Phytochemistry, 1971, Vol. 10, pp. 85 to 95. Pergamon Press. Printed in England.

THE BIOSYNTHESIS OF PHYTOSTEROLS IN *MUSA SAPIENTUM*

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(*Received* 21 *March* 1970)

Abstract—Mevalonic acid-2-^{14}C was administered to banana peel slices for time periods varying from 0·5 hr to 6 days. For each period the incorporation of label into squalene, triterpene esters, 4,4-dimethyl, 4α-methyl and 4-desmethyl sterols was determined. Such studies demonstrated the slow turnover of phytosterol precursors into the 4-desmethyl sterols. The order of labeling of triterpene intermediates was in accord with the proposed mode of phytosterol formation. Cycloartenol and 24-methylenecycloartanol were labeled at early incubation periods, the specific activity of the latter increasing with incubation time. After 2 hr, label began to appear in cycloeucalenol. The 4-desmethyl sterols were labeled after 8 hr, but only to an appreciable extent after 24 hr. For each incubation period incorporation of label into squalene and triterpene esters was also determined. The distribution of radioactivity between esterified cycloartenol, 24-methylenecycloartanol and cycloeucalenol differed from the distribution of radioactivity in the free triterpene pool. The significance of the latter results is at the moment obscure.

INTRODUCTION

THE FORMATION of triterpene intermediates during the biosynthesis of cholesterol in animal tissues is well documented, with lanosterol representing the cyclization product of squalene.[1–4] Although it was suggested that lanosterol may play a similar role in the formation of phytosterols,[5] this triterpene is found only rarely in plants.[6] The occurrence of cycloartenol[7,8] led to the suggestion that this triterpene may represent the primary cyclization product of squalene in plants and may be the initial triterpene precursor of the phytosterols.[9,10] Early studies indicated that cycloartenol could be labeled from 2-^{14}C-acetic acid and 2-^{14}C-mevalonic acid in a number of plants.[9–13] Experiments have also demonstrated that lanosterol cannot be converted to cycloartenol while the latter is incorporated into the phytosterols of *Solanum tuberosum*.[14] While such studies have indicated that lanosterol is probably not the usual phytosterol precursor, the enzymatic machinery of plants

* NSF Predoctoral Fellow. This work represents part of the requirements for the Degree of Doctor of Philosophy at St. Louis University.

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[7] H. R. BENTLEY, J. A. HENRY, D. S. IRVINE and F. S. SPRING, *J. Chem. Soc.* 3673 (1953).
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[13] P. BENVENISTE, L. HIRTH and G. OURISSON, *Compt. Rend. Lab. Carlsberg* **259**, 2284 (1964).
[14] H. H. REES, L. J. GOAD and T. W. GOODWIN, *Biochem. J.* **107**, 417 (1968).

is evidently capable of converting this triterpene to phytosterols. Thus, lanosterol has been converted to sterols in *Euphorbia peplus*,[15] and 24-methylenedihydrolanosterol was incorporated into the sterols of *Nicotiana tabacum*.[16] In addition, lanosterol, cycloartenol and 24-methylenecycloartanol have been converted to poriferasterol by *Ochromonas malhamensis*.[17] Therefore, although lanosterol is not an obligatory phytosterol precursor, the enzymatic systems involved in the formation of these substances are non-specific to the extent that unnatural substrates can be metabolized. Recent studies using *N. tabacum* have indicated that the order of labeling of triterpene intermediates is in accord with the series of transformations: squalene → cycloartenol → 24-methylenecycloartanol → cyclo-

