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THE EFFECT OF EXTRANEOUS HYDROCARBONS ON THE YIELD OF STEROIDAL SAPOGENIN FROM THE TUBER TISSUE OF *DIOSCOREA DELTOIDEA*

ROLAND HARDMAN and K. R. BRAIN*

Pharmacognosy Group, School of Pharmacy, Bath University of Technology, Bath BA2 7AY

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Abstract—The addition of hydrocarbons to incubating tuber tissue of *Dioscorea deltoidea* increases the sapogenin yield. The effect is relatively non structure specific; squalane, saturated *n*-alkanes and mono- and di-unsaturated *n*-alkenes are effective although some long chain ($>C_{28}$) *n*-alkanes reduce the yield. The added hydrocarbon disappears rapidly and 0·054% incorporation of label from 1-^{14}C-octadecane into diosgenin was obtained.

INTRODUCTION

THE DEMAND for diosgenin, the most important raw material for the production of pharmaceutical steroids, continues to rise.[1] In the commercial process the *Dioscorea* tubers are 'fermented'.[1] Squalane and a range of other hydrocarbons, when included in the fermentation medium, were found to be capable of further increasing the yield of diosgenin from a variety of plant material (*Dioscorea* tubers, Fenugreek seed, fruit parts of species of *Balanites*).[2,3] This work was first described in 1965.[2] About the same time, Corey and Russey using rat liver homogenate showed that squalane diminished the rate of conversion of squalene to sterol[4a] and that *n*-eicosane (C_{20}) was as equally effective as squalane.[4b] We now report our hydrocarbon experiments with the powdered dried tuber of *Dioscorea deltoidea* Wall., a commercial source of diosgenin.

A great number of micro-organisms are capable of degrading or assimilating hydrocarbons[5] and alkanes are very widely distributed in both the plant and animal kingdoms.[6] The metabolism of alkanes in animals has been investigated in a limited way.[4,7,8] We have examined the internal plant hydrocarbons (in contrast to the surface alkanes), using the seeds of species of *Balanites*[9] and the dried tuber of *D. deltoidea*,[10] with the commercial procedures employed in the isolation of diosgenin. A changing pattern of seed alkane during ripening of the *Balanites* fruits was disclosed[9] and a dynamic role for hydrocarbons

* Present address: Welsh School of Pharmacy, UWIST, Cardiff CF1 3NU, Wales.

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in plant metabolism was indicated.[10] The relevance of hydrocarbons to steroidal sapogenin metabolism in particular, is now considered.

RESULTS AND DISCUSSION

Increases in sapogenin yield on treatment with hydrocarbons could arise by synthesis or by release of pre-existent material. When squalane was present from the start of aqueous incubation of tuber powder of *D. deltoidea* it was found that the sapogenin yield, estimated by densitometric TLC[11] was increased.[3] The increase was concentration dependent: 10% (v/w) squalane gave a 7% increase and 20% (v/w) squalane a 14% increase, both after 72 hr, over the control incubation in the absence of squalane (Table 1a). If the squalane was

TABLE 1. INCUBATION OF TUBER POWDER OF *D. deltoidea* WITH SQUALANE FOR 72 hr

	Sapogenin assay (% m.f.b.)*	% Increase relative to control incubation
(a) *Small scale with densitometric TLC assay*		
Direct hydrolysis	4·44	—
Control incubation	4·79	—
With squalane 10% (v/w)	5·12	7
With squalane 20% (v/w)	5·47	14
With squalane 20% (v/w) added after incubation	4·82	—
(b) *Large scale; gravimetric yield*		
Direct hydrolysis	3·91	—
Control incubation	4·84	—
With squalane 20% (v/w)	5·73	18

* On moisture free basis.

added to the incubate after incubation, but before hydrolysis, then the sapogenin yield was the same as in the absence of squalane, indicating that incubation was a necessary part of the process. Confirmation of the effect of squalane was carried out by repeating the experiment on a larger scale with gravimetric recovery of the sapogenin. These large scale experiments showed an increase of 18% over the control incubation, after incubation with 20% squalane for 72 hr (Table 1b). The appearance, m.p., TLC characters and IR spectra of the crystals obtained from the incubations in the presence of squalane were of equal purity to those obtained in the absence of squalane.

