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THE EFFECT OF INCUBATION WITH CHLORINATED PHENOXY ACIDS ON THE YIELD OF STEROIDAL SAPOGENIN FROM THE FRUIT PARTS OF *BALANITES ORBICULARIS*

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Abstract—Incubation of the dried powdered fruit wall and the partially defatted seed of *Balanites orbicularis* Sprague (Balanitaceae) with water and chlorinated phenoxy acids, increased their yield of diosgenin and yamogenin by up to 20 per cent. The process was time and concentration dependent and close similarities were found between those phenoxy acids causing an increase in sapogenin yield and those with known auxin-like activity. No evidence was obtained from experiments with acetate-2-^{14}C to suggest that the increases in yield were caused by the biosynthesis of sapogenin. An investigation of the saponin content of the fruit wall showed that the phenoxy acid increased the endogenous saponase activity during the incubation.

INTRODUCTION

THE FRUITS of *B. orbicularis* Sprague (Balanitaceae) have been shown to contain commercially attractive quantities of diosgenin and yamogenin in the seed and in the fruit wall. Incubation of these powdered fruit parts with excess water at 37° afforded an additional sapogenin yield.[1] We have sought compounds,[2,3] which would further increase the yield of sapogenin by their influence on the incubation (fermentation) stage of the industrial procedure used for the extraction of sapogenin from plant material. Incubation with gibberellin and auxin-like compounds was shown to be an effective means of increasing the yield of sapogenin from dried, coarsely powdered plant material.[4] Antihypercholesteremics such as Atromid-S were also effective.[4,5] Considering these interesting links between sterol metabolism in animals, plant growth regulatory action and increase in steroidal sapogenin yield during incubation, the effect of a series of chlorinated phenoxy acids on the sapogenin yield of the fruit parts of *B. orbicularis* was investigated.

RESULTS AND DISCUSSION

The effect of aqueous incubation with penoxy acid on the sapogenin content of the powdered fruit wall of *B. orbicularis* was investigated using an i.r. method[6] to determine the sapogenin yields. Each compound was tested at three concentration levels (2×10^{-4},

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[1] R. HARDMAN and C. N. WOOD, *Phytochem.* **10**, 887 (1971).
[2] R. HARDMAN to National Research Development Corporation, London, Brit. Pat. 1136626 (1968).
[3] R. HARDMAN to National Research Development Corporation, London, Brit. Pat. Appl. 36711 (1966) and 39765 (1967).
[4] K. R. BRAIN and R. HARDMAN, *Phytochem.* in press (1971).
[5] C. N. WOOD, Ph.D. thesis, Bath (1970).
[6] K. R. BRAIN, F. R. Y. FAZLI, R. HARDMAN and A. B. WOOD, *Phytochem.* **7**, 1815 (1968).

2×10^{-5} and 2×10^{-6} M). The results were compared with control experiments performed at the same time in which the sapogenin yield rose steadily to a maximum after 48 hr incubation (Fig. 1) and beyond this time a further increase was not obtained. Of the compounds used *o*-chlorophenoxyacetic acid, *o*-nitrophenoxyacetic acid and 4-(2,4-dichlorophenoxy)butyric acid (2,4-Cl_2POB) had no significant effect on the sapogenin yield. *p*-Chlorophenoxyacetic acid (4-ClPOA), 2,4-dichlorophenoxyacetic acid (2,4-Cl_2POA), 2,4,5-trichlorophenoxyacetic acid (2,4,5-Cl_3POA) and 2,4,5-trichlorophenoxypropionic acid (2,4,5-Cl_3POP) all caused an increase in the maximum sapogenin yield by up to 20 per cent.

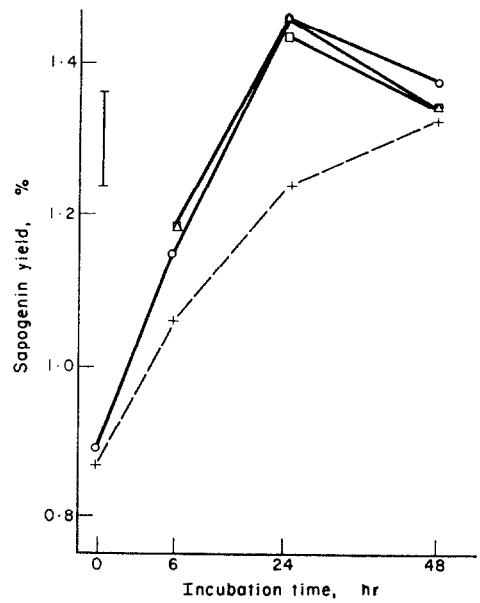

FIG. 1. THE EFFECT OF INCUBATION WITH 2,4,5-TRICHLOROPHENOXYACETIC ACID ON THE SAPOGENIN YIELD OF THE FRUIT WALL OF *B. orbicularis*.