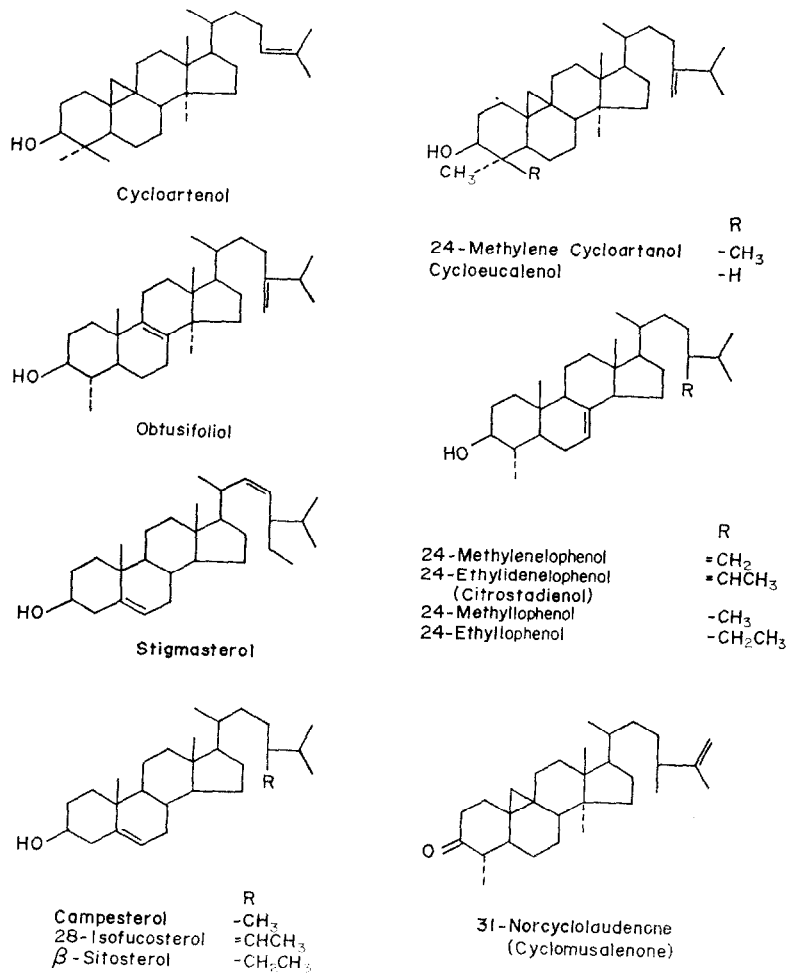

FIG. 1.

[15] D. J. BAISTED, R. L. GARDNER and L. A. MCREYNOLDS, *Phytochem.* **7**, 945 (1968).
[16] A. ALCAIDE, M. DEVYS, J. BOTTIN, M. FETIZON, M. BARBIER and E. LEDERER, *Phytochem.* **7**, 1773 (1968).
[17] J. HALL, A. R. H. SMITH, L. J. GOAD and T. W. GOODWIN, *Biochem. J.* **112**, 129 (1969).

eucalenol → obtusifoliol → lophenols → phytosterols.[18] The present investigations substantiate these results. The structures of these substances are illustrated in Fig. 1.

While preliminary investigations have indicated that the triterpene esters of some plants are rapidly labeled from 2-^{14}C-mevalonic acid, the distribution of radioactivity in the individual triterpene components was not determined.[19,20] In the present investigations, the identification of relatively large amounts of triterpene esters in banana peel and the fact that these esters are labeled from 2-^{14}C-mevalonic acid allowed a comparison of the distribution of radioactivity in both free and esterified triterpenes. The distribution of label differed in the two pools for all time periods studied. The possible biochemical significance of these findings is discussed in detail.

RESULTS

Banana peel is a rich source of triterpenes that have been postulated as biosynthetic intermediates of the phytosterols.[21] and represents an excellent tissue with which to study the formation and metabolism of these substances. Incubation of banana peel slices with 2-^{14}C-mevalonic acid for varying time intervals represented a means with which to study the turnover of squalene, triterpenes, triterpene esters and phytosterols. The incorporation of 2-^{14}C-mevalonic acid into neutral lipid was essentially linear for 14 hr after which it remained constant (Fig. 2). An aliquot of the lipid from each incubation (approximately 5 per cent) was chromatographed on silica gel using S-1. Ten R_f regions of the plates were

