Mechanism of Biosynthesis of Thio- β -D-glucuronides and Thio- β -D-glucosides

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1. The thio- β -D-glucosiduronic acids (thio- β -glucuronides) of *o*-aminothiophenol, diethylthiocarbamic acid, *p*-nitrothiophenol and thiophenol are formed biosynthetically in broken- and intact-cell preparations of mouse liver. 2. For this biosynthesis to occur in homogenates or microsomal fractions, UDP-glucuronic acid was required during incubation; glucose, glucuronic acid or UDP could not replace it. UDP was a product of the reaction. 3. The biosynthetic mechanism linking glucuronic acid to thiol and carbodithioic groups therefore requires UDP-glucuronyltransferase activity and resembles that forming the various types of *O*-glucuronides. 4. An analogous enzymic mechanism employing UDP-glucose synthesizes the thio- β -D-glucosides of diethylthiocarbamic acid and thiophenol in gut preparations of the mollusc *Arion ater*; this mechanism resembles that forming the *O*-glucosides. The thio- β -D-glucosides are formed also in intact cells. 5. As expected from the distribution of *O*-glycosides, *S*-glucuronides of these aglycones were not detectable with the invertebrate, nor were the *S*-glucosides with the vertebrate. 6. Despite their similar biosyntheses, *S*- and *O*- β -glycosides differ in susceptibility to hydrolysis by β -glycosidases. Rat preputial-gland β -glucuronidase hydrolysed thioglucuronides of *o*-aminothiophenol, diethylthiocarbamic acid and *p*-nitrothiophenol, hydrolysis being inhibited by glucarolactone; the thioglucuronide of thiophenol was not hydrolysed by preputial-gland or liver β -glucuronidase. The two *S*-glucosides resisted hydrolysis by β -glucosidase from almond emulsion.

β -D-Glucosiduronic acids, or glucuronides, result from a glycosidic linkage at C-1 of D-glucuronic acid with a wide range of chemical groups. These include several varieties of hydroxyl or carboxyl groups, as well as of amino and thiol functions (see Smith & Williams, 1966), linkage with aglycone being through an oxygen, a nitrogen and a sulphur atom respectively.

The various *O*-glucuronides are biosynthesized by the reaction:

(Clapp, 1956), 9-ethyl- and 9-butyl-mercaptapurine (Hansen *et al.*, 1963) and *NN*-diethylthiocarbamic acid (Kaslander, 1963, 1966; Strömme, 1965; Strömme & Eldjarn, 1966). They were isolated from human urine or from urine or tissues of rat or mouse; *NN*-diethylthiocarbamoyl thioglucuronide (the *S*-glucuronide of *NN*-diethylthiocarbamate) was found in rat liver extract (Strömme, 1965) and appeared in the medium after incubation of rat liver

where R-OH is the aglycone concerned (Dutton, 1966a).

N-Glucuronides may be biosynthesized by this mechanism or non-enzymically (see Dutton, 1966a); *N*-glucuronides differ markedly from *O*-glucuronides in several of their properties, and Marsh (1966) classes them as glucuronosylamines rather than glycosides.

The *S*-glucuronides may be expected to resemble the *O*-glucuronides but have been little studied. Those so far reported to be formed *in vivo* are of benzenethiol ('thiophenol'; Parke, 1952), benzothiazole-2-thiol

slices with the aglycone (Kaslander, 1966).

The *O*- β -glucosides of several phenols are synthesized in plants (Yamaha & Cardini, 1960; Dutton & Duncan, 1960) and invertebrates (Trivelloni, 1960; Smith & Turbert, 1961; Dutton, 1962) by an analogous UDP-glucosyltransferase. An *S*-glucoside of dimethylthiocarbamate has been formed by sliced plant tissue (Kaslander *et al.*, 1961).

The mechanism of biosynthesis of such thioglucuronides and thioglucosides has not been previously studied.

