Biochem. J. (1972) **128**, 491–497
Printed in Great Britain

The Formation of Glucosides of Isoflavones and of Some Other Phenols by Rabbit Liver Microsomal Fractions

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(*Received* 17 *January* 1972)

1. Rabbit liver microsomal fractions *in vitro* effected the transfer of glucuronic acid from UDP-glucuronic acid to biochanin A, formononetin, daidzein, genistein and equol. Only monoglucuronides were formed. 2. The same isoflavones were converted into monoglucosides when UDP-[6-^{3}H]glucose was substituted for UDP-glucuronic acid in the incubation medium *in vitro*. The glucosides were formed in much lesser yield than were the glucuronides. 3. The glucoside of genistein was identified as genistin (genistein 7-glucoside) by Sephadex chromatography and reverse isotope dilution. 4. The specificity of the glucuronyl- and glucosyl-transfer mechanisms was compared for a series of steroids and other phenols in addition to the isoflavones. It was concluded that separate transferases were responsible for the formation of the two types of glycosides.

A wide variety of phenolic and alcoholic compounds form glucuronides when incubated, in the presence of UDP-glucuronic acid, with microsomal fractions from animal tissues (Dutton, 1966). Under appropriate conditions *in vitro*, liver and kidney tissue from rabbits and from humans can also effect the transfer of glucose, galactose and *N*-acetylglucosamine from their respective uridine nucleotides to hydroxyl groups on steroid molecules (Collins *et al.*, 1970; Cable *et al.*, 1970; Williamson & Layne, 1971). The transfer of *N*-acetylglucosamine has thus far been demonstrated only to alcoholic hydroxyl groups in ring D of the steroid, but glucose and galactose may be transferred to either a ring-D alcoholic group or to the phenolic 3-hydroxyl group of the steroid oestrogens (Williamson *et al.*, 1971). In all the reported cases the sugar transferred and the acceptor group on the steroid were remarkably specific for the species studied, and it seems probable that each reaction is mediated by a specific transferase. In an attempt to discover whether or not these glycosyl-transfer reactions were specific for steroids, we tested a series of other biologically interesting phenols and alcohols as substrates in a system from rabbit liver *in vitro*. Of these, the isoflavones biochanin A (5,7-dihydroxy-4′-methoxyisoflavone), genistein (4′,5,7-trihydroxyisoflavone), formononetin (7-hydroxy-4′-methoxyisoflavone), daidzein (4′,7-dihydroxyisoflavone) and the isoflavone derivative equol [7-hydroxy-3-(4′-hydroxyphenyl)-chroman], as well as diethylstilboestrol and phenolphthalein, were found to form glucosides. The present paper reports a study of this reaction, and the results of attempts to form other glycosides of these materials *in vitro* with several rabbit tissues and with sheep liver.

Experimental

Materials

Isoflavones labelled with ^{14}C in position 4 were obtained from Professor R. H. Common, McGill University, Montreal, Que., Canada, and were the materials described by Tang & Common (1968). The specific radioactivities were determined in this laboratory by the radioactive assay of weighed samples, and were as follows (μCi/mg): biochanin A, 0.06; genistein, 0.02; daidzein, 0.38; formononetin, 0.05; equol, 0.02. Steroids, uridine nucleotides, chromatography materials, solvents and other reagents were those used in previous work (Collins *et al.*, 1970). Rabbits were mature female New Zealand Whites, and the sheep was a mature male crossbred.

Methods

General methods. Isoflavones were separated on t.l.c. in the systems used by Tang & Common (1968). The separation of glucuronides and glucosides was carried out in the systems described in detail by Collins *et al.* (1968) and Collins & Layne (1969). Radioactivity was measured by liquid-scintillation counting in a Nuclear-Chicago Unilux II spectrometer. The scintillation mixture consisted of 4g of 2,5-diphenyloxazole, 100ml of Bio-Solv BBS-3 (Beckman Instruments, Palo Alto, Calif., U.S.A.) and 900ml of toluene. Samples were counted for radioactivity in 10ml of this scintillator.

