are synthesized *in situ* in the plastid. It has been shown (Goodwin, 1958; Griffiths, Threlfall & Goodwin, 1964; Treharne, Mercer & Goodwin, 1966) that addition of labelled early precursors of terpenoids, such as [2-^{14}C]MVA§, to leaves of a variety of plant species results in heavy labelling of sterols and related compounds, which are synthesized outside the plastid. By contrast, during chloroplast development MVA is only insignificantly incorporated into chloroplast terpenoids such as β-carotene, the phytol side chains of the chlorophyll pigments and plastoquinone. These results led Goodwin & Mercer (1963) to suggest that terpenoid synthesis in developing seedlings was regulated by a combination of intracellular segregation of enzymes and specific membrane permeability. Isolated chloroplasts should therefore provide a system for the study of carotenogenesis that would enable the metabolic fate of labelled precursors to be traced without loss of label into sterols and other triterpenoids.

Chloroplasts prepared from a variety of plant species by conventional aqueous procedures, with gradient centrifugation, incorporated little label from [^{14}C]MVA or [^{14}C]acetate into carotenoids (Treharne, 1964). This failure was probably due in major part to the loss of water-soluble components from the plastids during isolation. Moreover, chloroplasts prepared by aqueous techniques are largely devoid of stroma and bounding membranes (Leech, 1964). Heber (1957) and Stocking (1959) described methods of isolation of chloroplasts in organic solvents, which overcome a number of the inherent difficulties of aqueous procedures, particularly the leaching of water-soluble enzymes from plastids.

This prompted the investigations, described below, to test the ability of chloroplasts isolated in organic solvents to incorporate MVA into carotenoids. Part of this investigation has already been briefly reported (Charlton, Treharne & Goodwin, 1966).

EXPERIMENTAL

Plant material. Seedlings of dwarf bean (*Phaseolus vulgaris* var. Lightning) were grown for 9–10 days at 27° in the light, and then dark-adapted for 2 days to decrease the starch content of the leaves. The dark treatment was carried out to prevent rupture of the lamellae and bounding membranes by starch grains in the chloroplasts during centrifugation of leaf homogenates. Midribs and large lateral veins were removed from the leaves, and the laminar tissue was sliced into pieces of approx. 1 cm.^{2} and immediately placed in cooled flasks surrounded by acetone–solid CO_2 mixture. The flasks containing the frozen tissue were attached to a freeze-drying apparatus housed in a deep-

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§ Abbreviation: MVA, mevalonic acid.

freeze, and the leaf material was freeze-dried for 2 days under vacuum and stored at −25° until required.

Preparation of chloroplasts. In each experiment 100mg. of freeze-dried leaf material was homogenized in carbon tetrachloride–hexane mixture (sp.gr. 1·34) in a hand-operated TenBroeck homogenizer. The homogenate was filtered through a pad of muslin and glass wool into a centrifuge tube and the filtrate adjusted to 10ml. with carbon tetrachloride–hexane (sp.gr. 1·34). Then 10ml. of carbon tetrachloride–hexane mixture (sp.gr. 1·32) was carefully layered on top, followed by 3ml. of hexane or 3ml. of carbon tetrachloride–hexane mixture (sp.gr. 1·30). The resulting gradient was then centrifuged at 12000*g* for 30min. at −5°, and the chloroplast layer pipetted out, mixed with hexane and centrifuged at 3000*g* for 5min. to sediment the chloroplasts. After the supernatant had been decanted off, the residual solvent was removed under a stream of N_2 and the chloroplast pellet dispersed in 1·0ml. of phosphate buffer, pH 7·4. The chloroplast suspension was subjected to ultrasonic disintegration for 1–2min. and portions of the ultrasonically treated material, containing about 3·5mg. of chlorophyll, were incubated in Thunberg tubes with [2-^{14}C]MVA. A supplement of ATP (10μmoles), $MgCl_2$ (20μmoles) and glutathione (20μmoles) was added to each tube giving a final volume of 1·2–1·4ml. and the preparations were incubated at 25–27° for 6hr. A control consisting of a boiled chloroplast suspension was used in every experiment.