The present work describes the mechanism of

synthesis of both thiolic and carbodithioic glucuronides and glucosides. Preliminary reports of this investigation have been published (Dutton & Illing, 1969; Illing & Dutton, 1970).

Experimental

Materials

Acceptor substrates. These were: *o*-aminothiophenol (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.), m.p. 26°C, chromatographically pure in two solvents; *o*-aminophenol (BDH Chemicals Ltd., Poole, Dorset, U.K.), re-sublimed twice, colourless; *NN*-diethylthiocarbamic acid, Na⁺ salt [Sigma (London) Chemical Co., London S.W.6, U.K.], chromatographically pure in two solvents; *p*-nitrothiophenol (Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.), recrystallized from acetone-ether (1:1, v/v) under N₂; *p*-nitrophenol (BDH), recrystallized from ethanol; phenol, analytical grade (BDH); thiophenol (BDH).

Donor substrates. These were: UDP-glucose (Sigma); UDP-[U-¹⁴C]glucose (273 mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.); UDP-glucuronic acid, NH₄⁺ salt (Sigma); UDP-[U-¹⁴C]glucuronic acid (302 mCi/mmol), NH₄⁺ salt (The Radiochemical Centre).

Other compounds. These were: *o*-aminophenyl-β-D-glucuronide (Aldrich Chemical Co.); L-cysteine hydrochloride (BDH); L-[³⁵S]cysteine, 36 mCi/mmol at 0 days (used within 7 days; The Radiochemical Centre); diethylthiocarbamoyl thio-β-D-glucuronide, Ca²⁺ salt, kindly given by Dr. C. W. Pluijgers, Utrecht, Holland, and freed of Ca²⁺ with K₂CO₃ before use; D-glucaro-(1→4)-lactone was prepared in solution by refluxing aq. potassium hydrogen glucarate (BDH) for 1 h (Levy, 1952); D-glucono-(1→5)-lactone (BDH); D-glucose (BDH); [U-¹⁴C]glucose, 273 mCi/mmol (The Radiochemical Centre); D-glucuronic acid, K⁺ salt (Corn Products Refining Co., Argo, Ill., U.S.A.). β-Glucosidase was from Sweet Almond Emulsin (Sigma Chemical Co.); β-glucuronidase was prepared from female rat preputial gland by the method of Levy *et al.* (1958), as far as the ammonium sulphate-precipitation stage.

Animals. Mice were mixed laboratory stock, and rats were Wistar strain, of either sex. Slugs were *Arion ater*, collected locally in spring and summer.

Methods

Tissue preparations. A 10% (w/v) homogenate of rat or mouse liver in 1 mM-EDTA, pH 7.0–7.5, was prepared in a glass-Teflon homogenizer. Slug gut was slit, rinsed and sonicated [as a 30% (w/v) suspension] by using a Dawe Soniprobe 1130A with $\frac{1}{4}$ in microtip. Debris was removed from these broken-cell preparations by centrifuging at 600g for 5 min.

Microsomal fractions of rodent liver were prepared by spinning 10% (w/v) homogenates in 0.25 M-sucrose–1 mM-EDTA, pH 7.0–7.5, at 8000g for 20 min; the pellet was discarded and then the microsomal fraction was spun down at 100000g for 30 min, washed and resuspended in sucrose–EDTA.

Sliced tissue was prepared as previously for rodent liver (Stevenson & Dutton, 1962) and for slug gut (Dutton, 1966b).

Reaction media and incubation. These are given in detail in the text under the respective substrates employed. All solutions of thiol substrates were made fresh daily under N₂; other substrates were stored frozen under N₂. All radioactivities were checked by counting of identical samples before incubation. Three controls were always run: with tissue, with acceptor substrate and with donor substrate respectively, added after incubation to the otherwise complete reaction medium.

Broken-cell preparations were shaken under N₂ at 37°C for 20 min (rodents) or at 25°C for 1 h (*Arion*). Slices were shaken under O₂+CO₂ (95:5) at 37°C for 1 h (rodents) or at 25°C for 3 h (*Arion*).