Replacement of the squalane by a series of mixed odd and even carbon number *n*-alkanes, from C_{14} to C_{28}, resulted in all except C_{24} giving a significant increase in sapogenin yield, estimated by the IR spectrometric method,[12] over the control containing no hydrocarbon (Table 2), whereas the longer chain C_{32}, C_{36} and C_{40} compounds caused a marked decrease. In several cases there were significant increases with the hydrocarbon without incubation (Table 2).

Introduction of mono- or di-unsaturation into the hydrocarbon did not have any marked effect, except that perhaps it reduced the effect of hexadecane (Table 3). Samples

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TABLE 2. TREATMENT OF TUBER POWDER OF *D. deltoidea* WITH SATURATED STRAIGHT CHAIN HYDROCARBONS

Added alkane	Direct hydrolysis		24 hr incubation	
	Sapogenin*	% Change†	Sapogenin*	% Change†
None (1)‡	5·33	—	5·22	—
n-C_{14}	5·16	−3	5·69	+9
n-C_{16}	5·35	—	6·65	+28
n-C_{18}	5·79	+9	5·75	+10
n-C_{20}	5·75	+8	6·13	+18
n-C_{21}	6·15	+15	5·63	+8
n-C_{22}	5·33	—	5·85	+12
n-C_{24}	5·27	—	5·18	—
n-C_{25}	5·59	+5	5·98	+15
None (2)‡	5·13	—	5·85	—
n-C_{26}	6·01	+17	6·15	+5
n-C_{28}	5·02	−2	7·38	+26
n-C_{32}	5·25	+3	4·04	−31
n-C_{36}	4·18	−18	4·04	−31
n-C_{40}	4·40	−14	5·01	−14

* Sapogenin percentage on a moisture free basis by IR method.
† Percentage change relative to control without alkane, by direct hydrolysis or incubation.
‡ The experiment with alkanes C_{14}–C_{25} was done at a different time from that with C_{26}–C_{40}; (1) and (2) are the respective controls.

TABLE 3. INCUBATION OF TUBER POWDER OF *D. deltoidea* WITH UNSATURATED HYDROCARBONS

Added alkene	Direct hydrolysis		24 hr incubation	
	Sapogenin*	% Change†	Sapogenin*	% Change†
None	4·26	—	4·28	—
n-C_{14}-1-ene	4·46	+5	4·62	+8
n-C_{16}-1-ene	4·36	+2	4·61	+8
n-C_{20}-1-ene	4·11	−4	4·75	+11
n-C_{22}-1-ene	4·13	−3	4·71	+10
n-C_{20}-1,19-diene	4·10	−4	4·81	+12

* Sapogenin percentage m.f.b. by densitometric TLC.
† Percentage change relative to control without alkene by direct hydrolysis or incubation.

containing these unsaturated compounds could not be estimated by the IR procedure as these molecules have strong absorbtion bands close to those used in the sapogenin assay.

In an attempt to determine whether the added hydrocarbon was, in fact, metabolized by the tuber tissue, the total hydrocarbon was recovered in a *n*-hexadecane experiment: recovery of total hydrocarbon was low being only 7·2% of the added *n*-hexadecane from the 24-hr incubation and even from the acid trated unincubated tuber the total hydrocarbon recovered was only 14·8% of the added *n*-hexadecane. GLC examination of the recovered hydrocarbon from the acid treated unincubated tuber experiment showed 70% of the total hydrocarbon to be *n*-hexadecane whereas that total hydrocarbon from the 24-hr incubation showed a negligible *n*-C_{16} peak and a major peak with an indicated chain length between C_{21} and C_{22}.

When 1-^{14}C-octadecane was used as the added hydrocarbon together with unlabelled *n*-octadecane in the incubate, there was incorporation into the sapogenin fraction to the

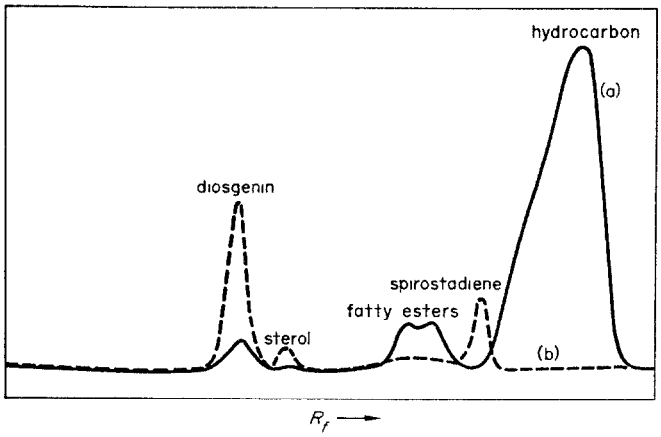

FIG. 1. METABOLISM OF 1-^{14}C-OCTADECANE IN A TUBER POWDER INCUBATE OF *D. deltoidea*. Extract chromatographed on Silica gel G with hexane: ethyl acetate 4:1. (a) Autoradiograph prepared from the plate and optical density scanned by transmission, ——. (b) Plate sprayed with $SbCl_3$–HCl reagent and heated at 110° for 30 min before transmission scanning, - - - - -.

