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THE EFFECT OF POST HARVEST APPLICATION OF GROWTH REGULATORS ON THE YIELD OF STEROIDAL SAPOGENIN FROM PLANT MATERIAL

ROLAND HARDMAN and K. R. BRAIN*
Pharmacognosy Group, School of Pharmacy, Bath University of Technology, Bath BA2 7AY

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Abstract—Incubation of harvested tuber of *Dioscorea deltoidea* Wall. and whole seed of *Trigonella foenum-graecum* L. with either synthetic or natural plant growth regulators increased the sapogenin yield by up to 35 per cent. The process is concentration and time dependent and is possibly linked to a role of the saponins.

INTRODUCTION

A GREAT deal of effort has been expended in improving the partial synthesis of pharmaceutical steroids from plant steroidal sapogenins such as diosgenin. On the other hand, little work has been carried out to obtain the maximum yield of the sapogenin from the plant material. In the face of increasing demands for steroidal pharmaceuticals[1] it is essential that the maximum use is made of the natural source by sophisticated work-up procedures.

As a part of the usual commercial extraction of diosgenin[1] from wild species of *Dioscorea*, the mashed tuber is 'fermented' for some time as this is reported to increase the yield.[2] In our experience the process is largely autolytic, and it will therefore be referred to as 'incubation'.

Blunden, Hardman and Wensley[3] showed that large increases in sapogenin yield could be obtained by incubating harvested plant material, from various species and morphological parts, in an excess of water. The process was enzymic and the endogenous enzymes could be replaced, at least partly, by cell wall degrading enzymes. The present work sought to further influence the sapogenin yield by the application of extraneous chemicals.[4] Chosen for these experiments were the tubers of *Dioscorea deltoidea* Wall. and the whole seed of *Trigonella foenumgraecum* L. (fenugreek) both of which afford as their principle sapogenins diosgenin and its epimer yamogenin.[5]

RESULTS AND DISCUSSION

Considerable work on steroid metabolism has been carried out in the search for compounds which will lower the high levels of blood cholesterol present in arteriosclerosis. Several of the available compounds have been studied in detail and shown to act on the

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

[1] R. HARDMAN, *Trop. Sci.* **11**, 196 (1969).
[2] Schering Corporation, *Brit. Pat.* 753,137 and 753,138 (1956).
[3] G. BLUNDEN, R. HARDMAN and W. R. WENSLEY, *J. Pharm. Pharmac.* **17**, 274 (1965).
[4] R. HARDMAN to National Research Development Corporation Brit. Pat. Appl. 39765/67.
[5] R. HARDMAN and E. A. SOFOWORA, *Phytochem.* **9**, 645 (1970).

biosynthesis of cholesterol.[6] Since cholesterol, or a closely related compound, has been implicated in sapogenin biosynthesis, one such cholesterol-blocking agent, 2-(*p*-chlorophenoxy)-2-methylpropionic acid ethyl ester, was incubated with the whole fenugreek seed for up to 24 hr. In fact after 24 hr the sapogenin yield from this experiment was about 20 per cent greater than that from a control experiment incubated without the ester.

2-(*p*-Chlorophenoxy)-2-methylpropionic acid ethyl ester can be considered as a derivative of the herbicidal phenoxyacetic acids. Therefore two of these synthetic plant growth regulators were tested against the yield of sapogenin from the powdered dried tuber of *D. deltoidea*. Whilst 2,4,5-trichlorophenoxyacetic acid showed an increase of 9 per cent, after 24 hr, *p*-chlorophenoxyacetic acid was inactive at the concentration used.