Assay of conjugates. The details of identification are described in the Results section. Radioactivity and colorimetric assays were checked against each other whenever practicable. For radioactivity assay the following general procedure was employed. Reaction media were incubated with UDP-[U-¹⁴C]-glucuronic acid, UDP-[U-¹⁴C]glucose or [U-¹⁴C]-glucose, and freed of protein and chromatographed; the chromatograms were scanned for radioactivity on a Pannax RTLS 1A scanner. Peaks representing (where present) radioactive conjugate, sugar nucleotide or sugar nucleotide breakdown products, were located on these chromatograms and identified by comparison with the mobilities of standards and suitable controls, run in parallel. All areas, test and control, at *R_F* values corresponding to the peaks were transferred to 10 ml of toluene-based scintillator fluid [500 ml of toluene (A.R.), 300 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 5 g of 2,5-diphenyloxazole (both from Nuclear Enterprises Ltd., Edinburgh, U.K.; scintillation grade)], and counted for radioactivity in a Nuclear-Chicago Unilux II scintillation counter for at least 10 min or to 1000 counts over background, whichever took the longer time. Efficiency was 70–80% with all substrates except when *o*-aminophenol and *o*-aminothiophenol were treated with a preliminary location spray with diazotization reagents, which lowered it to 55–60%. (A standard quench curve was derived by using Nuclear-Chicago ¹⁴C quenched standards in toluene scintillator fluid; by employing the channels-ratio method, counting efficiency was determined on each individual sample.) Aqueous material (e.g. sugar nucleotide solutions) was counted for radioactivity by using a naphthalene-based scintillator fluid [965 ml

of dioxan (A.R.), with 200g of scintillation-grade naphthalene, 7g of 2,5-diphenyloxazole and 300mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene].

Chromatographic solvent systems. These are referred to in the text by the following letters: A, methanol-water (9:1, v/v); B, butan-1-ol-acetic acid-water (8:3:3, by vol.); C, propan-1-ol-water (17:3, v/v); D, butan-1-ol-acetic acid-water (4:1:1, by vol.); E, butan-1-ol-acetic acid-water (8:1:3, by vol.); F, propan-1-ol-water-aq. NH_3 (sp.gr. 0.88) (10:1:1, by vol.); G, 2-methylbutan-2-ol-formic acid-water (3:2:1, by vol.); H, ethanol-1M-ammonium acetate, pH7.5 (3:7, v/v).

Identification tests on chromatograms or eluates. Diazotization. The chromatogram was sprayed with 0.2% NaNO_2 in 2M-HCl, dried in warm air and then sprayed with satd. aq. *N*-1-naphthylethylenediamine dihydrochloride; colour develops slowly in warm air.

Glucose oxidase test. Glucose oxidase (125mg; Sigma), peroxidase (0.5mg; Sigma) and 0.5ml of aq. 1% *o*-dianisidine (freshly prepared; BDH) were made up to 100ml with 0.5M-sodium phosphate buffer, pH7.0, and used as described by Huggett & Nixon (1957).

Lieberman test. This was Feigl's (1966a) modification; it detects a phenyl ring, except when a *p*-nitro group is present, by the formation of an indophenol dye.

Raney nickel test. Areas of chromatograms (the thin-layer cellulose was free of CaSO_4) were warmed in a small tube with 1.0ml of conc. HCl and 2-4mg of Raney nickel (BDH); moist lead acetate at the tube mouth blackened with H_2S produced from bi- or quadri-valent S (Feigl & Hagenauer-Castro, 1962).

Sodium azide-iodine test. Equal volumes of 0.78M- NaN_3 (BDH) and 0.20M-iodine soln. (BDH Volucan) were mixed before use and sprayed on chromatograms (Chargaff *et al.*, 1948; Feigl, 1966b); decoloration is caused by compounds with thiol, sulphide or diacyl-sulphide groups, but not those with thioether, disulphide, sulphone, sulphinic acid or sulphonic acid functions; inorganic compounds with bivalent S interfere.