Determination of glycoside formation. The procedure for the preparation of tissue homogenates and of microsomal fractions, and for the incubation of isoflavones with microsomal fractions, was the

same as that employed for steroids by Collins *et al.* (1970). The isoflavones, in amounts of 0.2–0.3 μmol, were added to the incubation tubes in methanol, and the solvent was removed under a stream of nitrogen. A solution of 0.5 μmol of the appropriate nucleotide in 2.0ml of 0.5M-tris–HCl buffer, pH8.0, and 0.3ml of microsomal suspension were successively added to the tubes, which were then incubated in a shaking water bath at 37°C for 1h. The contents of the tubes were extracted first with 5ml of benzene and then with 5ml of ethyl acetate.

In earlier work on steroids (Whittemore & Layne, 1965; Collins *et al.*, 1968) the measurement of the extent of glycoside formation was based on the fact that the steroid glycosides were not extracted from aqueous solution by chloroform, whereas the unconjugated steroid aglycones partitioned practically completely into chloroform from aqueous incubation media. The assay of the chloroform extract for radioactivity in the labelled aglycones, or by colorimetry or spectrophotometry in other cases, gave a measure of the amount of aglycone that was not conjugated, and, by difference, the amount of glycoside formed. This technique has been used repeatedly in this laboratory, although in more recent work (Collins *et al.*, 1970; Mellor & Layne, 1971) benzene was substituted for chloroform in the initial extraction. In the present work, this procedure was found reliable for diethylstilboestrol and *p*-nitrophenol, as well as for biochanin A, formononetin and equol. The more polar isoflavones daidzein and genistein, however, were not completely removed from the aqueous media by benzene, and assessment of the extent of glycoside formation by these compounds was made from thin-layer chromatograms of the ethyl acetate extract as described below.

Extraction of the incubation media with ethyl acetate, at the pH of the buffer for glucosides, galactosides or *N*-acetylglucosaminides, and at pH2.0 for glucuronides (Collins *et al.*, 1968), removes the intact glycosides. T.l.c. of both the benzene and the ethyl acetate extracts on silica gel H (E. Merck, Darmstadt, Germany) was employed as a check on the incubations by establishing whether the materials in the extracts were respectively unconjugated aglycone and glycoside. The t.l.c. systems employed for glucosides and glucuronides were respectively chloroform–methanol (89:11, v/v) and chloroform–propan-2-ol–formic acid (5:3:1, by vol.). Preliminary experiments carried out by this procedure provided evidence for the formation of glucuronides and glucosides, but not of galactosides or *N*-acetylglucosaminides by the isoflavones. Further investigation of glucoside formation was carried out by the modified procedure described below.

Improved assay for glucoside formation. Apart from the partition difficulties encountered with daidzein and genistein, the sensitivity of the assay for glycoside formation as described above was limited by the specific radioactivity of the labelled aglycones, which for the isoflavones was low, and by the sensitivity of the colorimetric assay for *p*-nitrophenol (Patel & Tappel, 1969). The availability of UDP-D-[6-^{3}H]glucose of high specific radioactivity (5.1Ci/mmol; Amersham/Searle Corp., Chicago, Ill., U.S.A.) permitted the incorporation of this material, diluted as required with the unlabelled nucleotide, into the incubation medium. Glucoside formation could therefore be measured as the amount of ^{3}H-labelled glycoside formed rather than by difference from the amount of labelled aglycone that remained after the incubation. The extraction of ^{3}H with ethyl acetate after the incubations was found to be a reliable measure of glycoside formation. Labelled glucose and excess of UDP-glucose remained in the aqueous medium. However, both the ethyl acetate extracts and the aqueous residues were examined by t.l.c. as a final check on the assay procedure.

The evidence for the formation of the glucosides shown in Tables 1 and 2 was obtained with labelled UDP-glucose. The incubation procedure was identical with that already described, except that the UDP-glucose added to each tube had a specific radio-

Table 1. *Transfer of glucuronic acid and of glucose from uridine nucleotides to various compounds by rabbit liver microsomal fractions*

For experimental details see the text. +, Formation of glycoside; –, no demonstrable formation of glycoside.