The phenoxyacetic acids are thought to act like the natural plant auxin indole-3-acetic acid (IAA), possibly by prevention of the destruction of endogenous hormone. Hence it was postulated that the natural compound might be active and this was investigated first in the

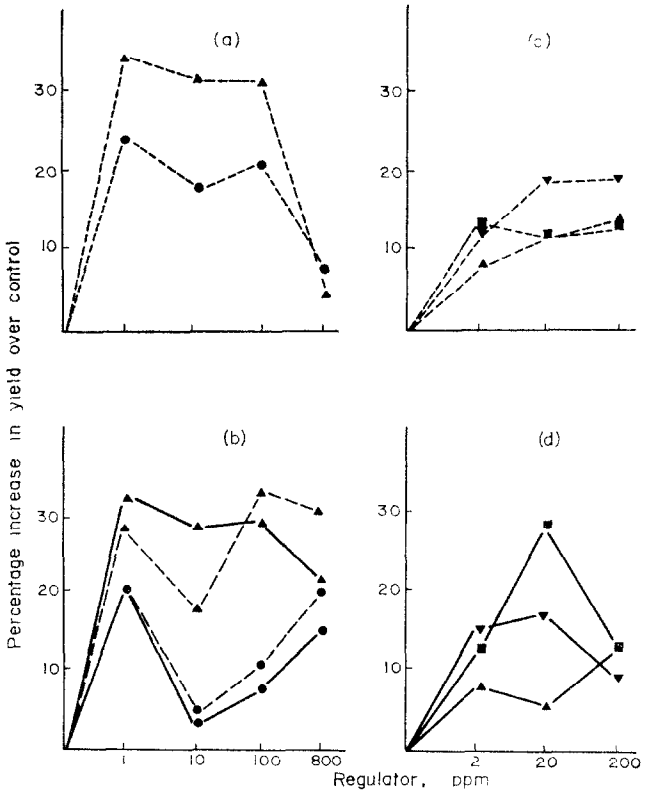

FIG. 1. INCREASE IN SAPOGENIN YIELD FROM SEED OF *T foenumgraecum* (a) AND (b) AND FROM TUBER OF *D. deltoidea* (c) AND (d) AFTER INCUBATION WITH PLANT GROWTH REGULATORS.
Samples were incubated, in an excess of water in the dark at 37°, for 6–72 hr in the presence of 1–800 ppm of natural plant growth regulators
Incubation time ● 6, ▲ 24, ▼ 48, ■ 72 hr
Regulator (a) and (c) — — — — IAA, (b) and (d) — — — — gibberellic acid (K salt), ——— gibberellin (A+X).

[6] R. M. HOCHSTER and J. H. QUASTEL, in *Metabolic Inhibitors. A comprehensive treatise*, Academic Press, London (1963).

seed system. In an incubation with *T. foenumgraecum* (Fig. 1a) as little as 1 ppm of IAA was sufficient to give rises in the total sapogenin of 25 per cent after 6 hr and 35 per cent after 24 hr. Increasing amounts of IAA eventually had a reverse effect.

In this seed system gibberellin (A+X) and gibberellic acid (K salt) showed bimodal responses after 6 hr, with maxima at 1 and 800 ppm (Fig. 1b). However, after 24 hr this double response was minimal although the overall increases were greater.

With the *D. deltoidea* tuber system and IAA the maximum increases were obtained after 48 hr with 20 and 200 ppm (Fig. 1c). At both 24 and 48 hr there was a tendency to increasing yield with rising concentration, whilst at 72 hr the yield was independent of concentration, but lower overall.

With gibberellin (A+X) in the tuber system there was a trend towards increasing yield with time but a concentration greater than 20 ppm was inhibitory (Fig. 1d). Incubation of the powdered dried tubers of a wild species of Mexican *Dioscorea* (probably *D. composita* Hemsl.) with 20 ppm gibberellin (A+X) produced an effect similar to that with *D. deltoidea* with a maximum increase of 20 per cent after 72 hr. Confirmation of these incubation increases in sapogenin yield was carried out by incubating larger samples of the same batch of powdered tuber of *D. deltoidea* with IAA and with gibberellin (A+X) and isolating the sapogenin. Increases of 7 per cent with 20 ppm IAA for 48 hr and of 35 per cent with 20 ppm gibberellin (A+X) for 72 hr incubation were obtained and the purity of the sapogenin isolated from these experiments was equal to that from the controls which had similar treatment at the same time but in the absence of the growth regulator.