Sugar test. This was the ammoniacal AgNO_3 test of Trevelyan *et al.* (1950) adapted for spraying.

Tollens' test. This was Paul's modification, quoted by Dutton (1966a), and performed on eluted areas of chromatograms.

Protein assay. This was Oyama & Eagle's (1956) modification of Lowry's method (Lowry *et al.*, 1951).

Results

o-Aminothiophenol as substrate

The reaction mixture contained 27mM-tris-HCl buffer, pH7.3, 7mM- MgCl_2 , 0.15mM-aminothiophenol, 0.75mM-ascorbate, 2.9mM-UDP-[U- ^{14}C]-

glucuronic acid (93 $\mu\text{Ci}/\text{mmol}$) and the microsomal fraction derived from 0.1ml of mouse liver homogenate, in a total volume of 0.3ml. An otherwise identical reaction mixture contained 0.15mM-*o*-aminophenol instead of its thiol analogue. Protein was precipitated by heating at 100°C for 2min under N_2 , and 100 μl of supernatant was chromatographed (t.l.c., silica gel) in solvent system A or B alongside authentic *o*-aminophenyl glucuronide.

As indicated in Table 1, the reaction mixture with *o*-aminophenol contained, after incubation, a radioactive compound with a diazotizable amino group that had the same mobility as authentic *o*-aminophenyl glucuronide. The radioactive and diazotizable compound formed after incubation of the *o*-aminothiophenol mixture had an R_F value closely similar to that of *o*-aminophenyl glucuronide but gave a positive Raney nickel test, consistent with it containing bi- or quadri-valent sulphur; it gave no evidence of thiolic sulphur by the NaN_3 - I_2 test. Free *o*-aminothiophenol possessed an R_F value different from this presumed conjugate and contained thiolic sulphur by the NaN_3 test.

Both of the presumed conjugates gave evidence of a sugar and, from Tollens' test, contained hexuronic acid. UDP-glucuronic acid or its breakdown products had different R_F values and did not interfere.

To determine whether the hexuronic acid was attached by a β -glucuronyl link to *o*-aminothiophenol, the reaction mixtures were incubated on a large scale ($\times 3$), protein was precipitated as described above and the supernatant was chromatographed as a band in solvent system A. The presumed conjugate, located by its radioactivity, was eluted with 1.0ml of water, and 0.1ml of eluate placed in each of three tubes. Tube (1) contained 0.1ml of 0.2M-sodium acetate buffer, pH5.5, and 0.1ml of water; tube (2) contained 0.1ml of β -glucuronidase preparation, in the acetate buffer, and 0.1ml of water; tube (3) was identical with tube (2) but contained 0.1ml of 50mM-glucarolactone, the specific inhibitor of β -glucuronidase, instead of the water. After incubation for 3h at 37°C, the tubes were heated at 100°C for 2min, the protein was spun down and all of the supernatant chromatographed on silica gel in solvent systems A and B. The supernatants from all three incubated tubes contained the presumed radioactive conjugate but only that from tube (2) contained an additional radioactive area, which was positive for sugar, at the R_F of glucuronic acid. The supernatant from tube (2) gave rise on diazotization to an additional pink spot at the R_F of authentic *o*-aminothiophenol, and which, like *o*-aminothiophenol, gave a positive Raney nickel test. Parallel experiments in which *o*-aminophenol replaced *o*-aminothiophenol, confirmed the presence of a β -glucuronyl linkage in the conjugate formed.

Table 2 shows results of similar experiments where

Table 1. *Chromatography and identification of substrates and conjugates*

(?) indicates biosynthetic conjugate whose identity is to be established; 'presumed' indicates one prepared by biosynthetic methods known to yield the authentic conjugate; 'authentic' indicates pure authentic conjugate put through the isolation procedure in question. Sugar nucleotide-breakdown products were located by chromatography of the complete reaction mixture incubated without the acceptor substrate. Tests are described in the Experimental section and in the text; negative findings are omitted. The R_F values for solvents A and B are for t.l.c. on silica gel, and for solvents C-F, on cellulose or paper.