	Glucuronide	Glucoside
Oestradiol-17α	+	+
Oestradiol-17α 3-glucuronide	–	+
Biochanin A	+	+
Genistein	+	+
Formononetin	+	+
Daidzein	+	+
Equol	+	+
Diethylstilboestrol	+	+
p-Nitrophenol	+	–
Phenolphthalein	+	+
Epitestosterone	+	–
Testosterone	+	–
Cortisol	+	–
Oestriol	+	–
2,4-Dihydroxyphenyl *p*-methoxybenzyl ketone	+	+
2,4-Dihydroxyphenyl *p*-hydroxybenzyl ketone	+	+

Table 2. *Formation of conjugates of isoflavones by microsomal fractions from various tissues*

For experimental details see the text. Results were the same with biochanin A, formononetin, daidzein, genistein and equol. +, Formation of glycoside; −, no demonstrable formation of glycoside.

Source of microsomal fractions	Nucleotide added to incubation medium: UDP-glucuronic acid	UDP-glucose	UDP-*N*-acetyl glucosamine	UDP-galactose
Rabbit				
Liver	+	+	−	−
Kidney	−	−	−	−
Small intestine	+	−	−	−
Large intestine	−	−	−	−
Sheep				
Liver	+	−	−	−

activity of 10Ci/mol. For the [^{14}C]isoflavones, strong evidence for glucoside formation was provided by the presence of both ^{14}C and ^{3}H in the glycoside. Recrystallization experiments were based on ^{3}H; the ^{14}C contribution to the ^{3}H counts was measured in each case and was negligible at the ^{3}H/^{14}C ratio used, which was approx. 250:1.

Enzymic hydrolysis of conjugates. The identity of presumptive glucuronides and glucosides obtained by incubation with tissue microsomal preparations was further examined by subjecting the conjugates to hydrolysis by β-glucuronidase (Ketodase; Warner–Chilcott Labs., Morris Plains, N.J., U.S.A.) or β-glucosidase (almond emulsin; Sigma Chemical Co., St. Louis, Mo., U.S.A.). The release of the aglycone by the appropriate enzyme, and the inhibition of this hydrolysis by saccharo-1,4-lactone for glucuronides and by glucono-1,5-lactone for glucosides, was taken as evidence for the identity of these conjugates. The procedures for carrying out the hydrolysis and inhibition experiments were those described in detail by Williamson & Layne (1970).

Preparation of genistin. Genistin (4′,5,7-trihydroxyisoflavone 7-β-D-glucopyranoside) was prepared by extracting commercial soya-bean meal with methanol as described by Walter (1941). Pale-yellow plates were obtained on crystallization from 80% (v/v) ethanol. The material was recrystallized three times from the same solvent.

Sephadex chromatography of isoflavone glucosides. Gel filtration on Sephadex, by the procedure described for other plant glucosides by Repas *et al.* (1969), was used to provide preliminary evidence for the identity of the radioactive glucoside of genistein with authentic genistin. For this purpose 129500d.p.m. of the material formed by the incubation of genistein with rabbit liver microsomal fractions in the presence of UDP-[6-^{3}H]glucose was mixed with 1130d.p.m. of the glucoside of daidzein obtained in a similar way. These two glucosides were distinguishable from each other by the ^{3}H/^{14}C ratio, which was 250:1 for the genistein glucoside and 49:1 for the daidzein glucoside. The radioactive materials were mixed with 1mg of authentic genistin obtained from soya-bean meal and the mixture was applied to a column (0.9cm×55cm) of Sephadex G-25. The sample was applied in water–methanol (9:1, v/v) and the column was eluted with the same solvent. Fractions (2.5ml) were collected. The radioactivity present in each fraction was measured. The E_{258} of each fraction was also measured, and the weight of genistin was calculated by comparison with a linear standard curve established with crystalline genistin.

Identification of genistin by isotope dilution. A mixture of 18.55mg of crystalline genistin and 188727d.p.m. of the tritiated genistein glucoside was acetylated as described by Walter (1941). The product was crystallized three times from ethyl acetate–hexane (2:1, v/v). After each crystallization, the crystals were dried for 5h under vacuum, and a sample of approx. 1mg was removed. This sample was accurately weighed and the ^{3}H content was assayed. The specific radioactivity of the crystals was determined from these measurements.