Extraction and separation of terpenoids. Incubation mixtures were treated with boiling ethanol and saponified by adding KOH (final concn. 6%, w/v) (Goodwin, 1955). Unsaponifiable material was extracted into diethyl ether and the extracts were chromatographed on columns of Brockmann grade III alumina, eluting with light petroleum (b.p. 60–80°) and increasing proportions of diethyl ether in light petroleum.

The light-petroleum fraction was chromatographed on thin layers of Kieselgel G impregnated with Rhodamine 6G, with light petroleum (b.p. 60–80°) or with 5% (v/v) benzene in light petroleum. This fraction was also examined by reversed-phase chromatography. Authentic samples of squalene and phytoene were chromatographed simultaneously as reference compounds and located under u.v. light.

Column fractions eluted with 8% (v/v) and 14% (v/v) ether in light petroleum were chromatographed on thin layers of Kieselgel G with chloroform, and also examined by reversed-phase chromatography on Whatman no. 1 paper impregnated with liquid paraffin, with 80–90% (v/v) acetone in water.

Preparation and examination of chemical derivatives. The unsaturated hydrocarbons, eluted in the light-petroleum fraction, were hydrogenated in ethanol, after addition of carrier compounds, in a micro-hydrogenation apparatus for 15min. with platinum oxide as catalyst. The reduction products were extracted into ether and examined on thin layers of Kieselgel G with 5% (v/v) benzene in light petroleum and by reversed-phase chromatography. Hydrogenation products were also identified by gas–liquid chromatography on a 5% (w/w) SE30 column on Chromosorb W in a Varian Aerograph.

The fractions eluted with 8% (v/v) ether in light petroleum were acetylated and the products chromatographed in the reversed-phase systems described.

Radioassay. Radioactive spots were located by exposing the dried chromatograms to fast X-ray film (Kodirex; Kodak Ltd.). Paper chromatograms and Neatan polymers of thin layers were counted automatically in the Actigraph II instrument (Nuclear–Chicago model C1001A); the detector was a thin end-window gas-flow counter with butane+helium (1·3:98·7) gas mixture. ^{14}C-labelled compounds were also counted in a Packard Tri-Carb liquid-scintillation spectrometer (series 314E with automatic control and sample changer, model 500D).

RESULTS

Preliminary investigations indicated that 'non-aqueous' chloroplasts, without rupture by ultrasonic treatment or osmotic 'shock', incorporated no label from [2-^{14}C]MVA into unsaponifiable material. Ultrasonically treated chloroplasts, on the other hand, were active but required a cofactor supplement of ATP, magnesium chloride and glutathione for significant conversion of MVA into terpenoids. Initial experiments were designed to determine the optimum concentrations of cofactors required by the chloroplast system.

Cofactor requirements

ATP as cofactor. Suspensions of ruptured chloroplasts, containing approx. 3·5mg. of chlorophyll, were incubated in phosphate buffer, pH 7·4, containing magnesium chloride (20μmoles) and glutathione (20μmoles), with various amounts of ATP. The reactions were started by addition of [2-^{14}C]MVA (1μc) to each tube and continued for 6hr. at 25–27°. Radioassay of the unsaponifiable material extracted from the incubation mixtures showed that no label was incorporated in the absence of ATP. Maximum incorporation (2% of available radioactivity) into non-saponifiable material was obtained with a supplement of 10μmoles of ATP, but increasing the concentration of ATP further resulted in a progressive suppression of the level of incorporation.

Mg^{2+} *as cofactor.* Similar experiments were conducted in which the concentration of magnesium chloride was varied over the range 0–70μmoles, keeping the amounts of ATP (10μmoles) and glutathione (20μmoles) in the supplement constant. Analysis of the unsaponifiable fraction showed that no label was incorporated in the absence of Mg^{2+} and that maximum incorporation was obtained with 20μmoles of magnesium chloride. Increasing the concentration of magnesium chloride above 20μmoles resulted in a progressive lowering of incorporation (as shown in Fig. 1). The results obtained from the ATP and Mg^{2+} studies indicate that the Mg^{2+}/ATP ratio is important for the chloroplast system to exhibit maximum activity. Fractionation of the unsaponifiable material on