Compound	Solvent ...	R_F						Locating or identifying tests
		A	B	C	D	E	F	
<i>o</i> -Aminothiophenol	Free substrate	0.75	0.95	—	—	—	—	Diazotization; $\text{NaN}_3\text{-I}_2$ Raney nickel
	(?) Thio- β -glucuronide	0.65	0.50-0.60	—	—	—	—	Diazotization; β -glucuronidase; radioactivity; Raney nickel; Tollens'
<i>o</i> -Aminophenol	Free substrate	0.80	0.87	—	—	—	—	Diazotization
	Presumed β -glucuronide	0.60	0.50-0.60	—	—	—	—	Diazotization; radioactivity; sugar; Tollens'
Glucuronic acid	Authentic β -glucuronide	0.60	0.50-0.60	—	—	—	—	Diazotization
UDP-[U- ^{14}C]glucuronic acid		0.40	0.30	—	—	—	—	Sugar; Tollens'
		0.10	0-0.10	—	—	—	—	Radioactivity; sugar; Tollens'
UDP-[U- ^{14}C]glucuronic acid-breakdown products		0.10-0.35	0-0.30	—	—	—	—	Radioactivity; sugar; Tollens'
Diethyldithiocarbamic acid	Free substrate	—	—	0.90	Trace near solvent front	—	—	$\text{NaN}_3\text{-I}_2$
	(?) Thio- β -glucoside	—	—	0.90	0.80	—	—	Glucose liberated; $\text{NaN}_3\text{-I}_2$; radioactivity; Raney nickel
	(?) Thio- β -glucuronide	—	—	0.26-0.30	0.60	0.55	—	β -Glucuronidase; $\text{NaN}_3\text{-I}_2$; radioactivity; sugar; Tollens'
	Authentic thio- β -glucuronide	—	—	0.26-0.30	0.60	0.55	—	
<i>p</i> -Nitrophenol	Free substrate	—	—	0.90	0.90	—	—	Yellow spot with glycine buffer
	Presumed β -glucuronide	—	—	0.50	0.60	—	—	Radioactivity; sugar; Tollens'
<i>p</i> -Nitrothiophenol	Free substrate	—	—	0.90	0.90	—	—	$\text{NaN}_3\text{-I}_2$
	(?) Thio- β -glucuronide	—	—	0.50	0.60	—	—	Aglycone oxidation; β -glucuronidase; radioactivity; Raney nickel; sugar; Tollens'

Phenol	Free substrate	—	—	0.90–1.00	0.90–1.00	—	—	Lieberman
	Presumed β -glucoside	—	—	0.80	0.70	—	—	Glucose liberated (acid and β -glucosidase); Lieberman
	Presumed β -glucuronide	—	—	0.70	0.60	—	—	β -Glucuronidase; Lieberman; radioactivity; reducing sugar; Tollens'
Thiophenol	Free substrate	—	—	0.90–1.00	0.90–1.00	—	0.90–1.00	NaN ₃ -I ₂ (trace)
	(?) Thio- β -glucoside	—	—	0.80	0.70	—	—	Resists acid hydrolysis; Lieberman; radioactivity; Raney nickel
	(?) Thio- β -glucuronide	—	—	0.70	0.60	—	0.40	Resists β -glucuronidase; Lieberman; radioactivity; Raney nickel; reducing sugar; Tollens'
Glucose		—	—	0.40	0.40	—	—	Radioactivity; reducing sugar
Glucuronic acid		—	—	0.15–0.40 (t.l.c.)	0.20–0.40	—	—	Reducing sugar; Tollens'
				0.05–0.10 (paper)				
UDP-[U- ¹⁴ C]glucose		—	—	0	0	—	—	Radioactivity; sugar
UDP-[U- ¹⁴ C]glucose-breakdown products		—	—	0–0.30 (trace)	0–0.30 (trace)	—	—	Radioactivity
UDP-[U- ¹⁴ C]glucuronic acid		—	—	0	0	—	—	Radioactivity; sugar; Tollens'
UDP-[U- ¹⁴ C]glucuronic acid-breakdown products		—	—	0–0.20	0–0.20	—	—	Radioactivity where applicable; sugar; Tollens'