Samples of fruit wall were incubated with water at 37° and sapogenin yields were estimated using the i.r. method.[6]

(+ — — —) Controls, (○————) Phenoxy acid concentration 2×10^{-4} M, (△————) Phenoxy acid concentration 2×10^{-5} M, (□————) Phenoxy acid concentration 2×10^{-6} M, (I) Minimum significant range.

The three concentration levels of 2,4,5-Cl_3POA investigated were equally effective for increasing the sapogenin yield (Fig. 1). In contrast the most effective concentration of the other phenoxy acids was 2×10^{-6} M. This is apparent when the averages of the results for 4-ClPOA, 2,4-Cl_2POA and 2,4,5-Cl_3POP, at each concentration level, are compared with the control experiments (Fig. 2). The results for each separate compound were similar to the averaged results. Higher concentrations of these phenoxy acids (2×10^{-5} and 2×10^{-4} M) did not significantly change the usual increase in sapogenin yield obtained by incubation alone. The low effective concentrations of phenoxy acid encountered in this work are typical of auxin-like activity and it is interesting to note that higher concentrations

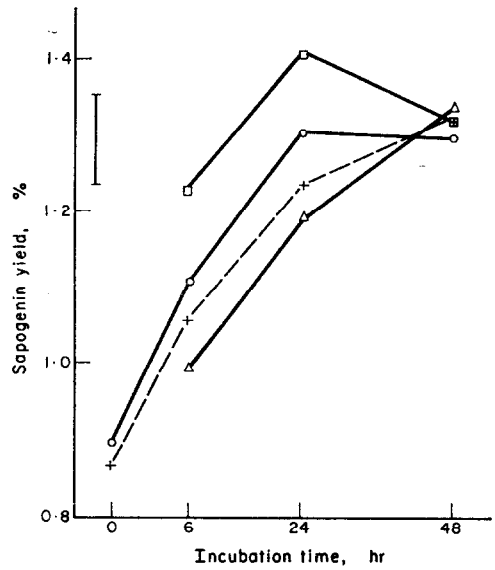

FIG. 2. THE EFFECT OF INCUBATION WITH PHENOXY ACIDS ON THE SAPOGENIN YIELD OF THE FRUIT WALL OF *Balanites orbicularis*.

The graphs represent the average of the results for *p*-chlorophenoxyacetic acid, 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxypropionic acid. Samples of fruit wall were incubated with water at 37° and sapogenin yields were estimated using the i.r. method.[6]

(+ — — —) Controls, (○————) Phenoxy acid concentration 2×10^{-4} M, (△————) Phenoxy acid concentration 2×10^{-5} M, (□————) Phenoxy acid concentration 2×10^{-6} M, (I) Minimum significant range.

of auxin have been shown to be ineffective.[7] The maximum sapogenin yields were obtained after 24 hr of incubation with phenoxy acid and these were greater than the maximum yields obtained after 48 hr of incubation without growth regulator (Fig. 1). In the presence of phenoxy acid the sapogenin yield fell back after 48 hr incubation to a level similar to that of the control experiments. It is unlikely that this was due to the degradation of the phenoxy acid because when 2,4,5-Cl_3POA was intermittently added (after 6, 12 and 24 hr of incubation) to the fruit wall incubation mixture, no additional sapogenin was obtained after 48 hr of incubation. This fall back in sapogenin yield may reflect an attempt to normalise the spirostan concentration in the plant system by the metabolism of sapogenin equivalent in amount to that formed in the presence of phenoxy acid. In three subsequent experiments incubation of the fruit wall of *B. orbicularis* for 24 hr with 2,4,5-Cl_3POA (2×10^{-5} M) afforded 8, 13 and 20 per cent of additional sapogenin.[5] In each case TLC examination showed that this consisted of diosgenin and yamogenin as obtained in the control experiments. Changes, not marked, in the relative proportions of diosgenin and yamogenin did occur but these could not be correlated with increased sapogenin yields.