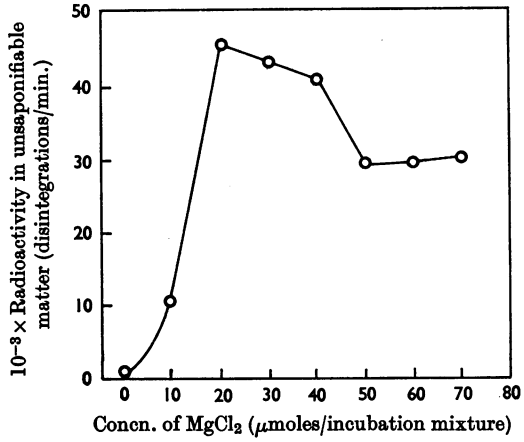

Fig. 1. Effect of concentration of Mg^{2+} on incorporation of [2-^{14}C]MVA into unsaponifiable material by isolated chloroplasts. The ultrasonically treated chloroplasts (0·35 mg. of chlorophyll), supplemented with ATP (10 μmoles) and glutathione (20 μmoles), were exposed to MVA (2 μc) for 6 hr. at 25–27°.

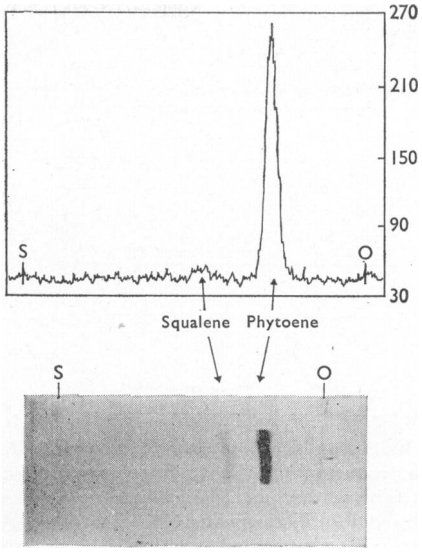

Fig. 2. Thin-layer separation of the light-petroleum fraction (F1) of unsaponifiable material from chloroplasts incubated with [2-^{14}C]MVA, with corresponding radioautograph. Radioscan conditions: count rate range: 300 counts/min.; collimator slit width: $\frac{1}{16}$ in.; integration time: 40 sec.; scanning speed: 1$\frac{1}{2}$ in./hr. O, Origin; S, solvent front.

Brockmann grade III alumina revealed a similar pattern of distribution of label irrespective of the concentrations of ATP and Mg^{2+}; the different amounts of cofactors in the supplements merely caused variations in the level of incorporation of label from MVA into terpenoids.

Mn^{2+} *as cofactor*. A supplement of manganese chloride was equally as effective as magnesium chloride in promoting incorporation of MVA. Replacement of Mg^{2+} by Mn^{2+} did not enhance incorporation of label nor did a mixture of the two ions have any synergistic effect on the activity of the system.

Glutathione as cofactor. Replacement of glutathione by L-cysteine resulted in a lower incorporation of label from [2-^{14}C]MVA into terpenoids.

All subsequent experiments were carried out with ultrasonically treated chloroplast suspensions, containing approx. 3·5 mg. of chlorophyll, supplemented with ATP (10 μmoles), magnesium chloride (20 μmoles) and glutathione (20 μmoles).

Identification of labelled compounds

Experiments were carried out to characterize the labelled compounds in the unsaponifiable material by combining the contents of a number of tubes containing the chloroplast system that had been exposed to [2-^{14}C]MVA (2 μc) for 6 hr. The combined incubation mixtures were saponified and the unsaponifiable material was chromatographed on columns of alumina. Four fractions were collected: fraction F1, eluted with light petroleum; fraction F2, eluted with 7% (v/v) ether in light petroleum; fraction F3, eluted with 14% (v/v) ether in light petroleum; fraction F4, eluted with ether and ethanol.

Examination of labelled compounds in fraction F 1. The light-petroleum fraction contained approx. 30% of the total radioactivity incorporated and was chromatographed on thin layers of Kieselgel G with light petroleum, with authentic phytoene and squalene as reference compounds. The compounds were located under u.v. light and the chromatogram was separated into zones that were systematically eluted and radioassayed. The zone that co-chromatographed with squalene (R_F 0·7) contained only 1% of the radioactivity, whereas the phytoene zone (R 0·3) accounted for 70% of the radioactivity on the chromatogram. The remaining radioactivity was associated with the origin, which probably reflected decomposition of the fraction during application to the thin layer.

