Biosynthesis of Porphyrins and Related Macrocycles. Part 11.[1] Studies on Biosynthesis of the Phytyl Chain of Chlorophyll *a* by Use of Carbon-13

By **Edward H. Ahrens, jun.**,† **D. Clive Williams**, and **Alan R. Battersby**,* University Chemical Laboratory, Lensfield Road, Cambridge CB2 1EW

A procedure is developed for the isolation on a small scale of pure phytol from cells of *Euglena gracilis*, and conditions are found for the separation of a crystalline phytyl carbamate from the corresponding derivative of geranylgeraniol. Dark-grown cells of *E. gracilis* are shown to contain only negligible quantities of phytol, but on illumination they incorporate acetate efficiently into the phytyl chain of chlorophyll; the low dilution achieved allows experiments with [1-^{13}C]acetate. The eight sites of ^{13}C enrichment in the resultant phytol are located by n.m.r. Presumably because of poor transport, the incorporation of mevalonate into phytol is very low.

PHYTOL (1), having the illustrated 7*R*,11*R*-stereochemistry,[2] is the hydrophobic esterifying alcohol of chlorophyll *a* (3) and of chlorophyll *b* (4). These pigments are collectors of light for photosynthesis, one of the most intriguing energy conversions in the physical world, and the ' fatty ' phytyl group has a function in the organisation of this complex process.[3,4]

Surprisingly, though the biosynthesis of the chlorophyll macrocycle has been intensively studied,[5,6] much less was known about the pathway to phytol; little definitive could be said about it even as late as the mid-sixties.[7] Furthermore, phytol is difficult to degrade to allow the unambiguous isolation of particular carbon atoms for radio-assay. As a result, the labelling patterns are not known for various [^{14}C]phytols isolated from the interesting tracer experiments which had previously been carried out.[8,9]

It seemed to us that the ideal way to study the biosynthesis of phytol would be by labelling the precursors of interest with carbon-13. This, in principle, should allow direct determination of all the sites of ^{13}C enrichment in the resultant phytol. However, success depended on the following practical hurdles being surmounted: (*a*) development of a suitable biological system that biosynthesises phytol from administered ^{13}C-labelled precursors without excessive dilution, (*b*) establishment of an isolation procedure for phytol on a small scale leading to a pure crystalline derivative, and (*c*) assignment of ^{13}C signals from this derivative followed by incorporation experiments with ^{13}C-labelled precursors and study of the resultant phytol derivative by n.m.r. Clearance of these hurdles, discussed more fully below, also lays the base for future studies.

The Biological System.—The plan to avoid excessive dilution of the phytol biosynthesised in our experiments was to grow the organism, *Euglena gracilis*, in the dark through several generations until virtually colourless cells were available. Such cells are known [10] to start a rapid synthesis of chlorophyll when they are illuminated (after a lag phase of *ca.* 20 h). We found that the lag phase lengthened as the cells passed through an increasing number of generations in the dark. The colourless cells were expected [10] to be essentially free from chlorophyll and this was confirmed spectroscopically. However, it was also important to prove the absence of significant amounts of phytol, whether free or in esterified form; the sensitive isolation procedure described in the following section was used. No phytol could be detected in the colourless cells, in free form or after alkaline hydrolysis, by direct visualisation on the t.l.c. plates nor by g.l.c. (after trimethylsilylation) of the extract from the area of the plates corresponding to the phytol standard. An equivalent quantity of green *Euglena* cells yielded *ca.* 20 mg of phytol and even 10% of this amount would have been detected readily in the colourless cells. This result for the colourless cells agrees with similar findings for dark-grown tobacco leaves [11] and callus tissue of *Kalanchoë crenata*,[12] and all are of interest in relation to the control and timing [13] of the biological production of phytol and chlorophyllide *a* (5) for construction of chlorophyll *a* (3).

Isolation of Pure Phytol.—It was essential for our subsequent work with ^{14}C- and ^{13}C-labelled materials to be

† On sabbatical leave from the Rockefeller University, New York, U.S.A.

[1] Part 10, A. R. Battersby, E. McDonald, R. Hollenstein, M. Ihara, F. Satoh, and D. C. Williams, *J.C.S. Perkin I*, 1977, 166.

[2] J. W. K. Burrell, L. M. Jackman, and B. C. L. Weedon, *Proc. Chem. Soc.*, 1959, 263; P. Crabbé, C. Djerassi, E. J. Eisenbraun, and S. Liu, *ibid.*, p. 264.

[3] ' The Chlorophylls,' ed. L. P. Vernon and G. R. Seely, Academic Press, New York, 1966.

[4] J. J. Katz, W. Oettmeier, and J. R. Norris, *Phil. Trans., Ser. B*, 1976, **273**, 227.