The response to gibberellin (A+X) with the *D. deltoidea* tuber system at the two lower concentrations was dynamically different to the response to IAA, and this was probably due to a different mode or site of action. Increases in the sapogenin yield on incubation can arise by two main routes, (i) synthesis and (ii) release of pre-existent materal.

(i) Synthesis may be from a primary metabolite or from a close precursor. Synthesis of sapogenin from acetate-2-^{14}C, but not from mevalonate, has been shown in a homogenate of tuber of *Dioscorea floribunda*,[7] and synthesis of sapogenin from acetate-2-^{14}C has been demonstrated in both the powdered dried fruit wall and the defatted crushed seed of *Balanites orbicularis* Sprague using the incubation procedures described in this paper.[8] Joly *et al.*[9] have isolated Δ^5-furostene-3β-22, 27-triol-3β-chacotrioside-27β-*O*-glucopyranoside from *Dioscorea floribunda* and have shown that this can be cyclized by the plant to diosgenin.[10] In addition cholesterol,[11] or a closely related compound,[12,13] has been implicated in sapogenin biosynthesis.

The incubation of tuber with gibberellin (A+X) showed no maximum with respect of time and the sapogenin yield was still rising after 72 hr. However, there was a lag period of 24 hr before any increase was observed. It is possible that the increase in yield in the presence of gibberellin (A+X) may be due more to the synthesis of new sapogenin, rather than the release of pre-existent material, and that the lag period is the time taken for the synthesis of new enzymes or the activation of those already present.

(ii) Sapogenins exist in the plant mainly as their glycosides, the saponins, and the free

[7] E. Heftmann, R. D. Bennett and J. Bonner, *Arch. Biochem. Biophys.* **92,** 13 (1961).
[8] C. N. Wood, Ph.D. thesis, Bath (1970).
[9] R. A. Joly, J. Bonner, R. D. Bennett and E. Heftmann, *Phytochem.* **8,** 857 (1969).
[10] R. A. Joly, J. Bonner, R. D. Bennett and E. Heftmann, *Phytochem.* **8,** 1445 (1969).
[11] R. D. Bennett and E. Heftmann, *Phytochem.* **4,** 577 (1965).
[12] R. Tschesche, H. Hulpke and R. Fritz, *Phytochem.* **7,** 2021 (1968).
[13] R. A. Joly, J. Bonner, R. D. Bennett and E. Heftmann, *Phytochem.* **8,** 1709 (1969).

sapogenins are released only after enzymic or acid hydrolysis.[14] The glycosidic link may be to the 3β-position as in dioscin and also to the 27β-position as in the furostene named above.[9] Blunden and Hardman[14] investigated the parameters of 'fermentation' of tubers of *D. hondurensis* R. Knuth. They showed that the increase in sapogenin yield was an enzymic process and that the endogenous enzyme system could be replaced, at least in good part, by commercial cellulase and pectinase. Within the plant cell wall cellulose is not simply a homopolymer[15] and there can be covalent bonds to non-cellulosic sugars. These bonds could arise by specific enzymic action, or as a result of random chemical transglycosidation, and it is possible that the sugar part of a saponin could be incorporated into the cell wall in this manner.

The concentration of saponin in certain morphological parts may reach very high levels. For example the seeds of *Yucca brevifolia* contain up to 18 per cent saponins,[16] and the tubers of *Dioscorea composita* have yielded as much as 13 per cent of diosgenin (equivalent to over 30 per cent of the triglycoside dioscin.)[17] At these high levels they must exert some influence on the cell metabolism, unless they are removed, perhaps by binding with cell wall components.

Indole-3-acetic acid has been shown to have a variety of effects on intact plant tissue.[18] During expansion of the cell wall the increasing osmotic pressure in the cell stretches the wall and produces tension in the wall polymers. It has been suggested that IAA has a 'priming' action, by causing the breakdown of some of the polymer crosslinks, and the polymer then takes up a new form with new crosslinks formed in a less strained configuration. The effect of increasing concentrations of IAA on the yield of sapogenin from *D. deltoidea* indicates that there is a maximum value for the increased yield. It has been proposed that some of the sapogenin may be linked into the polymer structure of the cell wall via a glycosidic link and it is further suggested that IAA facilitates the release of this linked quantity of material by exposing it to the hydrolytic action of enzyme or acid.