Similar chromatograms of the light-petroleum fraction were prepared and radioautographed; Neatan polymers were also prepared and scanned for radioactivity. Fig. 2 shows the radioscan and a photograph of the radioautograph; the main radioactive band corresponds to phytoene and the minor band to squalene.

To confirm the identity of the major labelled

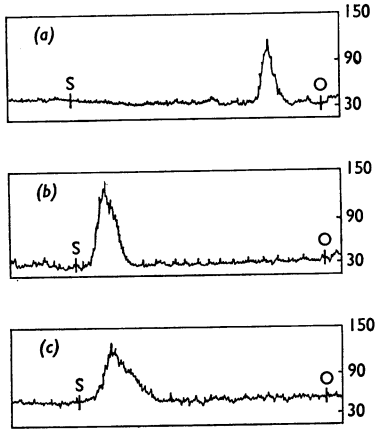

Fig. 3. Radioscan of thin-layer chromatograms of hydrogenation products of the light-petroleum fraction (F1) of unsaponifiable material and of [^{14}C]phytoene. (*a*) Authentic [^{14}C]phytoene; (*b*) hydrogenated [^{14}C]phytoene ([^{14}C]lycopersane); (*c*) hydrogenated fraction F1 of incubation mixture. Count rate range: 300 counts/min.; collimator slit width: $\frac{1}{16}$ in.; integration time: 40 sec.; scanning speed $1\frac{1}{2}$ in./hr. O, Origin; S, solvent front.

component, authentic [^{14}C]phytoene (obtained from carrot root slices and *Phycomyces blakesleeanus* exposed to [2-^{14}C]MVA) and the labelled component (to which carrier phytoene had been added) were hydrogenated. The products of the two hydrogenations were chromatographed on thin layers; radioautographs and Neatan polymers scanned in the Actigraph revealed only one active band. This indicated that the hydrogenated products of both authentic phytoene and the light-petroleum fraction of the incubation mixtures were chromatographically identical. Radioscans of [^{14}C]-phytoene and the hydrogenation products are shown in Fig. 3. The result was confirmed by reversed-phase chromatography and by gas–liquid chromatography, the hydrogenated compounds from the light-petroleum fraction having a mass peak identical with that of lycopersane (hydrogenated phytoene). At no time was any radioactivity detected in zones of the chromatograms that corresponded to lycopersene or phytofluene, which were clearly separable from phytoene in the chromatographic systems described. The gas–liquid chromatography ruled out the possibility of the highly labelled compound being dehydrosqualene since the hydrogenation product co-chromatographed with lycopersane and not with squalane. Thus it is concluded that the major radioactive compound in the light-petroleum fraction, synthesized from MVA, is the unsaturated C_{40} compound phytoene.

Investigation of fraction F2. The compounds eluted from alumina with 8% (v/v) ether in light petroleum were chromatographed on thin layers of Kieselgel G with chloroform as developer, on reversed-phase thin layers and on paper chromatography. In all systems employed only one major radioactive component was detected, and this co-chromatographed with authentic phytol. The acetylation product of the labelled compound co-chromatographed with phytyl acetate in the systems described. No radioactivity was detected in the phytosterol zones of the chromatograms.

Investigation of fraction F3. Chromatographic separations on thin-layer systems demonstrated that no radioactivity eluted with 14% (v/v) ether in light petroleum was associated with the phytosterol zones. The labelled components (two bands) remain unidentified.

Effect of further supplements to the chloroplast system

Addition of nicotinamide nucleotides. The effect of a supplement of reduced and oxidized forms of the nicotinamide nucleotides on incorporation of MVA by the chloroplast system was investigated. Incubations were carried out for the usual 6 hr. at 25–27° and extracted unsaponifiable material was fractionated on alumina columns. The results of the radioassays of the column fractions are presented in Table 1. Addition of nicotinamide nucleotides did not stimulate incorporation of label into unsaponifiable material; indeed the oxidized forms decreased the incorporation by approx. 30%. The distribution of radioactivity in the column fractions, by comparison with the standard incubation mixture (Table 1, column 1), was the same when NADH was added, but the pattern was different with NADPH, NAD^+ and $NADP^+$. Radioautography of thin-layer separations of the light-petroleum fractions on Kieselgel G with 5% (v/v) benzene in light petroleum revealed several noteworthy features, which are presented in Fig. 4. In the presence of NAD^+ or $NADP^+$ all the radioactivity in the light-petroleum fraction was located in the phytoene zone whereas squalene was insignificantly labelled. By contrast, under identical conditions of incubation, supplements of NADH or NADPH resulted in significant labelling of squalene as well as radioactivity in the phytoene zone. No radioactivity was detected in the lycopersene zone or in the phytofluene zone.