[5] Reviewed by A. R. Battersby and E. McDonald, in ' Porphyrins and Metalloporphyrins,' ed. K. M. Smith, Elsevier, Amsterdam, 1975, 2nd edn.

[6] O. T. G. Jones, *Phil. Trans., Ser. B*, 1976, **273**, 207; G. W. Kenner, J. Rimmer, K. M. Smith, and J. F. Unsworth, *ibid.*, p. 255 and references in both surveys.

[7] J. Lascelles, ' Tetrapyrrole Biosynthesis and its Regulation,' Benjamin, New York, 1964, p. 76; G. S. Marks, ' Heme and Chlorophyll,' van Nostrand, Princeton, 1969, p. 148.

[8] *Inter alia* (*a*) F. G. Fischer, G. Märkl, H. Hönel, and W. Rüdiger, *Annalen*, 1962, **657**, 199; (*b*) K. J. Treharne, E. I. Mercer, and T. W. Goodwin, *Biochem. J.*, 1966, **99**, 239; (*c*) C. Costes, *Phytochemistry*, 1966, **5**, 311; (*d*) D. N. Skilleter and R. G. O. Kekwick, *ibid.*, 1970, **9**, 153; (*e*) J. M. Charlton, K. J. Treharne, and T. W. Goodwin, *Biochem. J.*, 1967, **105**, 205; and *cf.* M. J. Buggy, G. Britton, and T. W. Goodwin, *Phytochemistry*, 1974, **13**, 125.

[9] A. R. Wellburn, K. J. Stone, and F. W. Hemming, *Biochem. J.*, 1966, **100**, 23c; see also A. R. Wellburn, *Phytochemistry*, 1968, **7**, 1523.

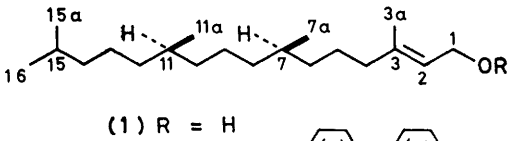
[10] M. Brody in ' The Biology of Euglena,' ed. D. E. Buetow, Academic Press, New York, 1968, p. 219.

[11] S. Shimizu, H. Fukushima, and E. Tamaki, *Phytochemistry*, 1964, **3**, 641.

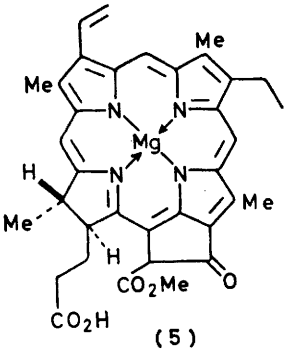
[12] A. K. Stobart, N. R. Weir, and D. R. Thomas, *Phytochemistry*, 1969, **8**, 1089.

[13] C. Liljenberg, *Physiol. Plant.*, 1974, **32**, 208 and references therein.

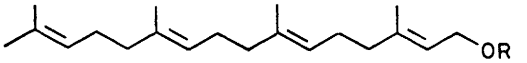
certain of the purity of the phytol isolated from *Euglena gracilis*. This was not a trivial problem because of the rich supply of carotenoids in this organism, including polar ones. Further, any contamination of the isolated phytol with geranylgeraniol (6) or farnesol would have been disastrous for the determinations of dilution values; such problems have caused difficulties in earlier studies (personal communication from Dr. A. R. Wellburn and ref. 8*e*). After much experimentation and and help from colleagues (see acknowledgements), the following procedure was developed which proved reproducibly effective; 15 runs were carried out successfully.



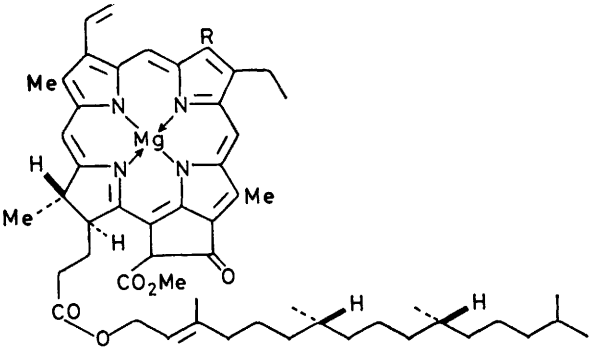
(1) R = H
(2) R = CO·NH



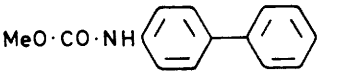
(5)



(6) R = H
(7) R = CO·NH



(3) R = Me
(4) R = CHO



(8)

The methanol extract from the cells was treated with aqueous dioxan until precipitation of the chlorophylls [14] was complete; the solution contained the unwanted polar carotenoids. After removal of the magnesium from the chlorophylls, the resultant pheophytins were freed from non-polar carotenoids on alumina and then they were hydrolysed with methanolic alkali. Phytol (1) was thus obtained containing minor impurities which were removed by fractionation twice under different conditions using p.l.c. on silica gel impregnated with silver nitrate and, importantly, treated with detergent.[15] Finally, the chemically pure material was converted into its crystalline biphenyl-4-ylcarbamate derivative (2) to allow proof of radiochemical purity.