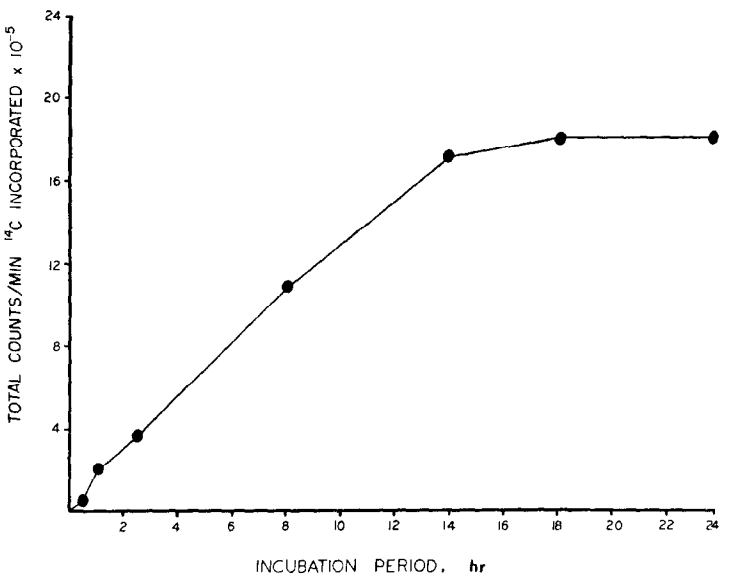

FIG. 2. RATE OF INCORPORATION OF 2-^{14}C-MEVALONIC ACID INTO THE NEUTRAL LIPIDS OF BANANA PEEL.

The tissue slices (5 gm) were incubated with 5 μc of the radioactive substrate for the periods indicated.

[18] P. BENVENISTE, M. J. E. HEWLINS and B. FRITIG, *European J. Biochem.* **9**, 526 (1969).
[19] D. R. THRELFALL, W. T. GRIFFITHS and T. W. GOODWIN, *Biochem. J.* **92**, 56P (1964).
[20] R. J. KEMP, A. S. A. HAMMAN, L. J. GOAD and T. W. GOODWIN, *Phytochem.* **7**, 447 (1968).
[21] F. F. KNAPP and H. J. NICHOLAS, *Phytochem.* **8**, 207 (1969).

scraped off, eluted and counted. The results are shown in Fig. 3 as the percentage of the total radioactivity recovered from the plate in each R_f region. The low background radioactivity in areas of the plates where no terpenoid material was found indicated that there was no randomization of the substrate and that little autoxidation had occurred. After 0·5 hr

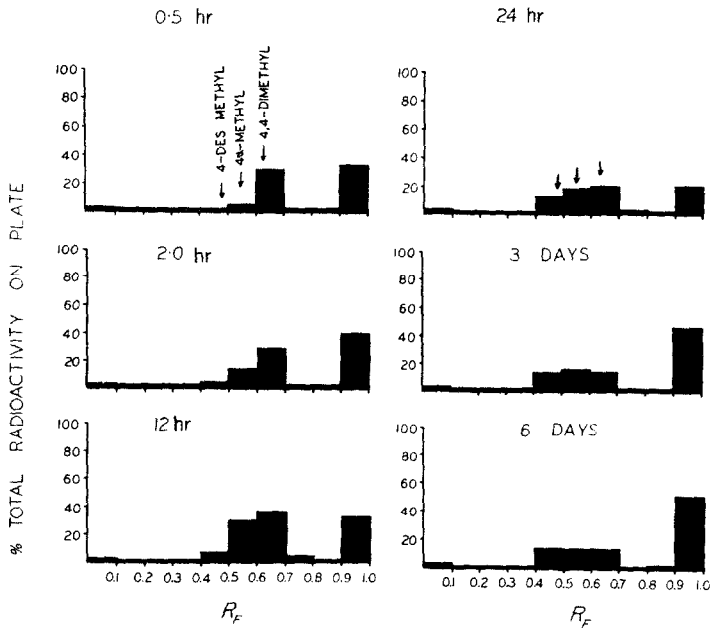

FIG. 3. TLC OF ALIQUOTS OF THE NEUTRAL LIPID MATERIAL FROM BANANA PEEL SLICES (5 gm) INCUBATED WITH 5 μc OF 2-^{14}C-MEVALONIC ACID.