It has been shown[19–23] that saponins and sapogenins have growth promoting activities. Hydrolysis of the saponins releases sugars into the cell causing a rise in osmotic pressure and this could contribute to cell expansion. However this does not explain the action of sapogenins unless these are converted first to saponins. The increased yield of sapogenin in the presence of added plant growth regulator may be merely a chance occurrence, unrelated to the normal metabolism of the intact tissue, but on the other hand the sapogenin/saponin level may be under the control of the naturally occurring plant growth regulators, which probably exist normally in the tissue.

EXPERIMENTAL

Plant Material

Dried tuber of *Dioscorea deltoidea* Wallich was obtained from Seth Panchhi Ram and Co., Kuth Growers, Manali, Kulu Hills, India and reduced to a coarse powder in a disintegrator. The Mexican sample of *Dios-*

[14] G. BLUNDEN and R. HARDMAN, *J. Pharm. Pharmac.* **15**, 273 (1963).
[15] S. M. SIEGEL, in *The Plant Cell Wall*, Pergamon Press, Oxford (1962).
[16] A. M. WOODBURY, M. E. WALL and J. J. WILLAMAN, *Econ Botany* **15**, 79 (1961).
[17] M. E. WALL, J. W. GARVIN, J. J. WILLAMAN, Q JONES and B. G. SCHUBERT, *J. Pharm. Sci.* **50**, 1001 (1961).
[18] J BONNER and J. E VARNER, in *Plant Biochemistry*, Academic Press, London (1965)
[19] J. BALANSARD and F. PELLESSIER, *Compt. Rend. Ser. Biol* **137**, 455, 461, 523 and 763 (1943), **139**, 1098 (1945).
[20] G. HELMKAMP and J. BONNER, *Plant Physiol.* **28**, 428 (1953).
[21] J. C. VENDRIG, *Nature* **203**, 1301 (1958).
[22] E. C. NORD and G. R. VANATTA, *Forest Sci.* **6**, 350 (1960).
[23] S. KELLER, *Z. Botan.* **48**, 32 (1960)

corea tuber was obtained from Laboratorios Julian de Mèxico, S.A. Whole seed of *Trigonella foenumgraecum* L., of Moroccan origin, was obtained from the London Spice Market.

Chemicals

2-(*p*-Chlorophenoxy)-2-methylpropionic acid ethyl ester was obtained from I.C.I. Pharmaceuticals, Macclesfield; the other compounds were of commercial origin.

Quantitation

The *Trigonella* samples were estimated by i.r. spectrometry (S.D. 2·6%)[24] and the *Dioscorea* samples by densitometric TLC (S.D. 1·1%).[25]

Incubation Procedures

In the control experiments duplicate samples of 5 g of seed or 2·5 g of tuber were incubated, in the dark at 37°, with 50 ml of water for the specified time before the addition of 10 ml conc. HCl and hydrolysis and extraction as reported previously.[24]

In the additive experiments the compound was dissolved in a suitable solvent and aliquots pipetted into flasks. The solvent was removed at low temperature on a rotary vacuum evaporator before the addition of the plant material and treatment as above.

Large Scale Experiments

For these 100 g of coarse powder of the same batch of dried tuber of *D. deltoidea*, of moisture content 5·5%, was used. Extraction was essentially similar to that for the small samples except that, before extraction with petrol, the acid insoluble residue was powdered in a hand mill so that all particles passed B.S.S. No. 10. The sapogenin was crystallized from the petrol extract after evaporation of the solvent to small volume and examined for purity by TLC, m.p. and i r. spectral data. All the products were mixtures of diosgenin (> 90%) together with its C_{25}-epimer yamogenin and traces of spirostadienes.

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[24] K. R. BRAIN, F. R. Y. FAZLI, R. HARDMAN and A. B. WOOD, *Phytochem.* **7**, 1815 (1968).
[25] K. R. BRAIN and R. HARDMAN, *J. Chromatog.* **38**, 355 (1968).