Wellburn *et al.*[16] had shown that phytol (1) and *trans*,-*trans*-geranylgeraniol (6) are separable by reversed-phase t.l.c. on kieselguhr. Fortunately, a clean separation of the corresponding biphenyl-4-ylcarbamates (2) and (7) was also possible under similar conditions; this was more convenient for the ^{14}C studies than separation of the alcohols.

N.m.r. Studies and Labelling Experiments.—The standard sample of phytol for n.m.r. studies was obtained by fractionation of commercial material using the foregoing methods and the product was shown to be homogeneous by g.l.c. Its ^{13}C n.m.r. spectrum agreed closely with that reported by Allerhand *et al.*[17] and by Closs

^{13}C Signals from phytol (1) and its derivative (2) measured in $CDCl_3$ (δ_C in p.p.m. from Me_4Si)

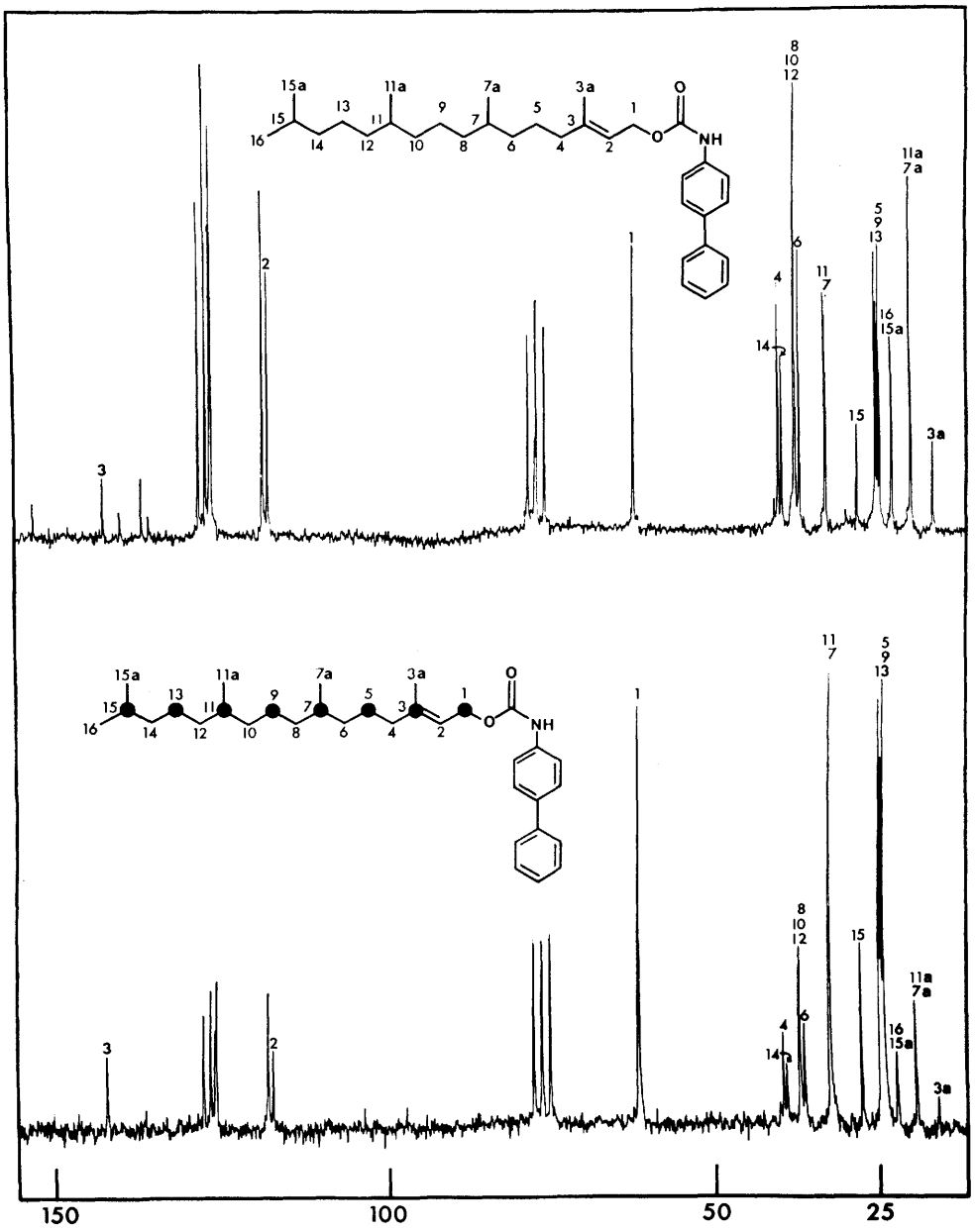
Carbon no.	Phytol (ref. 17) δ_C	Phytol (present work) δ_C	Carbamate (2) [b] δ_C
1	59.39	59.37	62.07
2	123.4	123.15	118.03
3	139.9	139.95	142.96
4	39.95	39.87	39.86
5	25.28	25.17	25.07
6	36.80	36.68	36.67
7	32.80	32.70	32.68
8	37.49	37.37	37.37
9	24.56	24.48	24.47
10	37.55	37.37	37.37
11	32.89	32.78	32.80
12	37.42	37.37	37.37
13	24.85	24.80	24.77
14	39.50	39.38	39.36
15	28.04	27.98	27.95
3a	16.17	16.17	16.43
7a, 11a	19.80	19.74	19.72
15a	22.64	22.63	22.60
16	22.72		22.68
>CO of carbamate [a]			159.39