Results

Formation of glucuronides and glucosides

Glucuronides. In addition to a series of other compounds (Table 1), biochanin A, genistein, formononetin, daidzein and equol gave evidence for the formation of glucuronides when incubated with rabbit liver microsomal fractions in the presence of UDP-glucuronic acid. No formation of conjugates was observed when UDP-glucuronic acid was omitted

from the incubation medium. The products all behaved as monoglucuronides on t.l.c. on silica gel H in chloroform–propan-2-ol–formic acid (5:3:1, by vol.). This system has been used to distinguish mono- and di-conjugates of steroids (Collins *et al.*, 1968). The incubation of the conjugates with β-glucuronidase (Ketodase) led to the release of the respective aglycones. This hydrolysis was almost completely inhibited by saccharo-1,4-lactone at a concentration of 5mg/ml (Williamson & Layne, 1970).

Glucosides. The substitution of UDP-glucose for UDP-glucuronic acid in the incubation medium led to the formation of conjugates of the isoflavones and of diethylstilboestrol and phenolphthalein (Table 1). These behaved as monoglucosides on t.l.c. in chloroform–methanol (89:11, v/v). No evidence for the formation of diglycosides was obtained, and no conjugates were formed when UDP-glucose was omitted from the incubation medium. The conjugates were hydrolysed by almond emulsin, and this hydrolysis was inhibited by glucono-1,5-lactone (Williamson & Layne, 1970).

Other glycosides. No evidence was obtained for the formation of galactosides or *N*-acetylglucosaminides of the isoflavones or of the other compounds listed in Table 1, with the exception of oestradiol-17α (Williamson *et al.*, 1971).

Glycoside formation by various tissues

Microsomal fractions prepared from several rabbit tissues and from sheep liver were tested for their ability to form glycosides of the isoflavones. The results (Table 2) show that rabbit liver and small intestine, as well as sheep liver, formed isoflavone glucuronides, whereas glucosides were formed only by rabbit liver. None of the tissues formed galactosides or *N*-acetylglucosaminides of the isoflavones. These results were the same whether biochanin A, genistein, formononetin, daidzein or equol was the substrate used.

Quantitative comparison of glucuronide and glucoside formation

The assays for glycoside formation gave results on duplicate samples that were reproducible to within ±5%. This permitted the estimation, with reasonable confidence, of the amounts of isoflavone glucuronides and glucosides formed in parallel experiments with the same microsomal preparation from rabbit liver. The results are shown in Table 3. The amounts of glucuronide formed were in all cases much larger than those of glucoside. In each of these experiments portions of the ethyl acetate extracts obtained after incubation, as well as of the residual aqueous layer, were subjected to t.l.c. In no case was radioactive material found at an R_F value other than those of the unchanged isoflavone or the appropriate glycoside. This indicated that the results in Table 3 represent the specific measurement of glycoside formation.

Sephadex chromatography

The separation of the radioactive glucosides of daidzein and genistein is shown in Fig. 1. The material eluted between fractions 27 and 35 of the Sephadex G-25 chromatogram had a $^3H/^{14}C$ ratio (46:1) that was almost the same as that of the daidzein glucoside applied. The genistein glucoside ($^3H/^{14}C$ ratio >220:1) was eluted between fractions 40 and 67, and coincided with the authentic genistin as measured by u.v. spectroscopy. Two peaks eluted in the first 25 fractions contained ^3H but no detectable ^{14}C. They were believed to be breakdown products of [^{3}H]glucose, but were not further studied.

Recrystallization of acetylated genistin

Table 4 shows the results of the recrystallization of the acetylated mixture of radioactive genistein glu-

Table 3. *Comparison of the rates of formation of glucuronides and glucosides of isoflavones by rabbit liver homogenates*

Substrate	Conjugate formed (nmol/h per mg of protein)	
	Glucuronide	Glucoside
Biochanin A	80	2.3
Genistein	55	3.0
Formononetin	74	1.3
Daidzein	39	0.5
Equol	100	0.7

Table 4. *Recrystallization of an acetylated mixture of authentic genistin (4',5,7-trihydroxyisoflavone 7-β-D-glucopyranoside) and tritiated conjugate formed by the incubation of genistein and UDP-[6-^{3}H]glucose with rabbit liver microsomal fractions*

For experimental details see the text. Calculated specific radioactivity = 5633d.p.m./mg.