Addition of electron acceptors. Further supplements of FAD, FMN and ferricyanide were investigated as possible electron acceptors to aid the dehydrogenation step from phytoene to phytofluene. The results obtained (Table 1) demonstrated that addition of any of these compounds to the

Table 1. *Effect of supplements on the incorporation of* [2-^{14}C]*MVA into terpenoids of isolated chloroplasts*

The chloroplast system, containing chlorophyll (0·35 mg.) and supplemented with ATP (10 μmoles), $MgCl_2$ (20 μmoles) and glutathione (20 μmoles), was exposed to DL-[2-^{14}C]MVA (2 μc).

	$10^{-3}\times$ Radioactivity in unsaponifiable matter (disintegrations/min.)				
	Total	Light-petroleum fraction (F1)	8% Ether in light petroleum (F2)	14% Ether in light petroleum (F3)	Ether and ethanol (F4)
Control chloroplast system	48·0	16·94	5·44	20·52	3·20
Chloroplast system + NAD^+ (5 μmoles)	31·7	5·14	5·14	9·40	4·30
Chloroplast system + NADH (5 μmoles)	47·9	13·42	3·40	17·28	5·30
Chloroplast system + $NADP^+$ (5 μmoles)	34·2	7·16	2·28	14·22	3·56
Chloroplast system + NADPH (5 μmoles)	44·4	8·78	2·76	21·06	2·58
Chloroplast system + FAD (5 μmoles)	44·5	11·28	4·50	17·24	4·24
Chloroplast system + FMN (5 μmoles)	44·6	10·12	2·72	19·82	6·04
Chloroplast system + $Fe(CN)_6^{3-}$ (5 μmoles)	44·5	12·16	2·66	16·68	5·56

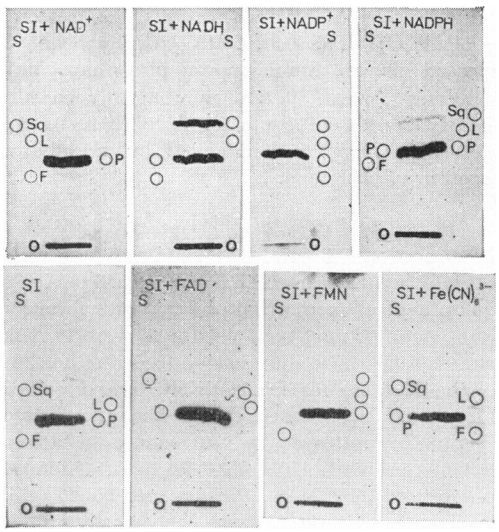

Fig. 4. Effect of various supplements on incorporation of [2-^{14}C]MVA into the light-petroleum fraction (F1) of unsaponifiable material by isolated chloroplasts. SI, Standard incubation mixture of chloroplasts + ATP, Mg^{2+} and glutathione. O, Origin; S, solvent front; P, phytoene; Sq, squalene; F, phytofluene; L, lycopersene.

standard chloroplast system had no effect on the level of incorporation of label from MVA into the unsaponifiable material. Fig. 4 shows radioautographs of thin-layer separations of the light-petroleum fractions that revealed an identical pattern of distribution of radioactivity with that of the control incubation mixture; virtually all the radioactivity was located in the phytoene zone and none could be detected in the phytofluene zone.

Effect of addition of fluoride and iodoacetamide. A supplement of potassium fluoride (30 μmoles) to the chloroplast system had no stimulating effect on incorporation of MVA into unsaponifiable material. Addition of 10 mg. of iodoacetamide resulted in complete inhibition of incorporation of label into the unsaponifiable fraction.