[a] The methyl carbamate (8) showed δ_C 153.81 (>CO) and 52.32 (MeO). [b] The signals from the aromatic rings appeared at δ_C 140.40, 137.17, 136.12, 128.55, 127.50, 126.80, 126.61, and 118.80; *cf.* those for the methyl carbamate (8) at δ_C 140.37, 137.01, 136.36, 128.60, 127.53, 126.64, and 118.94.

[14] K. Iryami, N. Ogura, and A. Takamiya, *J. Biochem.* (*Japan*), 1974, **76**, 901.
[15] A. Gloe and N. Pfennig, *Arch. Microbiol.*, 1974, **96**, 93.
[16] A. R. Wellburn, J. Stevenson, F. W. Hemming, and R. A. Morton, *Biochem. J.*, 1967, **102**, 313.
[17] R. A. Goodman, E. Oldfield, and A. Allerhand, *J. Amer. Chem. Soc.*, 1973, **95**, 7553.

et al.,[18] and we relied on their careful assignments of the resonances (see Table). Spectra taken with single-frequency off-resonance decoupling and the effect of derivatisation on chemical shifts were in full agreement with their assignments. The Table shows that conversion of phytol (1) into the carbamate (2) causes a significant change of chemical shift only for carbon atoms 1, 2, 3, and 3a. The decision as to which signals in the low-field region correspond to the two aromatic rings of the carbamate (2) was assisted by measuring the ^{13}C spectrum of the methyl carbamate (8).

A study was next made of the extent to which [1-^{14}C]-acetate undergoes dilution during its incorporation into phytol by the biological system developed above. The labelled precursor was added to the suspension of colourless cells which were then illuminated to initiate chlorophyll synthesis. Conditions were found which afforded a *ca.* 20% specific incorporation of [1-^{14}C]acetate into phytol (1) corresponding to a biological dilution with roughly four parts of unlabelled material. Such a small dilution readily allows ^{13}C studies; therefore, [1-^{13}C]-acetate at 65 atom % enrichment, in admixture with a suitable quantity of [1-^{14}C]acetate, was administered to the *Euglena gracilis* cells in the same way. The isolated



^{13}C N.m.r. spectra of the phytyl carbamate (2) in $CDCl_3$; upper spectrum at natural abundance, lower spectrum that of sample obtained by incorporation of [1-^{13}C]acetate on illumination of dark-grown *Euglena gracilis*

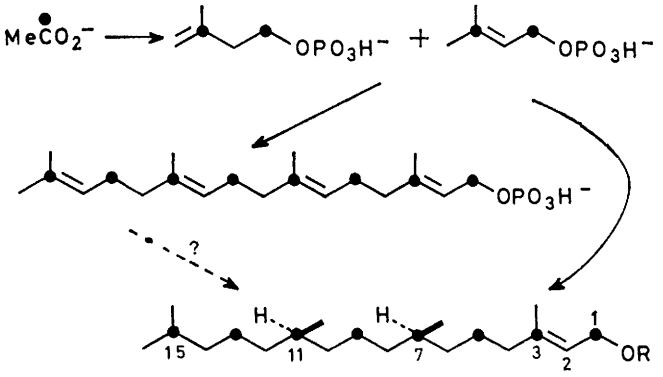
[18] S. G. Boxer, G. L. Closs, and J. J. Katz, *J. Amer. Chem. Soc.*, 1974, **96**, 7058.