The solvent system was 2,2,4-trimethyl pentane–ethyl acetate–acetic acid, 40:20:0·4 (by vol.).

essentially all of the radioactivity was associated with the 4,4-dimethyl triterpenes (R_f 0·62) and the hydrocarbons and triterpene esters (solvent front). The percentage radioactivity at the solvent front remained nearly constant for 24 hr and then slowly increased up to 6 days. After 2 hr label appeared in the 4α-methyl triterpene region (R_f 0·54, cycloeucalenol) and this increased up to 12 hr and then remained essentially constant. Only a small percentage of radioactivity was associated with the 4-desmethyl sterols even after 24 hr (R_f 0·46). This could be accounted for by the slow turnover of phytosterol precursors and thus explain the marked accumulation of these substances in banana peel. From 24 hr to 6 days the percentage of radioactivity in each of the three sterol classes was essentially the same (approximately 20 per cent). While the radioactivity in the triperpene ester and squalene area decreased steadily for the first 24 hr, it then increased to three times this value after 6 days. Material in the R_f 0·82 represents the 4α-methyl triterpene ketone[21] which has now been shown to be 31-norcyclolaudenone.[22] The biosynthesis of this triterpene has been studied.[23] This substance was labeled to only a small extent compared to the significant amounts of radioactivity that were incorporated into the other triterpenes at all incubation periods.

[22] F. F. KNAPP, Ph.D. Thesis, St. Louis University (1970).
[23] F. F. KNAPP and H. J. NICHOLAS, *Phytochem.* **10**, 97 (1971).

Since material at the solvent front represented both hydrocarbons and triterpene esters, the remaining lipid from each incubation was chromatographed using S-1 and material at the solvent front eluted from the adsorbant. This material represented both hydrocarbons and triterpene esters and was re-chromatographed in a system (S-2) which separated these two components. In the system benzene-petroleum ether (20:80, v/v) squalene had an R_f of 0·90 and the triterpene esters R_f 0·26. Ten R_f regions of the plates were scraped off and material in the R_f 0·20–0·30 and 0·85–0·95 regions eluted and aliquots counted. Silica gel from the other areas were scraped directly into counting vials, scintillation fluid added and the radioactivity determined. There was essentially no radioactivity found in these other regions, indicating that the system separated the two components with a minimum of breakdown products. The percentage of the total radioactivity in each of these two com-

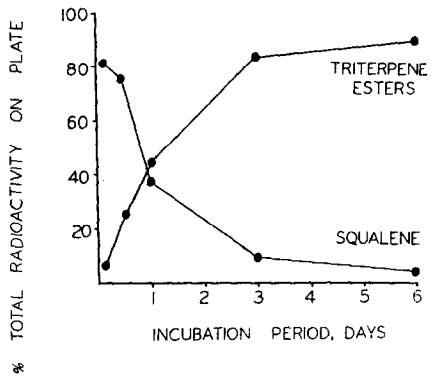

FIG. 4. DISTRIBUTION OF RADIOACTIVITY IN SQUALENE AND TRITERPENE ESTERS FROM BANANA PEEL SLICES (5 gm) INCUBATED WITH 5 μc OF 2-^{14}C-MEVALONIC ACID.

Material at the solvent front was eluted from the chromatograms of the crude neutral lipid (Fig. 2). These fractions were then re-chromatographed in the system benzene–petrol, 20:80 (v/v).

ponents recovered from the TLC plates is plotted in Fig. 4. After short incubation periods nearly all of the radioactivity was associated with squalene. The percentage of label in this hydrocarbon rapidly declined, indicating turnover to other metabolites. Radioactivity in the triterpene esters steadily increased, reaching a maximum at about 3 days. These fractions were set aside for further analysis.

Radioactivity which chromatographed in the squalene region was shown to be associated with only this hydrocarbon by GLC analysis using the radioactive monitoring system. The labeled squalene from the incubations was combined and an aliquot run on GLC. These results are illustrated in Fig. 5. There was only one radioactive peak that co-chromatographed with the large mass peak for squalene. The series of smaller mass peaks represent a homologous series of *n*-alkanes as shown by comparison with standards. The remainder of the labeled squalene was combined with 100 mg of carrier material and dissolved in acetone cooled in an ice bath. HCl gas was passed through the solution for 3 hr. The squalene hexahydrochloride precipitate was then crystallized to constant specific activity from acetone as indicated in Table 1.