The response of the sapogenin yield to 2,4,5-Cl_3POA was not limited to the samples of fruit wall. Incubation of the partially defatted seed from the same batch of fruits of *B. orbicularis* with 2,4,5-Cl_3POA for 24 hr afforded a 12 per cent increase in the maximum sapogenin yield.

[7] A. C. LEOPOLD, *Plant Growth and Development*, McGraw-Hill, New York (1964).

There appears to be a close connection between those compounds exhibiting auxin-like activity and those effecting an increase in sapogenin yield. The auxin-like activity of 79 different phenoxy acids was investigated by Åberg who found that substitution of a chlorine atom in the *para* position of the benzene ring augmented such activity.[8] The *ortho* substituted isomer was shown to have only weak auxin-like activity. Incubation of the fruit wall of *B. orbicularis* with *o*-chlorophenylacetic acid, 2,4-dichlorophenol and 3-hydroxypropionic acid had no significant effect on the sapogenin yield. Thus it would appear that both the side chain and the aromatic moiety of the phenoxy acid are necessary for increase in sapogenin yield.

The exact mode of action of auxin in plants is not yet known but it seems likely that plant growth regulators influence the synthesis of nucleic acid and hence the mechanisms by which enzymes are made in the cell.[9,10] In this way auxin is potentially capable of controlling most of the metabolic processes of the cell. It is not inconceivable that the induction of enzymes could lead to the increased biosynthesis of sapogenin. Alternatively a mechanism involving the enzyme release of pre-existent bound sapogenin could be involved.

The Role of Enzyme

Samples of the powdered fruit wall of *B. orbicularis* were suspended in water and immediately autoclaved at 110° for 30 min, a process sufficient to destroy the enzyme present in the plant tissue. The sapogenin yields of the autoclaved mixtures, determined after incubation without and with 2,4,5-Cl_3POA (2×10^{-4} M), were virtually identical and little different from the yield of a non-autoclaved, non-incubated sample of fruit wall (1·05 per cent). These observations confirm the role of enzyme in increasing the yield of sapogenin during incubation without and with phenoxy acid. Auxin is capable of causing cell wall expansion within a period of 30 min[11] but in the case of dried powdered plant material, used in these experiments, the additional increase in sapogenin yield was achieved only after up to 24 hr of incubation. A latent period for the activation of the metabolic systems in the dried powdered plant material on the addition of water is not unreasonable whether auxin is absent or present.

The Role of Synthesis

Samples of the powdered fruit wall of *B. orbicularis* were incubated with water containing acetate-2-^{14}C. Of the total activity fed 5·5 per cent was recovered in the petroleum-soluble crude sapogenin extract of the control experiment whereas the sapogenin extract from the phenoxy acid treated tissue retained 7·0 per cent. The sapogenin fractions were isolated and purified to constant activity by acetylation and recrystallisation (Table 1). These incorporations demonstrated the biosynthesis of lipophilic material. The specific activity of the sapogenin isolated from the phenoxy acid treated fruit wall was very similar to that isolated in the control experiment. In a similar experiment samples of partially defatted seed, prepared from the same fruits of *B. orbicularis* were incubated with acetate-2-^{14}C for 48 hr. Once again the specific activity of the sapogenin isolated from the 2,4,5-Cl_3POA treated tissue (13·8 counts/min/μmole) was the same as that from the corresponding control experiment (Table 1). Incubation of the fruit wall of *B. orbicularis* with choles-

[8] B. ÅBERG, in *Plant Growth Regulators*, p. 219, Iowa State University Press (1963).
[9] L. D. NOODEN, *Plant Physiol.* **43**, 140 (1968).
[10] A. W. GALSTON and P. J. DAVIES, *Science* **163**, 1288 (1969).
[11] Y. MASUDA, *Plant Cell Physiol.* **10**, 1 (1969).