[^{13}C,^{14}C]phytol was diluted with *ca.* two parts of unlabelled phytol for ease of handling and the derivative (2) was then rigorously purified. The ^{14}C label showed that the specific incorporation (*i.e.* before our addition of unlabelled material) had been 19%, in agreement with the pilot experiment.

The foregoing [^{13}C, ^{14}C]carbamate (2) was checked for the presence of geranylgeranyl carbamate (7) by the method already described. Less than 0.3% of the total radioactivity was in the band corresponding to (7), and 99.7% remained in the phytyl carbamate (2). Thus the n.m.r. work could be undertaken with confidence.

The ^{13}C n.m.r. spectra of unlabelled phytyl carbamate (2) and of the foregoing ^{13}C,^{14}C-labelled material are shown in the Figure. In comparing the upper spectrum with the lower, one should recognise that the former is of stronger intensity throughout. The signals from the aromatic rings act as internal standards; these lie downfield from that of C-2 and are not numbered on the spectra. It is then evident that only the signals corresponding to the carbon atoms marked ● on the lower diagram have enhanced intensity, the average increase being *ca.* 4.5 fold.

These results demonstrate the specific incorporation of [1-^{13}C]acetate into phytol, and all eight labelled sites are



SCHEME Incorporation of acetate into the phytyl group of chlorophyll *a*; R = chlorophyllide *a* residue

pin-pointed. The pattern found supports the operation of the normal terpenoid pathway to geranylgeranyl pyrophosphate [19] with subsequent reduction. In agreement with this, administration of (3*RS*)-[2-^{14}C]mevalonate to the *Euglena* cells followed by work-up and purification of the phytol by the above searching method gave labelled phytyl carbamate (2). The incorporation was low (specific incorporation 0.07%), almost certainly owing to difficulties in penetration of the labelled material to the site of synthesis.

There has been considerable recent interest [20] as to whether geranylgeranyl pyrophosphate is an intermediate on the pathway to the phytyl group of chlorophyll *a*, and there is evidence in dark-grown barley [13] supporting the initial formation of the geranylgeranyl ester of protochlorophyllide. This ester can subsequently be converted into chlorophyll *a*.[13] These and other problems require further work, and it is hoped that the methods reported here will be helpful for these future studies.

EXPERIMENTAL

Except as noted below, general directions are given in ref. 21, save that evaporations were carried out at <40 °C. Spectra were recorded using a pulse width of 70 μs, with a sensitivity enhancement half the aquisition time (0.8 s) and a spectral width of 5 000 Hz. Radioactive samples were assayed on a Packard Tricarb liquid scintillation counter (model 3 320) with internal standardisation using [^{3}H]- or [^{14}C]-hexadecane.

Production of Colourless Cells of Euglena gracilis.—The source and type of cells and the modified medium [22] for their growth are given in ref. 23. Normal techniques were used for sterilisation, for protection of growth vessels, and for sterile transfer of solutions.

The cells were grown at 25 °C in stirred medium in Erlenmeyer flasks (250 ml leading to 2.5 l) in the dark for 4—5 days; in all experiments the inoculating cell suspension represented 10% of the volume of medium to which it was added. After the above time, the cell concentration was *ca.* 10^6 cells per ml determined as follows. A sample of the cell suspension (10 ml) was treated with aqueous formaldehyde (4 drops of 37% v/v) and part (1 ml) was diluted to 100 ml with isotonic solution. The cells were then counted using a Coulter counter (Coulter Electronics Ltd., Dunstable, Beds.), the average of five readings being taken. These accurate cell counts were used to calibrate optical density measurements on an undiluted sample at 700 nm, so allowing more rapid, but rougher, estimations of cell counts during cell growth.

Cells were grown through at least eight generations before use as below.

Illumination of Colourless Cells and Administration of Labelled Precursors.—The cells from five 2.5 l flasks were collected at 6 000 rev min^{-1} at 4 °C on an M.S.E. ' High Speed 18 ' centrifuge fitted with a continuous action rotor. The resultant cell paste was then resuspended in a resting medium [24] (2.5 l) to which cyclopentane-1,2,3,4-tetracarboxylic acid (2.5 g l^{-1}) had been added to assist the penetration of the precursors.[25] The resting medium contained 0.054M-mannitol, 0.01M-$MgCl_2$, and 0.01M-KH_2PO_4, and was adjusted to pH 3.6.

The cell suspension was stirred in a 5 l Erlenmeyer flask between two parallel sets of fluorescent light tubes (total 4 × 40 W, Cryselco ' warm white '). The labelled precursor

[19] G. Popják and J. W. Cornforth, *Biochem. J.*, 1966, **101**, 553 and references therein; G. P. Moss, Chem. Soc. Specialist Periodical Report, Terpenoids and Steroids, 1971, vol. 1, p. 221, and references therein.