Since the radioactivity in the 4,4-dimethyl triterpene region represented both cycloartenol and 24-methylenecycloartanol (and perhaps other unidentified triterpenes), it was necessary to determine the distribution of radioactivity in these components. This was determined in relation to the incorporation into cycloeucalenol. From the chromatograms

TABLE 1. RECRYSTALLIZATION OF SQUALENE HEXAHYDROCHLORIDE

Crystallization	Yield, mg	Specific activity, cpm/mg
3	78·40	254
5	72·40	273
7	67·70	285

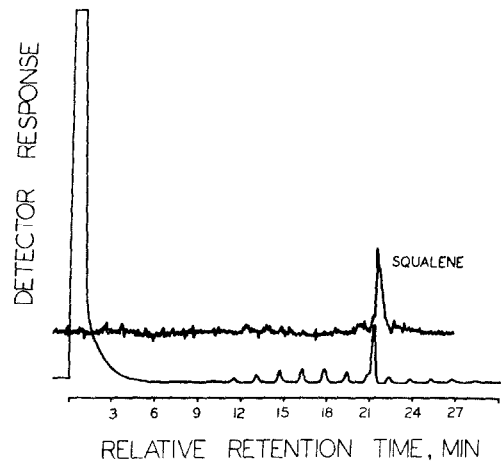

FIG. 5. GLC ANALYSIS WITH THE RADIOACTIVE MONITORING SYSTEM OF ^{14}C-LABELED SQUALENE. Squalene fractions for each of the incubation periods were eluted from the chromatograms described in Fig. 4. These were combined and an aliquot then analyzed. The upper curve represents the radioactive monitor tracing.

of the remaining lipid the 4,4-dimethyl and 4α-methyl triterpene regions were eluted together. The 4-desmethyl regions were eluted and set aside for GLC analysis. The triterpene fractions were chromatographed using the radioactive monitoring system. The results are shown in Fig. 6. After 0·5 hr there were essentially equal amounts of label in cycloartenol and 24-methylene cycloartanol. The specific activity of this material for incubation periods less than 0·5 hr was too low to demonstrate the initial labeling of cycloartenol. After 2 hr there was a small amount of radioactivity found in cycloartenol and cycloeucalenol had become significantly labeled. By 24 hr there was again only a small amount of radioactivity found in cycloartenol as compared to the other two components. After 3 and 6 days the distribution of label in cycloeucalenol and 24-methylenecycloartanol was essentially equal. Chromatography of the 4-desmethyl sterol fractions in the same manner indicated the early labeling of β-sitosterol (Fig. 7). For incubation periods less than 8 hr the specific activity of this material was too low for these analyses. It should be noted that although all of the radioactivity seemed to be associated with β-sitosterol for the early incubations, stigmasterol accounts for greater than 90 per cent of the total sterol mass.[21,24] After 24 hr and especially 3 and 6 days radioactivity appeared in campesterol and stigmasterol. These results may lend support to the observed conversion of β-sitosterol to stigmasterol in *Digitalis lanata*.[25]

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[25] R. D. BENNETT and E. HEFTMANN, *Steroids* **14**, 403 (1969).

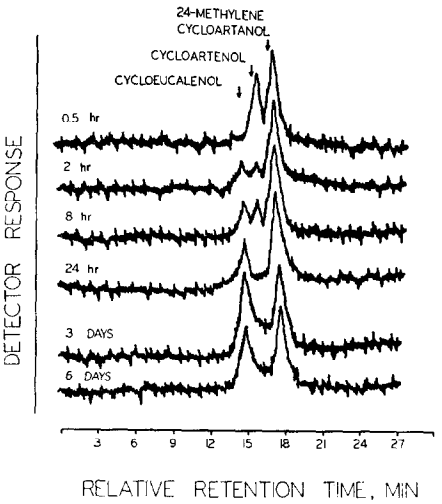

FIG. 6. RADIOACTIVE TRACINGS OF THE GLC ANALYSIS OF FREE TRITERPENE ALCHOHOLS LABELED FROM 2-^{14}C-MEVALONIC ACID IN BANANA PEEL.
The 4,4-dimethyl and 4α-methyl triterpene regions of chromatograms of the crude neutral lipid were eluted and aliquots then analyzed.