Effect of addition of chloroplasts isolated in aqueous media. To investigate the possibility of loss of integrity and organization of chloroplasts during isolation in organic media, attempts were made to enhance incorporation of MVA into phytoene, and possibly into the subsequent polyenes on the biosynthetic pathway, by mixing chloroplasts isolated in aqueous media with the non-aqueous system. It was hoped that the aqueous procedure might provide a site of synthesis and the non-aqueous preparation the necessary enzymes. The system of mixed chloroplasts, however, resulted in a lower level of incorporation than with the non-aqueous system alone, and no radioactivity was incorporated into phytofluene.

Effects of incubation conditions

Time of incubation. The effect of the length of the incubation period on the level of incorporation and distribution of label in the unsaponifiable material was investigated during 1–22 hr. exposure to [2-^{14}C]MVA. The results showed that incorporation

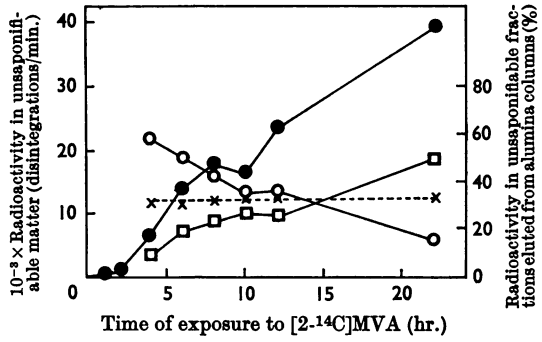

Fig. 5. Effect of incubation time on the incorporation of [2-^{14}C]MVA into terpenoids by isolated chloroplasts (see the text for experimental conditions). ●, Radioactivity (disintegrations/min.) in unsaponifiable matter; ○ percentage radioactivity in light-petroleum fraction; ×, percentage radioactivity in fractions eluted with 8% (v/v)+14% (v/v) ether in light petroleum fractions; □, percentage radioactivity in ether fraction.

of label into the unsaponifiable fraction progressively increased with time of exposure to the labelled substrate; after a 10hr. incubation approx. 2% of the available radioactivity in [2-^{14}C]MVA was incorporated into unsaponifiable material, rising to 4% with a 22hr. exposure. Fig. 5 shows the curve of incorporation of label with time. Fractionation of the unsaponifiable material obtained after various times on Brockmann grade III alumina revealed three facts. First, though incorporation of radioactivity progressively increased with time, the percentage of radioactivity eluted from the columns with light petroleum was at a maximum after 4hr. exposure and thereafter progressively decreased (see Fig. 5). Secondly, if the major labelled compound in the light-petroleum fraction were a triterpenoid precursor, a corresponding increase in the fractions eluted with 8% (v/v) and 14% (v/v) ether in light petroleum might be expected with time. However, the radioactivities of these fractions (see Fig. 5) remained at a virtually constant level over the whole period of exposure. Thirdly, the decrease in the radioactivity in the light-petroleum fraction, reflected by a decrease in label of the phytoene band, was accompanied by a corresponding increase in the percentage label of the fraction eluted with ether from the columns, as shown in Fig. 5. The labelled compounds in the ether fraction remain to be identified, but it is noteworthy that this fraction contained the xanthophyll pigments.

Effect of light. A comparison of the effect of light of rather low intensity and complete darkness on MVA incorporation into the chloroplast system was carried out. The results showed that the levels of radioactivity incorporated and the distribution of label in the unsaponifiable material were identical under the two conditions. Thus it may be concluded that the conversion of MVA into terpenoids by isolated chloroplasts, supplemented with ATP, Mg^{2+} and glutathione, is independent of light of moderate intensity.

Anaerobic conditions. Further experiments revealed that the level of incorporation of MVA by the chloroplast system was unaffected when incubations were carried out in an atmosphere of nitrogen. Under anaerobic conditions, however, the label located in the light-petroleum fraction and in the phytoene band was greater than with incubation in aerobic conditions.

Incubation of chloroplast system with [^{14}C]phytoene

Investigations already described indicated that, though the isolated chloroplasts effectively incorporated MVA into phytoene, no label could be detected in the phytofluene zone, even after addition of electron acceptors. [^{14}C]Phytoene was obtained from carrot root slices exposed to [2-^{14}C]MVA for 48hr., and purified by successive chromatography on columns of alumina and magnesium oxide–Celite (5:2, w/w) and by thin-layer separation on Kieselgel G. The purified [^{14}C]phytoene was dispersed as an emulsion in phosphate buffer containing Tween 80 and incubated with ultrasonically treated chloroplasts. A 100% recovery of added radioactivity was achieved, but no label was located in phytofluene.