[20] (*a*) A. R. Wellburn, *Phytochemistry*, 1970, **9**, 2311 and references therein; see also (*b*) J. J. Katz, H. H. Strain, A. L. Harkness, M. H. Studier, W. A. Svec, T. R. Janson, and B. T. Cope, *J. Amer. Chem. Soc.*, 1972, **94**, 7938; and (*c*) H. Brockmann, G. Knoblach, I. Schweer, and W. Trowitzsch, *Arch. Microbiol.*, 1973, **90**, 161.

[21] A. R. Battersby, D. A. Evans, K. H. Gibson, E. McDonald, and L. Nixon, *J.C.S. Perkin I*, 1973, 1546.

[22] C. C. Greenblatt and J. A. Schiff, *J. Protozool.*, 1959, **6**, 23.

[23] A. R. Battersby, G. L. Hodgson, E. Hunt, E. McDonald, and J. Saunders, *J.C.S. Perkin I*, 1976, 273.

[24] A. I. Stern, J. A. Schiff, and H. T. Epstein, *Plant Physiol.*, 1964, **39**, 220.

[25] J. A. Schiff, personal communication; also H. F. Mallette, *J. Bacteriol*, 1967, **94**, 283 and K. M. Tamburo and S. Hutner, *J. Protozool.*, 1971, **18**, 667.

(see later) was added at the start of the illumination; when acetate was administered the pH rose to 6.1. At the point corresponding to the centre of the flask, the light intensity was ≫200 foot candles. After a lag phase which varied between 20 and 60 h, chlorophyll synthesis occurred linearly for *ca.* 120 h; the number of cells did not increase and, for the experiments with labelled acetate, the pH of the medium fell gradually from 6.1 to 4.5. The fully greened cells were collected as above for extraction of chlorophyll.

Chlorophyll production was assayed by diluting a sample (1 ml) of the cell suspension with acetone (4 ml) and measuring absorbance (Z) at 663 nm. The chlorophyll content [26] (mg l^{-1}) is then given by $5Z \times 10^3/90.8$ (90.8 is the specific absorption coefficient for chlorophyll *a* in 1 : 4 water–acetone [27]).

Purification of Commercial Phytol and Preparation of the Carbamate (2).—Silica gel G p.l.c. plates (Merck No. 5 717, 2 mm thickness, 20 × 20 cm) were dipped into an aqueous detergent solution [15] [1% w/v of ' Sun ' detergent (Lever Bros. Ltd.)] for 1 min, then, after draining, were left until they no longer appeared shining wet. They were then dipped into aqueous silver nitrate (5% w/v) for 1 min, drained, and air-dried for 1 h before drying in the oven at 80° for 2 h.

Silver nitrate plates prepared without detergent were finally dried at 120 °C for 16 h and they remained useful for several weeks when stored in the dark at *ca.* 20 °C.

Commercial phytol was first fractionated on untreated p.l.c. plates using 2 : 3 (v/v) diethyl ether–light petroleum (b.p. 40—60 °C). The phytol band was detected in long wavelength u.v. light after spraying with aqueous 0.01% Rhodamine G. The phytol band was extracted with diethyl ether and re-run on silver nitrate plates with 3 : 1 benzene–ethyl acetate, and the pure material was distilled (bulb-to-bulb) at *ca.* 145 °C and 2 mmHg. The trimethylsilyl ether of this product showed a single peak on g.l.c. (20% ethylene glycol succinate column at 185 °C; retention 0.69 relative to methyl palmitate); *m/e* 296 (M^+, 21%), 278 (13), 196 (13), 123 (72), 111 (17), 95 (32), 81 (64), and 71 (100); *cf.* refs. 28 and 20*b*.

The pure phytol and an equal weight of biphenyl-4-yl isocyanate were dissolved in diethyl ether, the solution was evaporated, and the residue was heated at 100 °C for 1 h. The residue was extracted repeatedly with boiling light petroleum (b.p. 60—80 °C) and the filtered solution was evaporated. Fractionation by p.l.c. on silica gel G plates using benzene gave the *phytyl biphenyl-4-ylcarbamate* (2), which was eluted with chloroform and crystallised from chloroform with addition of a little acetone (yield of first crop >60%), m.p. 56—56.6° (Found: C, 80.6; H, 10.2; N, 2.8. $C_{33}H_{49}NO_2$ requires C, 80.6; H, 10.05; N, 2.85%); $\lambda_{max.}$ 271 nm (ε 26 910); *m/e* 491 (M^+, 7%), 447 (7), 278 (3), 222 (9), 213 (39), 195 (36), and 169 (100).