Crystallization	Sp. radioactivity of crystals (d.p.m./mg)
1	3648
2	3674
3	3479

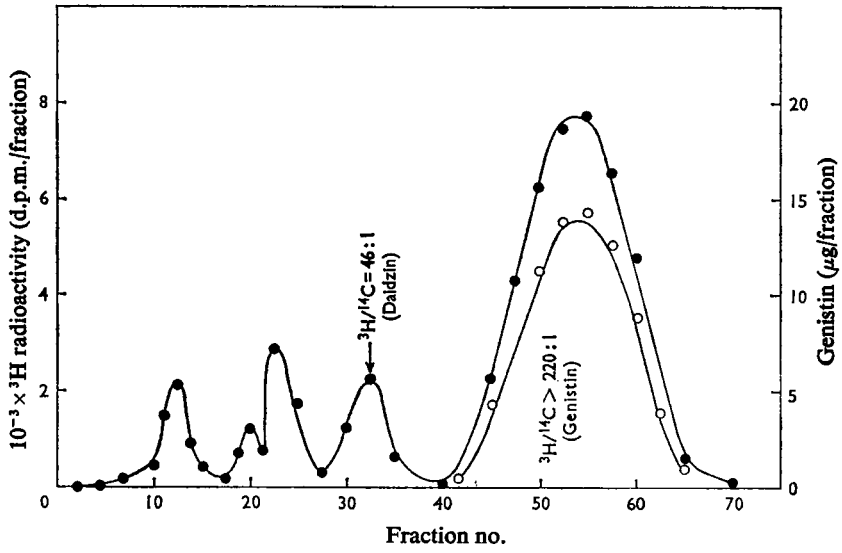

Fig. 1. *Chromatography on Sephadex G-25 of a mixture of authentic genistin (genistein 7-glucoside) and of radioactive glucosides of genistein and daidzein formed by rabbit liver microsomal fractions*

For experimental details see the text. ^3H (●) and E_{280} (○) were measured in each fraction (2.5ml). ^{14}C was measured in pooled fractions of each peak. The $^{14}C/^3H$ ratio of the presumptive daidzein glucoside was 49:1, and that of the presumptive genistein glucoside was 250:1.

coside and authentic genistin. Constant specific radioactivity was attained after the first crystallization.

Discussion

The formation of steroid glucuronides under the conditions used in this work has been previously demonstrated (Collins *et al.*, 1968, 1970). The results on steroid glucuronide formation in Table 1 are included for purposes of comparison, and as a check on the viability of the microsomal preparations. The observation that the isoflavones biochanin A, genistein, formononetin and daidzein, as well as their degradation products, namely equol, 2,4-dihydroxyphenyl *p*-methoxybenzyl ketone and 2,4-dihydroxyphenyl *p*-hydroxybenzyl ketone, all form glucuronides is novel as far as we are aware, but it is not unexpected. Only monoglucuronides were formed, and with formononetin the site of glucuronide formation must be the 7-hydroxyl group, since no other position is available. The structure of the glucuronides was not further studied.

The results in Table 1 confirm previous findings (Williamson *et al.*, 1971) that oestradiol-17α and oestradiol-17α 3-glucuronide form glucosides when incubated with rabbit liver microsomal fractions in the presence of UDP-glucose, whereas oestriol and non-phenolic steroids such as cortisol and testosterone do not. The present findings establish that the isoflavones and their phenolic degradation products, as well as diethylstilboestrol and phenolphthalein, act as acceptors for glucose in the system *in vitro*. As with the glucuronides, only monoconjugates were formed, and in formononetin, the sugar must be attached to the 7-hydroxyl group. The glucoside of genistein had identical mobility on Sephadex G-25 with genistin, the structure of which has been firmly established by the work of Walz (1931) as the 7-glucoside of genistein. The acetylated mixture of genistin and the tritiated genistein glucoside crystallized to a constant specific radioactivity (Table 4), which was 65% of the value calculated for the mixture before acetylation. This indicates that at least a very large percentage of the tritiated material was genistin, and provides further firm evidence that rabbit liver microsomal fractions transfer glucose to the 7 position of genistein. It seems probable that this is the site of the glycosidic linkage in the other isoflavones as well.