Table 2. *Effect of β -glucuronidase on *o*-aminothiophenol conjugate*

The radioactive conjugate, believed to be of glucuronic acid and *o*-aminothiophenol, was incubated as described in the text with (1) buffer only, (2) buffer + β -glucuronidase, (3) buffer + β -glucuronidase + glucaro-(1 $\rightarrow$ 4)-lactone. A portion from each mixture was chromatographed, and the radioactive peaks observed at areas corresponding to unchanged conjugate and to liberated glucuronic acid were removed and counted for radioactivity as described in the text. Counting efficiency was 70–72%. Final concn. of glucarolactone solution was 17mM in Expt. 1 and 25mM in Expt. 2.

Incubation method	Radioactivity (d.p.m.)					
	Expt. 1			Expt. 2		
	(1)	(2)	(3)	(1)	(2)	(3)
Conjugate	245	0	110	189	0	152
Glucuronic acid	0	250	134	0	176	9
% of total radioactivity remaining in conjugate	100	0	45	100	0	95

the radioactivity of areas at the R_F values of conjugate and free glucuronic acid were measured. These results indicate complete liberation of glucuronic acid from the conjugate by active β -glucuronidase.

From the above evidence, the conjugate formed with *o*-aminothiophenol as substrate may be considered to be the thio- β -glucuronide of *o*-aminothiophenol.

Diethyldithiocarbamic acid as substrate

The reaction mixture contained 52mM-tris-HCl buffer, pH7.3, 14mM-MgCl₂, 10mM-*NN*-diethyldithiocarbamic acid, 4.2mM-UDP-glucuronic acid and 0.1ml of mouse liver homogenate (10%, w/v) in a total volume of 0.50ml. After incubation, acetic acid (0.1ml) was added and the mixture was extracted with 3 $\times$ 3ml of butan-1-ol. The butanol extracts were evaporated at 50°C *in vacuo* to a gum, re-dissolved in 0.1ml of methanol-water (1:1, v/v), and 50 μ l samples were chromatographed in solvent systems C, D and E on paper or thin-layer cellulose (Table 1).

The NaN₃-I₂ test revealed a new sulphur-containing spot additional to that of the substrate in solvent system C (no substrate spot was observed with systems D and E, the sulphur presumably being removed as CS₂ under these conditions: Martin, 1953; Bode, 1954; Strömme, 1965). This new spot had the same R_F values in the three solvent systems as authentic diethylthiocarbamoyl thioglucuronide subjected to the same isolation procedure; in systems C and D its R_F value was that reported by Kaslander (1963) for the biosynthetic compound. A positive NaN₃-I₂ reaction is consistent with the R-C(S)-S-R sulphur linkage present in the *S*-glucuronide of diethyldithiocarbamic acid.

Hexuronic acid was detected in the new spot and was coincident with radioactivity when UDP-

[U-¹⁴C]glucuronic acid was donor substrate; the labelled sugar nucleotide and its breakdown products from control incubations did not interfere (Table 1).

When the presumed conjugate was treated with β -glucuronidase in the presence and the absence of glucarolactone, by the procedure described above for the thioglucuronide of *o*-aminophenol, it behaved exactly as did authentic diethylthiocarbamoyl thio- β -glucuronide. Chromatography of hydrolysis products in solvent system C revealed free diethyldithiocarbamate (NaN₃-I₂ test) and sugar, unless glucarolactone had been present. Table 3 illustrates a similar experiment in which β -glucuronidase specifically lessens radioactivity at the R_F of the conjugate, and increases radioactivity at the area of glucuronic acid.