TABLE 1. THE EFFECT OF 2,4,5-TRICHLOROPHENOXYACETIC ACID ON THE METABOLISM OF ACETATE-2-^{14}C DURING INCUBATION OF THE FRUIT PART OF *Balanites orbicularis*. RECRYSTALLISATION OF THE SAPOGENIN ACETATE TO CONSTANT ACTIVITY

	Activity of the sapogenin, counts/min/μmole			
	From the fruit wall		From the seed	
Solvent	Control	With phenoxy acid	Control	With phenoxy acid
Acetone	4·61 ± 0·07	4·44 ± 0·07	78·3 ± 1·2	60·4 ± 0·9
Acetone	2·22 ± 0·03	2·93 ± 0·04	20·2 ± 0·3	17·1 ± 0·3
Acetone	2·37 ± 0·04	2·41 ± 0·04	16·2 ± 0·2	13·4 ± 0·2
Hexane	2·39 ± 0·04	2·45 ± 0·04	14·9 ± 0·2	13·7 ± 0·2
Hexane	—	—	13·8 ± 0·2	13·3 ± 0·2
Methanol	—	—	13·8 ± 0·2	13·8 ± 0·2

terol-4-^{14}C and with cholestenone-4-^{14}C did not afford labelled sapogenin whether phenoxy acid was present or absent. Thus no evidence was obtained to suggest that the auxin-induced increase in sapogenin yield was effected by an increased biosynthesis of spirostan. The synthesis of sapogenin from labelled acetate has been demonstrated using homogenates of fresh tuber of *Dioscorea floribunda*[12] but the present work shows that a low rate of synthesis of sapogenin does occur during the incubation of dried powdered plant material with water.

The Effect of Phenoxy Acid on Enzyme

The sapogenin affording endogenous enzyme of *Yucca glauca* can be largely replaced by commercial cellulase and by pectinase.[13] Hardman and Sofowora[14] have obtained similar results with the fruit wall of *B. aegyptiaca* and proposed that the wall contains a type of saponin which can only be released by cell wall disrupting enzymes. Much time has been devoted to investigating the effect of auxin on cell wall expansion and hence growth, and auxin has been shown to cause the induction of cellulase,[15] pectinase[16] and pectinesterase.[17] These cell wall degrading enzymes cause the breakdown of some cell wall component leading to cell wall loosening and hence to the initiation of cell elongation. It is possible that disruption of the cell wall, under the influence of auxin-like compounds, could result in the release of bound saponin and thus account for the increase in sapogenin yield.

Steroidal sapogenins exist in the plant mainly as their glycosides, the steroidal saponins and the free aglycones are isolated only after enzymic or acid hydrolysis. Consequently the amount of free sapogenin present in plant material could give a measure of the extent of saponase activity. All of the spirostan contained in a methanol extract of the fruit wall of *B. orbicularis* was shown, by TLC, to be in the form of saponin. After incubation of samples of the fruit wall of *B. orbicularis*, the tissue mixtures were filtered and not refluxed with 2N acid. The spirostan contained in the filtrate as saponin accounted for between 10 and 20 per

[12] E. HEFTMANN, R. D. BENNETT and J. BONNER, *Arch. Biochem. Biophys.* **92**, 13 (1961).
[13] G. BLUNDEN, R. HARDMAN and W. R. WENSLEY, *J. Pharm. Pharmac.* **17**, 274 (1965).
[14] R. HARDMAN and E. A. SOFOWORA, *Planta Med.* in press.
[15] A. H. DATKO and G. MACLACHLAN, *Plant Physiol.* **43**, 735 (1968).
[16] E. TANIMOTO and Y. MASUDA, *Physiol. Plantarum*, **21**, 820 (1968).
[17] M. J. MACEY, *Physiol. Plantarum*, **18**, 368 (1965).

cent of the total spirostan obtained from the fruit wall by incubation and acid hydrolysis. The remaining spirostan in the filter cake was extracted with methanol and consisted of saponin and free sapogenin. It is reasonable to assume that the free sapogenin was precipitated after the action of endogenous saponase. The ratio of saponin–sapogenin was estimated using densitometric TLC. In the control experiments (no phenoxy acid present) the ratio (of areas beneath the densitometric peaks) of saponin to sapogenin was 1:0·75 after 24 hr incubation, and 1:1·8 after 48 hr incubation. Incubation with 2,4,5-Cl_3POA gave the corresponding ratios of 1:1·7 and 1:2·3. Thus the phenoxy acid caused an increase in free sapogenin within 24 hr incubation and the effect was maintained at 48 hr incubation. This would indicate that phenoxy acid also promotes saponase activity during incubation.