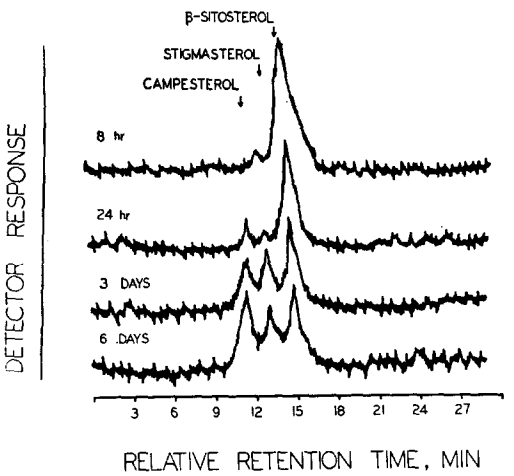

FIG. 7. RADIOACTIVE TRACINGS OF THE GLC ANALYSIS OF THE FREE PHYTOSTEROLS LABELED FROM 2-^{14}C-MEVALONIC ACID IN BANANA PEEL.
The sterols were eluted from chromatograms of the crude neutral lipid and aliquots then analyzed.

The triterpene ester fractions from each incubation period were saponified to yield the ^{14}C-labeled triterpene alcohols, which were then analyzed using the GLC-radioactive monitoring system. Technical problems made it impossible to analyze the intact esters for radioactivity by these methods. These results are reproduced in Fig. 8 and are strikingly different from the distribution of radioactivity in the free triterpene pool. While only a small amount of label was found in cycloartenol after 24 hr (Fig. 6), significant radioactivity was present in esterified cycloartenol even after 6 days. The triterpenes obtained from the 0·5, 2

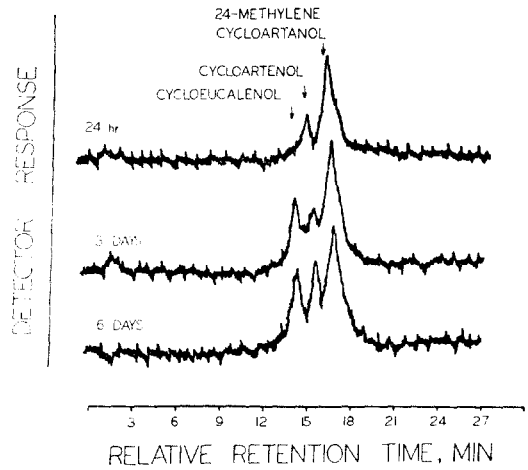

FIG. 8. RADIOACTIVE TRACINGS OF THE GLC ANALYSIS OF TRITERPENES OBTAINED BY HYDROLYSIS OF THE ESTER FRACTIONS PURIFIED BY TLC.

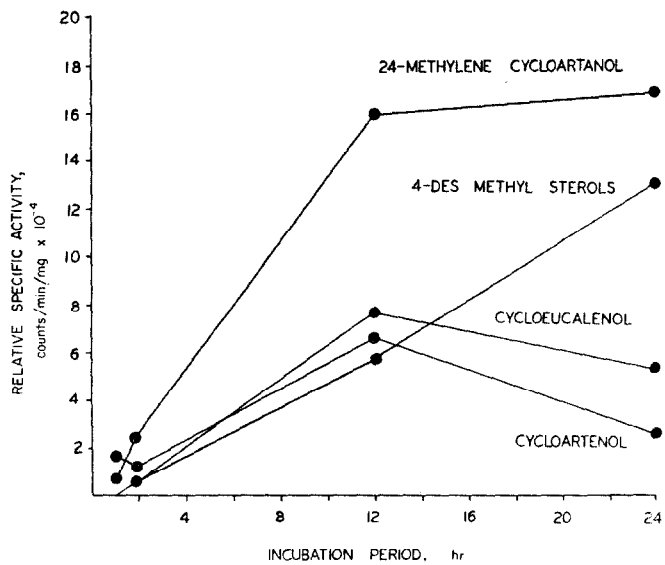

FIG. 9. RELATIVE SPECIFIC ACTIVITY OF PHYTOSTEROLS AND TRITERPENE ALCOHOLS LABELED FROM 2-^{14}C-MEVALONIC ACID IN BANANA PEEL.

and 12 hr ester fractions were not labeled sufficiently for these analyses. A plot of the relative specific activities of each of the components for each incubation period is shown in Fig. 9.