Effect of pretreatment of leaves by etiolation

Illumination of etiolated seedlings stimulates carotenoid synthesis, which is an integral part of the development of primordial plastids into functional chloroplasts. Dwarf-bean seedlings were grown in darkness for 9 days, illuminated for a further 24hr. and the leaf tissues freeze-dried. The developing chloroplasts were isolated by the non-aqueous procedure, ruptured by ultrasonic treatment, supplemented with ATP, Mg^{2+} and glutathione and exposed to [2-^{14}C]MVA for 6hr. at 25–27°. The incorporation of label into the unsaponifiable material was raised to 7% and the pattern of distribution of label was similar to that obtained with isolated mature chloroplasts. The fraction eluted from alumina with light petroleum contained approx. 45% of the total incorporated radioactivity, which was located entirely in the phytoene zone of the thin-layer separation; no radioactivity was located in the squalene zone.

DISCUSSION

Chloroplasts isolated in non-aqueous media are devoid of their outer lipid membrane and of some chloroplast pigments (Stocking, 1959). The loss of soluble protein is, however, far less than from

chloroplasts prepared in aqueous media. One of the main difficulties associated with the non-aqueous procedure is the possible inactivation of some enzymes through prolonged contact with organic solvents. Evidence has been presented here that non-aqueous chloroplasts are capable of converting the early key intermediate, MVA, into phytoene.

The fact that the isolated chloroplasts were capable of carrying out this conversion only after rupture by ultrasonic treatment (or osmotic 'shock') indicated that *in vitro* the chloroplast membrane is essentially impermeable to MVA. This result is confirmed by Rogers, Shah & Goodwin (1966) in studies of the conversion of MVA into phosphorylated derivatives by isolated chloroplasts, and supports the proposal that the regulation of terpenoid synthesis involves specific membrane permeability (Goodwin & Mercer, 1963).

The chloroplast system required a supplement of ATP, Mg^{2+} and glutathione to exhibit maximal incorporation of label from [2-^{14}C]MVA. The results of the cofactor requirement studies indicated that a stoicheiometric relationship exists between the ATP and Mg^{2+} concentrations and that imbalance of this ratio leads to inhibition of the system. This same situation is found with plant phosphofructokinase (Dennis & Coultate, 1966), brain phosphofructokinase (Lowry & Passonneau, 1966) and hexokinase (Uyeda & Racker, 1965); it may be a kinase regulatory mechanism in plants.

Seedlings and crude plant preparations incorporate much more label from [2-^{14}C]MVA into squalene, sterols and other triterpenoids than into phytoene and other carotenoids. With the chloroplast system, label from [2-^{14}C]MVA is incorporated into a compound that from the evidence presented has been identified as phytoene. No lycopersene, or any radioactivity in the zone corresponding to this compound, was detected in any of the investigations, which supports the theory that this compound is not an obligatory precursor of carotenoids (see Goodwin, 1965). The small amount of radioactivity located in squalene may be due to a limited ability of chloroplasts to carry out the synthesis, or to the contamination of the chloroplasts through adsorption of enzymes of cytoplasmic origin. No radioactivity was detected in the phytosterols in any experiment.

Anderson & Porter (1962*a*,*b*) demonstrated that the incorporation of [^{14}C]terpenyl pyrophosphates into phytoene, by soluble extracts of carrot root and tomato plastids, was enhanced by addition of $NADP^+$ to the incubation system, and decreased by a supplement of the reduced nicotinamide nucleotide. With the chloroplast system described, conversion of MVA into phytoene was not enhanced by addition of oxidized nicotinamide nucleotides or FAD, FMN or ferricyanide. The failure to stimulate incorporation into phytoene by these supplements would support the view that no oxidation–reduction step is involved in phytoene biosynthesis. The failure of NADH or NADPH also to stimulate phytoene synthesis supports this view. However, these cofactors did stimulate squalene synthesis, so that the failure to stimulate synthesis of the squalene homologue lycopersene (15,15′-dihydrophytoene) under these conditions lends further support to the view that this compound is not a carotenoid precursor.