Methyl biphenyl-4-ylcarbamate (8) was prepared by heating biphenyl-4-yl isocyanate (195 mg) in methanol (10 ml) under reflux for 2 h. The residue from evaporation of the filtered solution was extracted with hot benzene and the soluble material was crystallised from the minimum volume of benzene by addition of light petroleum (b.p. 60—80 °C); the product had m.p. 126—126.5° (Found: C, 73.8; H, 5.75; N, 6.3. $C_{14}H_{13}NO_2$ requires C, 74.0; H, 5.75; N, 6.15%), $\lambda_{max.}$ 270 nm (ε 25 510); *m/e* 227 (M^+ 74%), 195 (55), 149 (30), and 91 (100).

Derivatisation of Geranylgeraniol.—This alcohol was a 2 : 1 mixture of all-*trans* material with the *trans,trans,*2-*cis*-isomer. The carbamate, containing mainly (7), was prepared as for phytol; it was amorphous (Found: C, 81.5; H, 9.0; N, 2.6. Calc. for $C_{33}H_{43}NO_2$: C, 81.6; H, 8.9; N, 2.9%); *m/e* 485 (M^+, 0.23%), 441 (2), 332 (7), 272 (29), 213 (32), and 195 (100).

This material (R_F 0.72) was readily separable from the phytyl carbamate (R_F 0.43) on reverse phase plates using 1 : 4 (v/v) water–acetone. The plates were prepared from a blended mixture of Merck Kieselguhr G (100 g) and water (140 ml). After they had been air-dried for 15 h, they were dipped into a solution of paraffin oil in light petroleum [16] (5% v/v; b.p. 60—80 °C) and air-dried for at least 4 h.

Isolation of Phytol from Euglena gracilis.—(*a*) *Isolation of pheophytins.* The green cell paste (typically 60 g) from centrifugation was frozen, then thawed overnight at 4 °C, and finally the suspension was heated in boiling water at 100 °C for 2 min. The subsequent steps as far as the alkaline hydrolysis stage under (*b*) were carried out in the dark or in green light.[29] Methanol was added to the cell suspension (to total vol 1 l); after 20 min with occasional stirring, the mixture was filtered twice.

The clear filtrate containing typically 115 mg of chlorophyll was mixed with one seventh of its volume of dioxan,[14] and to the stirred solution water was added dropwise until particles separated; usually the volume of water added was 16% of the total methanol–dioxan volume. The suspension was kept at 4 °C for at least 3 h, then filtered (repeatedly if necessary) until a clear filtrate (discarded) and a green pigment paste on the filter were obtained. The latter, now containing typically 90 mg of chlorophylls, was dissolved in 2 : 1 v/v chloroform–methanol (*ca.* 250 ml) and treated dropwise with conc. hydrochloric acid (1 ml).

The now brown solution of pheophytins was overlayered with water (250 ml) in a separating funnel and kept for *ca.* 20 h; this [30] removed water-soluble materials whilst avoiding emulsions. Evaporation of the lower layer gave the crude pheophytins. These were fractionated on alumina (100 g; activity 5) on a column packed in 1 : 3 v/v dichloromethane–light petroleum (b.p. 40—60 °C), the same solvent being used to add the pigments to the column. Elution with the same mixture (100 ml) was followed with 1 : 1 dichloromethane–light petroleum. Non-polar carotenoids *etc.* (*ca.* 700 mg) appeared in the fractions up to about 450 ml, followed by the dark grey band of pheophytin *a* (typically 70 mg). This product from the appropriate fractions was shown spectroscopically [31] to be essentially free from carotenoids.

(*b*) *Purification of phytol.* The foregoing pheophytin fraction in methanol (18 ml), containing potassium hydroxide (0.45 g) and light petroleum (4 ml; b.p. 40—60 °C), was heated under reflux for 2 h. The subsequent steps were carried out under normal lighting. The green solution was partitioned between water (100 ml) and light petroleum (100 ml; b.p. 40—60 °C), and the aqueous phase was run into a second separating funnel containing light petroleum (100 ml) and then equilibrated. The upper layer in the first funnel was swirled with saturated aqueous sodium

[26] C. A. Price and E. F. Carell, *Plant Physiol.*, 1964, **39**, 862.
[27] L. P. Vernon, *Analyt. Chem.*, 1960, **32**, 1144.
[28] C. Lijenberg and G. Odham, *Physiol. Plant.*, 1969, **22**, 686.
[29] J. A. Schiff, *Methods Enzymol.*, 1972, **24**, 321.
[30] J. Folch, M. Lees, and G. H. Sloane Stanley, *J. Biol. Chem.*, 1957, **226**, 497.
[31] F. C. Pennington, H. H. Strain, W. A. Svec, and J. J. Katz, *J. Amer. Chem. Soc.*, 1964, **86**, 1418.

hydrogen carbonate (100 ml). The lower phases from the two funnels were then transferred, that in the first into the second and that in the second into a third funnel. Fresh light petroleum (100 ml) was added to the third funnel and water (100 ml) to the first and all three funnels were equilibrated. In this countercurrent way, four aqueous phases were passed through four light petroleum phases; the latter were combined and washed thrice with water. After drying, the petroleum was evaporated off to leave an oil (typically 33 mg).