The conjugate formed with diethyldithiocarbamic acid as substrate can therefore be considered identical with authentic diethylthiocarbamoyl thio- β -glucuronide.

p-Nitrothiophenol as substrate

The reaction mixture contained 27mM-trisHCl-buffer, pH7.3, 0.20mM-*p*-nitrothiophenol (or, in parallel experiments, 0.20mM-*p*-nitrophenol), 2.9mM-UDP-glucuronic acid and mouse liver microsomal preparation derived from 0.08ml of a 10% (w/v) homogenate, in a total volume of 0.30ml. After incubation, 0.5ml of 0.2M-trichloroacetic acid was added and the mixture was centrifuged; 0.5ml of supernatant was then added to 1.0ml of glycine buffer, pH9.0 (containing 0.27M-glycine, 0.17M-NaHCO₃ and 0.12M-NaCl), and unconjugated *p*-nitrothiophenol or *p*-nitrophenol measured from the E_{410} and E_{400} respectively (Hétu & Gianetto, 1970) against the usual controls. Disappearance of free phenol was always greatest when UDP-glucuronic

Table 3. *Hydrolysis by β-glucuronidase of conjugates of glucuronic acid and diethylthiocarbamic acid or thiophenol*

Substrates were incubated with UDP-[U-¹⁴C]glucuronic acid and a mouse liver homogenate; each radioactive conjugate was isolated and treated with β-glucuronidase. The conditions are described in the text. The supernatant from this last incubation mixture was then chromatographed and radioactive areas were located and counted. β-Glucuronidase was from rat preputial gland, except for Expt. 6, when it was from rat liver.

Expt. no.	Substrate	Radioactive area corresponding to	Radioactivity of area (d.p.m.)			Hydrolysis by β-glucuronidase (%)	Inhibition by glucarolactone of hydrolysis (%)
			Buffer only	Buffer + β-glucuronidase	Buffer + β-glucuronidase + glucarolactone (17 mM)		
1	Diethylthiocarbamic acid	Conjugate	267	54	207	80	72
2		Conjugate	183	44	157	76	81
3		Conjugate	2300	274	1946	88	83
		Glucuronic acid liberated by β-glucuronidase	(0)	2158	244	(100)	88
4	Thiophenol	Conjugate	853	867	871	0	0
5		Conjugate	2453	2594	2615	0	0
6		Conjugate	6138	5857	5733	4	0

acid had been present during incubation, suggesting formation of an *O*- or *S*-glucuronide.

Incubations were repeated with 2.9mM-UDP-[U-¹⁴C]glucuronic acid (93 μ Ci/mmol). Samples chromatographed on thin-layer cellulose (Table 1) in solvent system C or D gave rise to a new radioactive peak only when either acceptor substrate had been present in the complete reaction mixture.

The presumed conjugate derived from the complete mixture with *p*-nitrothiophenol as substrate gave evidence of sulphur, as indicated by a Raney nickel test. Since the NaN₃-I₂ test was positive only at the *R_F* value of free *p*-nitrothiophenol the sulphur in the conjugate was probably linked to glucuronic acid.

Evidence for glucuronic acid in the conjugate area of chromatograms came from the presence of radioactivity, sugar and a positive Tollens' test (Table 1). The β -glucuronyl linkage of the conjugate was detected as for the previous examples, by demonstrating specific hydrolysis by β -glucuronidase. When deproteinized supernatant from such hydrolytic incubations was chromatographed in solvent C, the liberated glucuronic acid was identified from its *R_F* value, its radioactivity and a positive reaction in the sugar test. To confirm the β -glucuronyl linkage and establish

the aglycone of the thioglucuronide as *p*-nitrothiophenol a colorimetric procedure was adopted (Table 4), which indicated the liberation of up to 96% of aglycone by β -glucuronidase, and up to 100% specific inhibition of this hydrolysis by glucarolactone. The orange-yellow alkaline solutions of the aglycone liberated by active enzyme was completely decolorized with H₂O₂ or by air bubbles at the same rate as similar solutions of authentic *p*-nitrothiophenol. Solutions of *p*-nitrophenol lost no colour under identical conditions.