The present work shows that the level of extractable steroidal sapogenin in the plant is influenced by exogenous growth regulator under *in vitro* conditions. This probably reflects the potential of plant growth regulator for controlling the steroid metabolism of the plant cell. The influence of chlorinated phenoxy acids on other related products of secondary metabolism in sapogenin-affording tissue is being further investigated.

EXPERIMENTAL

Plant Material

The separated whole seed and fruit wall were from the dried ripe fruits obtained from the Conservator of Forests, Entebbe, Uganda and authenticated after consultation with the East African Herbarium in Nairobi. Prior to use in experiments the seed was crushed and partially defatted by extraction in a soxhlet for 2 hr with light petroleum (b.p. 40–60°) so as to reduce the fixed oil content to about 10%. Plant material was then comminuted so that all of the powder used passed a No. 8 B.S. sieve. All yields are expressed on a moisture free basis.

Incubation Procedure

In the control experiments duplicate samples, 5 g, of either fruit wall or partially defatted seed were incubated at 37° with 100 ml water for the specified time. Afterwards 22 ml conc. HCl was added and the hydrolysis and extraction completed as previously described.[6] When phenoxy acid was added at the start of the incubation the required aliquots of an acetone solution of the compound were pipetted into the flasks. The acetone was removed under vacuum leaving the phenoxy acid as a thin film across the base of the flask. The plant material was added and the incubation and work up completed as just described. Phenoxy acid added after the start of incubation was introduced as aqueous doses sufficient to give a concentration of 2×10^{-5} M in 100 ml water. The crude sapogenin extracts were examined by TLC using hexane–EtAc as the solvent system.

Quantitation

The sapogenin yields were estimated by i.r. spectroscopy[6] (S.D. = 2·9%[5]). The significance of individual results was tested by the calculation of a minimum significant range from the residual variance estimate of the duplicate results (see Figs. 1 and 2).[18]

Tracer Experiments

Samples of powdered fruit wall, 20 g, were incubated for 48 hr with 15 μc of sodium acetate-2-^{14}C using the extraction and estimation methods described above. The sapogenin yield of the 2,4,5-Cl_3POA (2×10^{-4} M) treated tissue was 10 per cent higher than that of the control experiment. Aliquots of the crude sapogenin extracts were dissolved in toluene and used for scintillation counting. The remainder of the extracts were fractionated on alumina columns and the samples of crude sapogenin so obtained were acetylated and recrystallised to constant activity as shown in Table 1. Sapogenin samples, 8–10 mg, were counted in Analar toluene containing 41 mg P.P.O and 1 mg P.O.P.O.P. The efficiency of ^{14}C scintillation counting was 94·5 ± 1%. Pure sapogenin did not cause quenching but the quenching of crude sapogenin samples was corrected after counting the samples without and with a known quantity of reference hexadecane-1-^{14}C.

In a similar experiment 10 g samples of the partially defatted seed of *B. orbicularis* were incubated with 15 μc of sodium acetate-2-^{14}C. The work up of the sapogenin to constant activity is illustrated in Table 1.

[18] A. GOLDSTEIN, *Biostatistics*, Macmillan, New York (1964).

The Investigation of the Saponin Content

Powdered fruit wall, 10 g, was extracted with MeOH in a soxhlet for 24 hr. This saponin extract was examined by TLC on silica gel plates using butanol saturated with water as the solvent system. Spots were located by spraying with a 300%, w/v solution of $SbCl_3$ in conc. HCl. Duplicate samples, 10 g, of powdered fruit wall were incubated with 2,4,5-Cl_3POA (2×10^{-4} M) for 24 hr and 48 hr. The tissue suspensions were filtered and the filter cake was washed with water. Sufficient conc. HCl was added to the filtrate and washings to give a strength of 2N and the mixture was worked up and estimated for sapogenin as described above. The filter cake was dried in vacuo over silica gel and the powdered material was exhausted with MeOH in a soxhlet. The resulting solution of sapogenin and saponin was made up to 50 ml with MeOH and examined by densitometric TLC.[19] An aliquot of the methanolic extract was refluxed with 2N HCl and the sapogenin was recovered and estimated as before.

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[19] K. R. Brain and R. Hardman, *J. Chromatog.* **38**, 355 (1968).