extent of 0·054% of the original activity administered, as well as into other compounds in the petroleum-soluble fraction (Fig. 1).

These results suggest that the tuber tissue is capable of the assimilation of exogenous hydrocarbon, although the mechanism of the subsequent degradation and of the incorporation into steroidal compounds is unknown. It seems most likely that the octadecane is taken up as small units rather than as a whole, and the added hydrocarbon could be acting solely as an available carbon source. No attempt was made to exclude microbial contamination, as this was impractical under the industrial-type conditions intentionally employed, but no gross contamination was ever visible in the presence or absence of hydrocarbon during 30 months of experimentation. Furthermore the inclusion of the antibiotics griseofulvin, cephaloridine and penicillin did not prevent the normal rise in sapogenin on incubation.[13]

There has been little report on the metabolism of exogenous alkanes by higher plant tissue although their biosynthesis has been extensively studied.[14,15] The only reported experiments on the interrelation of steroids and alkanes are those of Badiello *et al.*[16] They obtained incorporation of the label from ^{14}C-myristic acid into 22,23-dihydrostigmasterol and into heptacosane in *Wistaria sinensis* but could not incorporate the label from cholesterol-4-^{14}C into heptacosane or from heptacosane into 22,23-dihydrostigmasterol. It is feasible that these reactions could be driven in the reverse direction, particularly in the presence of large quantities of alkane. However, synthesis seems unlikely to explain the increases in Table 2 obtained on treatment with hydrocarbon without incubation. We have earlier proposed that some of the sapogenin in tuber is in a bound form[17] and it is possible that it is this which is released under the action of the hydrocarbon. A simple solvent effect

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by the added hydrocarbon is unlikely but a preferential take-up of sapogenin into the organic phase after release could facilitate further release or synthesis of sapogenin in the aqueous phase. Alternatively it is possible that the hydrocarbon has a site-specific, but not structure-specific, action on a particular metabolic pathway. Hahn and Wagenknecht[18] found competitive inhibition by heptane against $NADH_2$ in the $NADH_2$ dehydrogenase of rat liver and concluded that this was probably due to participation at an apolar binding site on the enzyme. Stoppani *et al.*[19] have recently investigated the inhibition of NADH-oxidase by steroids and found that "the most effective steroid structure to inhibit electron transfer is a planar narrow hydrocarbon framework with relatively small, adequately oriented polar end-groups and a large intermediate hydrophobic area". These structural characteristics would cause inhibition of the main respiratory chain by interference at the NADH-flavoprotein site.[19]

A narrow hydrocarbon framework may be sufficient to produce a blocking action without the polar groups if the concentration is sufficiently high. On the other hand there is some correlation between the alkanes and the inert biological depressants which are effective as anaesthetics. These have practically no chemical reactivity and it is thought likely[20] that these nonstructure-specific depressants act by disorganization of the spatial relationship of consecutive enzymes in the respiratory pathway. If the main respiratory chain was blocked in this manner it is likely that alternative pathways would be used to provide energy. A possible function of the saponins in plant material is as a store of sugar as well as of steroid and it is possible that the need for energy could be partially satisfied by the hydrolysis of saponin, releasing the sapogenin as a by-product.[17]

EXPERIMENTAL

Plant Material

The tuber was obtained as *Dioscorea deltoidea* Wallich from Seth Panchhi Ram and Co., Kuth Growers, Manali, Kulu Hills, India. Macroscopically the rhizome agreed closely with the description of Prain and Burkill[21] for *D. deltoidea*. The dried material was reduced to a coarse powder in a disintegrator.