DISCUSSION

Other than the recently published experiments of Benveniste *et al.*,[18] this represents the only study of the simultaneous formation and metabolism of triterpene intermediates during

the biosynthesis of the phytosterols. Our results substantiate the data of these workers showing that the order of labeling of triterpene intermediates is in agreement with the proposed phytosterol biosynthetic scheme.[9,10] Of additional interest is the apparent conversion of β-sitosterol to stigmasterol (Fig. 7). This conversion was suggested by other workers who noted the decrease in specific activity of β-sitosterol while that of the latter increased.[26–28] These proposals have now been confirmed by the conversion of β-sitosterol-3-^{14}C to stigmasterol in *Digitalis lanata*.[25]

In addition to the removal of the 4α-methyl group, there are essentially four events which must occur during the conversion of cycloeucalenol to the phytosterols. These are the opening of the cyclopropane ring, isomerization of the nuclear double bond, removal of the C-14 methyl group and alkylation at C-24. Therefore, obtusifoliol, 24-methylenelophenol and 24-ethylidenelophenol or similar triterpenes undoubtedly are formed during this series of transformations.[6] Neither mass peaks nor radioactive metabolites with retention times similar to these triterpenes have been detected in banana peel. They do not accumulate in this tissue and if formed during phytosterol biosynthesis in banana peel must represent short-lived, enzyme-bound intermediates.

It is of interest that cycloeucalenyl and 24-methylenecycloartanyl palmitic acid esters have been identified in banana peel[21,24] and cycloartenyl palmitate has been isolated from this tissue and also from the seeds of *Strychnos nux-vomica*.[29] While it is known that cycloartenol and related triterpenes are esterified in a number of other plants, in most cases the fatty acid moities of the esters have not been identified. For example, hydrolysis of a crude fraction from birch wood yielded cycloartenol, 24-methylenecycloartanol and 24-ethylidenelophenol (citrostadienol).[30] Triterpenes are also esterified in *Zea mays*,[6,19,20] *Pisum sativum*,[6] grapefruit peel,[6,31] and tobacco[6] and the incorporation of 2-^{14}C-mevalonic acid into these esters was reported. In none of these cases, however, was either the distribution of radioactivity in the individual esterified triterpenes or a comparison of the distribution of label in free and esterified triterpenes made. The present investigation has demonstrated that the distribution of label in esterified triterpenes labeled from 2-^{14}C-mevalonic acid is different than the distribution in the free triterpene alcohols.

As other workers have mentioned, the formation of triterpene esters as intermediates during phytosterol biosynthesis does not seem reasonable.[6] This is primarily because it has been demonstrated that 3-ketones are formed during lanosterol demethylation in animals[32,33] and that such ketones are also formed during phytosterol formation, presumably during the C-4 demethylation process.[4,34] We have recently shown that such a ketonic intermediate is formed during the conversion of 24-methylenecycloartanol to cycloeucalenol in *M. sapientum*.[35] A mechanism has been proposed to explain the formation of a 3-ketone as part of the demethylation process.[23] The formation of 3-ketones does not necessarily eliminate the possibility that triterpene esters play some important physiological function in plants. The formation of esters as transfer products between intracellular organelles

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[31] B. L. Williams, L. J. Goad and T. W. Goodwin, *Phytochem.* **6**, 1137 (1967).
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[33] A. C. Swindell and J. L. Gaylor, *J. Biol. Chem.* **243**, 5546 (1968).
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would in itself constitute an important process. Certain triterpene esters exhibit some special physical properties which would make them well suited for living systems. Cycloartenyl palmitate, for instance, is known to form a well-defined cholesteric mesophase.[29,36,37] The occurrence and possible biological significance of liquid crystals in living systems is a relatively new area of interest, and has been discussed by several groups of workers.[29,38,39]