The lack of synthesis of phytofluene in the presence of NAD^+, $NADP^+$, FAD, FMN or ferricyanide by the chloroplast preparation suggests that this is due to the requirement of the system for a hydrogen acceptor not investigated, to the requirement for a combination of cofactors in a particular stoicheiometric relationship or, most likely, to the possibility that the isolation procedure disintegrated an essential structural unit present in the normal functional chloroplast of the intact plant. It is, however, significant that addition of the reduced nicotinamide nucleotides did not affect incorporation of label from [2-^{14}C]MVA into phytoene but did lead to a large increase of radioactivity in squalene. Whether the result reflects a real but small synthesis by the chloroplasts, or by an adhering cytoplasmic fraction, there is agreement with the observations of Popják, Goodman, Cornforth, Cornforth & Ryhage (1961) that the formation of squalene from two C_{15} terpenoids requires, and is stimulated by, reduced nicotinamide nucleotide. The work of Heber (1960*a*,*b*) indicates that non-aqueously prepared chloroplasts are contaminated with about 6% of cytoplasmic protein.

The conversion of MVA into phytoene by the isolated chloroplast system was independent of light and was, in fact, enhanced in anaerobic conditions, which agrees with the observations of Suzue (1961) and Anderson & Porter (1962*a*,*b*) that phytoene synthesis proceeds readily in the absence of oxygen.

Goodman & Popják (1960) suggested that optimum conversion of MVA into terpenyl pyrophosphates and higher terpenoids is achieved in the presence of fluoride ions, which are reputed to inhibit active phosphatase enzymes. However, addition of potassium fluoride to the chloroplast system caused a decrease in the level of incorporation. Iodoacetamide has been shown to inhibit the isomerase that converts isopentenyl pyrophosphate into $\beta\beta$-dimethylallyl pyrophosphate, before the formation of the first C_{10} terpenoid intermediate (Popják & Cornforth, 1960). Complete inhibition of the conversion of MVA into unsaponifiable material was obtained when iodoacetamide was added to the incubation mixtures.

Investigations of the effect of time on incorporation of MVA revealed that after a 4hr. exposure the percentage radioactivity in the compound identified as phytoene progressively decreased with a corresponding increase in the more polar components of the unsaponifiable material, which were not characterized. The major radioactive component in the fraction eluted from alumina with 8% (v/v) ether in light petroleum has been shown to co-chromatograph with phytol. The other labelled components in the fractions eluted with 8% (v/v) and 14% (v/v) ether in light petroleum remain to be identified, but did not correspond to phytosterols, and the percentage of radioactivity in these fractions remained constant and independent of the time of exposure to [2-^{14}C]MVA.

Kakutani, Suzue & Tanaka (1964) demonstrated the conversion of [^{14}C]phytoene into [^{14}C]β-carotene by extracts of *Sporobolomyces shibitanus*, and Beeler, Nandi & Porter (1962) reported a limited production of phytofluene from phytoene by isolated tomato plastids. The failure of the ultrasonically treated chloroplast system to convert phytoene into phytofluene may be due to inactivation of the necessary enzyme system by the organic solvents employed for isolation of the chloroplasts, or may be due to isomerization of the phytoene during purification procedures with consequent alteration of the metabolically active form, or to the high state of purity of our phytofluene. Goodwin & Williams (1966) carried out investigations with [^{14}C,^{3}H]MVA that indicated that phytoene is almost certainly an obligatory intermediate on the biosynthetic pathway of carotenoids. The failure of the chloroplast system to convert MVA and phytoene into phytofluene or later polyenes suggests inactivation of a certain factor during isolation. Heber & Tyszkiewicz (1962) demonstrated an enhancement of $^{14}CO_2$ fixation by a combination of chloroplasts isolated in aqueous and non-aqueous media, but our results indicated that such a combination proved ineffective in aiding conversion of MVA into carotenoids.

Exposure of isolated developing chloroplasts to MVA resulted in a fourfold increase of incorporation into unsaponifiable material compared with incorporation in mature chloroplasts, and such a system is likely to prove a useful tool for the study of carotenogenesis in plants, because it will allow more efficient use to be made of labelled terpenoid precursors.

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