The fact that the formation of *N*-acetylglucosaminides or of galactosides was not detected in this study (Table 2) indicates that the isoflavones are, at best, very poor acceptors for these sugars. However, the possibility that formation of these glycosides

might be demonstrated if the corresponding nucleotides were available in sufficiently high specific radioactivity remains open. In the same way, the results in Table 2 permit the conclusion that rabbit liver is much more active in the transfer of glucose to the isoflavones than are the other tissues studied, but they do not entirely rule out the formation of glucosides by these tissues.

The results raise the question of the nature of the enzymic mechanism for glucose transfer to the aglycones shown in Table 1. The possibility exists that the glucuronyl-transfer systems that operate to form glucuronides for excretion purposes might, in the presence of excess of UDP-glucose and in the absence of UDP-glucuronic acid, be capable of forming glucosides. This is an attractive hypothesis, particularly in view of the fact that in these experiments (Table 3) glucuronic acid transfer took place much more readily than did glucose transfer to compounds that formed both glucuronides and glucosides. Although there is without doubt a large family of substrate-specific glucuronyltransferases, some of which may also be able to utilize UDP-glucose to form glucosides, several compounds in Table 1, notably oestriol and *p*-nitrophenol, readily form glucuronides but cannot be shown to form glucosides. Williamson *et al.* (1971) have shown that the mechanism in rabbit liver microsomal fractions for glucose transfer to the 3-hydroxyl group of oestrone, oestradiol-17α or oestradiol-17β is, in contrast to the mechanism for glucuronic acid transfer, not entirely dependent on the presence of the appropriate uridine nucleotide. Further, although we have repeatedly failed to demonstrate the transfer by rabbit tissues of glucuronic acid to the alcoholic 17α-hydroxyl group of oestradiol-17α 3-glucuronide (Collins *et al.*, 1970), transfer of glucose to this group is readily shown (Table 1). In unpublished work the present authors have found that sheep liver also effects the transfer of glucose to oestradiol-17α 3-glucuronide, and an enzyme responsible for this transfer has been partially separated from steroid glucuronyltransferases and shown to behave differently towards solubilizing reagents than do these enzymes.

The above considerations indicate that glucosyl transfer to at least some of the aglycones studied is mediated by transferases distinct from those concerned with glucuronic acid transfer. The resultant glucosides have not thus far been found in excreta, with the exception of the double glycoside of oestradiol-17α (Williamson *et al.*, 1969), in which the 3-position of the steroid is linked to glucuronic acid. The absence of monoglucosides from urine and bile is not surprising, since they are poorly soluble in water. The fact that the isoflavones, some of which are oestrogenic in animals (Nilsson, 1962), are substrates for rabbit liver glucosyltransferase, coupled with the high specificity of the transferase for phenolic steroids, suggests a correlation between oestrogenicity and the formation of glucoside. This, however, seems unlikely, since oestradiol-17α, which is the substrate most readily combined with glucose by both rabbit and human (Williamson *et al.*, 1971; Williamson & Layne, 1971), is not a potent oestrogen. Further, although diethylstilboestrol is an extremely active oestrogen, it formed only small amounts of glucoside in the present experiments, and there is no evidence that phenolphthalein, which forms a glucoside (Table 1), has oestrogenic activity. Although the intact glucosides of these compounds have not been tested for oestrogenic effect, their action as hormones *per se* seems doubtful.

A more likely role for the steroid and other glucosides formed in animals would seem to lie in their possible function as metabolic intermediates. Steroid sulphates are metabolized without removal of the sulphate group (Wang & Bulbrook, 1968), and Williamson (1971) has obtained evidence for the presence in rabbit liver of a phenolic steroid 17α-dehydrogenase, which is much more active on oestradiol-17α 3-glucuronide than on the free steroid. The possibility exists that the attachment of glucose at a specific position may condition the nature and rate of further metabolism of the aglycone molecule.

During the preparation of this manuscript, Fevery *et al.* (1971) have reported the excretion in dog bile of glucose and xylose conjugates of bilirubin, and Wong (1971) has shown the formation of bilirubin glucoside by rat liver preparations *in vitro*. These results indicate that the formation of non-acidic glycosides in animals may be of more frequent occurrence than has hitherto been recognized.

We thank Dr. D. G. Williamson for assistance in the preparation of genistin, and the Medical Research Council of Canada for support through Grant MT-3287 to D. S. L.

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