EXPERIMENTAL

General

All solvents were distilled before use. Mevalonic acid-2-^{14}C as the dibenzylethylenediamine salt was purchased from Sigma Chemical Company. Determination of radioactivity was performed using an Ansitron liquid scintillation. Samples were counted in toluene solution with fluors in the usual manner. This instrument counted ^{14}C with 95 per cent efficiency. Triterpene esters were saponified in ethanol–benzene–water, 80:10:10 (by vol.) which contained 15% KOH.

Incubations and Preparation of Lipid Material

Bananas (*Musa sapientum*, L.) were purchased locally. For the short term incubations of 0·5, 2, 12 and 24 hr, 5 gm slices of banana peel were used. The under surface of the slices were scraped and etched with a razor to insure complete absorption of the substrate which was applied in water. The slices were moistened occasionally with distilled water throughout the incubation period. The tissue was turgid and had a normal appearance for up to about 36 hr. For the long term incubations of 3 and 6 days whole tissue was used. The peeling of the intact banana was punctured several times with a 5 mm dia. cork borer such that the borer only pierced the outer portion of the peel and did not enter the pulp. The substrate was applied to these areas and after absorption the flap of skin was replaced and covered with transparent tape to prevent drying.

To the tissue from the short term incubations 10 gm of carrier banana peel was added and the combined tissue then extracted overnight with ethanol. After evaporation to dryness the residue was dissolved in 200 ml ether and washed successively with 5% KOH and water. Evaporation of the solvent yielded the neutral lipid material (12–15 mg). For the long term incubations 15 gm of tissue in the immediate vicinity where the substrate was applied was removed and worked up in the same manner. Independent experiments indicated that not much radioactivity migrated from this area during the incubation period.

Thin-layer Chromatography

Glass plates (20 × 20 cm^2) were spread with silica gel H and samples were applied as bands from benzene solution. For the separation of the crude neutral lipid material the solvent system (S-1) was 2,2,3-trimethylpentane–EtOAc–HOAc, 80:40:0·4 (by vol.). In this system, the R_fs obtained were: steryl esters and hydrocarbons migrated to the solvent front; 31-norcyclolaudenone[22], R_f 0·82; 4,4-dimethyl triterpenes, R_f 0·62; 4α-methyl triterpenes, R_f 0·58; 4-desmethyl sterols, R_f 0·46. Triterpene esters and hydrocarbons were separated using benzene–petrol (1:4) (v/v) (S-2). The esters had an R_f of 0·26 and squalene, R_f 0·90. In all cases markers were run on the borders of the plates. These areas were sprayed with anisaldehyde reagent, the plates heated enough to detect the markers and material corresponding to these materials then scraped from the plate. Material was eluted with benzene. The absorbant was stirred with benzene and the slurry then centrifuged at low speed. The benzene was then decanted from the pellet. This procedure was repeated two times. From 70 to 90% of the original radioactivity was usually recovered from the plate by these procedures.

Gas–Liquid Chromatography

For these analyses a Barber–Colman Model 5000 instrument equipped with a radioactive monitoring system (Model 5190) was used. Samples were injected in hexane solution onto a 1% SE-30 column (6 ft, 4 mm I.D.). The carrier gas was Argon with a flow rate of 60 ml/min. Other conditions were as follows: flash heater, 295°; detector, 235°; column, programmed from 220 to 260° at a rate of 2°/min.

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The instrument was constructed such that only one part of the column effluent went to the mass detector while ten parts went to the proportional counter. Before entering the counting system the effluent was quenched with 10% Propane. The mass and radioactivity were recorded on separate units. In most cases the specific activity of the material chromatographed was high enough that no mass was detected while a large radioactive peak was recorded. In these cases authentic standards were chromatographed before and after the radioactive material. The retardation of the radioactive peak from the mass peaks is instrumental in origin.[40,41]

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