This product was fractionated using 2 : 3 diethyl ether–light petroleum (b.p. 40—60 °C) on the silica plates, previously described, which had been treated with silver nitrate and detergent. The phytol band (R_F *ca.* 0.30) was recovered from the plate with ether and was further purified on the same type of p.l.c. plate using 3 : 1 benzene–ethyl acetate (phytol R_F *ca.* 0.37). Phytol so obtained (typically 17 mg after being dried at 1 mmHg over P_4O_{10} and KOH pellets for 20 h) was converted as described earlier into its biphenyl-4-ylcarbamate derivative (typically 25 mg in two crops).

Radioactive samples were recrystallised from chloroform–acetone to constant specific activity. This product from the biosynthetic experiment with [1-^{14}C,^{13}C]acetate (3 g) (see later), was fractionated by p.l.c. on reversed-phase Kieselguhr G as described earlier; the results proving the absence of a significant amount of the geranylgeranyl derivative are reported in the main text.

A Search for Phytol in Dark-grown Cells of Euglena gracilis.—The cells (43 g wet wt.) were extracted by the standard method, save that acetone replaced methanol for the initial step. Evaporation of the solution left a residue which in 2 : 1 chloroform–methanol was overlayered with water and kept to remove hydrophilic materials as before. The organic layer yielded *ca.* 3 g of amorphous material in which no free phytol could be detected by t.l.c. as described earlier.

The remainder of this material was hydrolysed in the usual way, and the material (1.65 g) extracted into the light petroleum phase was chromatographed on alumina in light petroleum (b.p. 40—60 °C) containing steadily increasing proportions of dichloromethane (from 1 part in 3 to 3 parts in 4). In none of the 23 fractions could phytol be detected by t.l.c.

Experiments with Labelled Precursors.—(a) [1-^{14}C]*Acetate.* The quoted quantities here and for subsequent experiments refer to one standard suspension of colourless *E. gracilis* cells in 2.5 l of resting medium. The sodium [1-^{14}C]acetate (0.1 mCi) was diluted to 3 g with unlabelled sodium acetate before addition; the incubation time was 170 h in the light. This run yielded phytol (7.2 mg), part of which was diluted with unlabelled phytol; the mixture was purified to constant specific activity as the biphenyl-4-ylcarbamate derivative. The value found corresponded to 4.66×10^4 disint. min^{-1} mg^{-1} for the undiluted phytol (specific incorporation 26%).

An equivalent run, though with 1 g of sodium [1-^{14}C]-acetate (0.033 mCi), gave phytol (17 mg) of activity 6×10^3 disint. min^{-1} mg^{-1} (specific incorporation 3.7%). The ^{13}C studies were therefore based on addition of 3 g of sodium acetate.

(b) [1-^{14}C,^{13}C]*Acetate.* A mixture of sodium [1-^{13}C]acetate (2 g; 97.9 atom % ^{13}C) with sodium [1-^{14}C]acetate (1 g; 0.033 mCi) was used in a standard run with colourless cells, the incubation time in light being 126 h. The specific activity of the isolated phytol (14 mg), determined as above by dilution and preparation of the biphenyl-4-ylcarbamate, was 1.13×10^4 disint. min^{-1} mg^{-1} (specific incorporation 19%). The carbamate at constant specific activity was used for the ^{13}C n.m.r. spectrum.

(c) (3RS)-[2-^{14}C]*Mevalonate.* A solution, with a small immiscible layer, of (3*RS*)-mevalonolactone (2 g) in benzene (50 ml) was evaporated; the residue was mixed in benzene (50 ml) with (3*RS*)-[2-^{14}C]mevalonolactone (0.1 mCi, 10.9 mCi mmol^{-1}), and the solution was evaporated. One quarter of the residue was warmed with water and the mixture evaporated; the residue was then redissolved in water to give a clear solution. This was added to the standard run with *E. gracilis* cells and the system was illuminated for 180 h. The specific activity of the crystalline phytol derivative corresponded to a specific incorporation of 0.07% into the undiluted phytol (15 mg).

An equivalent run in which the labelled lactone (0.5 g) was heated at 40 °C with aqueous 0.01M-sodium hydrogen carbonate for 45 min before addition to a standard run gave phytol (27 mg) with a specific incorporation of 0.063%, as determined on the corresponding crystalline carbamate.

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