Sapogenin Extraction Procedure

The extraction procedure was essentially that of Blunden and Hardman.[22] Small plant samples, normally 2·5 g, in powdered form, were hydrolysed by heating for 2 hr under reflux with 2 N HCl, using 25 ml/g of dried plant material. The mixture was cooled and filtered, the acid-insoluble residue was washed (H_2O, 20 ml/g), and then neutralized with 40 ml/g of 5% (w/v) NH_4OH. The drained insoluble residue was extracted in a soxhlet with light petroleum (b.p. 40–60°) to exhaustion. The solvent was removed and the residue dissolved in sufficient $CHCl_3$ to give a total sapogenin concentration of 0·5–1·0 g/100 ml for the IR spectrometric, or 0·1 g/100 ml for the densitometric TLC assay. All experiments were carried out in duplicate and the mean value taken.

Modified Extraction Procedure in the Presence of Hydrocarbon

Samples of tubers were treated with hydrocarbon without any incubation period to ascertain if there was any immediate effect on sapogenin yield. The plant material was dispersed in 20 ml/g H_2O and the required quantity of hydrocarbon added, followed by conc. HCl, 4 ml/g, to make the solution 2 N. The whole was shaken mechanically for 5 min before hydrolysis and extraction as above.

Incubation of Plant Material

The plant material was shaken in 20 ml/g H_2O for 5 min before being set aside in the dark at 37°. At the end of the incubation procedure conc. HCl, 4 ml/g, was added and the sample hydrolysed and extracted as above. Where hydrocarbon was included in the medium this was added before the shaking stage.

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Quantitation of Sapogenin

In the small samples the sapogenin was estimated by the technique of Brain *et al.*[12] which involves the measurement of the specific IR absorption of the spirostan nucleus, or the densitometric TLC procedure of Brain and Hardman,[11] which measures the sapogenin after chromatographic separation. The precision of the IR method was 2·9% and of the densitometric TLC procedure 1·1%

Large Scale Experiment with Squalane

Powdered tuber, 100 g, of *D. deltoidea* was boiled under reflux with 730 ml of 2 N HCl for 2 hr. The mixture was cooled to room temp., the acid-insoluble matter collected, washed (H_2O), and then with 5% NH_4OH until it was alkaline. The insoluble residue was dried at 60° for 16 hr and powdered to B.S.S. No 10. The powder was extracted in a soxhlet apparatus with light petroleum (b.p. 40–60°), 1200 ml, to exhaustion (48 hr). The petroleum extract was set aside for 16 hr at room temp. before the crystalline sapogenin was collected and washed with petroleum. The mother liquor and washings were bulked and concentrated to a volume of 200 ml to afford a second crop of sapogenin.

A further sample was hydrolysed in the presence of squalane as follows. Powdered tuber, 100 g, in 300 ml H_2O and 20 ml squalane was shaken for 5 min, the sides of the flask washed with 300 ml of water. Conc. HCl, 130 ml, was added, and the extraction continued as above.

In the samples which were incubated, both with and without squalane, the flask was shaken immediately and again for 2 min after 22 hr.

Recovery of n-*Hexadecane*

The petroleum extract was evaporated to dryness and the hydrocarbon isolated by chromatography on an alumina column. The hydrocarbon was examined by GLC under the following conditions: Varian Aerograph 1527-C, dual 5 ft × ⅛ in. o.d. stainless steel 5% SE 30 on AW DMCS Chromosorb W columns programmed from 120° to 300° at 2°/min. Injection temp. 235°, detector temp. 325°, detector dual f.i.d., carrier N_2. A tentative carbon number was assigned to each peak on the basis of the retention times of coinjected standards and the peak areas measured from peak height × peak width at half peak height.

*Incorporation of Octadecane-*1-^{14}C

Octadecane-1-^{14}C, 100 μc (added first in benzene which was then removed under N_2) tuber powder, 20 g, 400 ml H_2O, unlabelled octadecane (4 ml) were mixed by shaking for 2 min then set aside in the dark at 37° for 72 hr before extraction by the normal procedure. TLC examination of the petroleum extract showed the trace given in Fig. 1. An autoradiograph of this plate was prepared by exposure to Ilford Industrial X-ray film.

The petroleum extract was saponified and the unsaponifiable matter chromatographed on an alumina column. The hydrocarbon fraction was eluted first to yield 3·43 g, $1·05 \times 10^8$ dis/min, specific activity $3·5 \times 10$ dis/min/mg (47·3% of the activity originally administered). The sapogenin fraction was eluted and recrystallized from acetone to constant specific activity, 0·96 g, $1·20 \times 10^5$ dis/min, specific activity $1·25 \times 10^2$ dis/min/mg (0·054% of the activity originally administered).

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