The conjugate formed with *p*-nitrophenol as substrate therefore appears to be the thio- β -glucuronide of *p*-nitrothiophenol.

Thiophenol as substrate

Reaction mixtures contained 52mM-tris-HCl buffer, pH 7.3, 14mM-MgCl₂, 0.40mM-thiophenol (or phenol in parallel experiments), 2.9mM-UDP-[U-¹⁴C]glucuronic acid (93 μ Ci/mmol) and 0.08ml of a 10% (w/v) mouse liver homogenate, in a total volume of 0.30ml. After incubation, protein was precipitated by heating at 100°C for 2min; 0.1ml of supernatant was chromatographed on thin-layer cellulose in

Table 4. *Hydrolysis of p-nitrothiophenol conjugate by β -glucuronidase*

Tubes (a), (b) and (c) were incubated in duplicate with the full synthetic reaction mixture as described in the text to form the *p*-nitrothiophenol conjugate; another pair (d) were incubated with UDP-glucuronic acid added only after the incubation. Incubation was terminated by heating for 2min at 100°C. To tubes (a) and (d), 0.3ml of 0.1M-sodium acetate buffer, pH 5.5, was then added, to (b) 0.2ml of buffer + 0.1ml of β -glucuronidase in buffer, and to (c) 0.1ml of buffer + 0.1ml of β -glucuronidase in buffer + 0.1ml of boiled 0.05M-glucarate in the buffer. All tubes were incubated under N₂ for 3h at 37°C, and then treated with 0.5ml of 0.2M-trichloroacetic acid. The free *p*-nitrothiophenol present was then assayed by adding 0.5ml portions to 0.5ml of glycine buffer (see the text) and measuring *E*₄₁₀. Tube (d) contained the amount of free *p*-nitrothiophenol originally added as substrate; readings (d) minus (a) gave the amount of *p*-nitrothiophenol conjugated during the synthetic incubation in tubes (a), (b) and (c); readings (b) minus (a), and (c) minus (a), gave the *p*-nitrothiophenol liberated by β -glucuronidase alone and in the presence of glucarolactone respectively. Duplicates differed by less than $\pm 5\%$ and the means are given below for each of two separate experiments.

	Expt. I	Expt. II
<i>p</i> -Nitrothiophenol (nmol)		
Conjugated during synthetic incubation	14.0	12.5
Liberated during hydrolytic incubation with β -glucuronidase	13.5	10.5
Liberated during hydrolytic incubation with β -glucuronidase + glucarolactone (final concn. 10mM)	2.0	0
Hydrolysis of conjugate (%)		
During hydrolytic incubation with β -glucuronidase	96	84
During hydrolytic incubation with β -glucuronidase + glucarolactone	14	0
Inhibition of conjugate hydrolysis by glucarolactone (%)	85	100

solvent systems C, D and E (Table 1). Radioactive presumed conjugates containing either thiophenol or phenol were located on chromatograms at similar R_F values; they were absent from control reaction mixtures.

The presumed conjugates contained radioactivity derived from UDP-[U- 14 C]glucuronic acid and gave a positive Tollens' test. When treated by the Lieberman test the presumed phenol conjugate rapidly turned blue, becoming purple on warming. This colour appeared more slowly with a comparable sample of the thiophenol conjugate and not at all in blank areas of the chromatograms.

The conjugate containing thiophenol gave a negative $\text{NaN}_3\text{-I}_2$ test. A positive reaction at the R_F value of thiophenol itself (Table 1) indicated that the free aglycone had not wholly volatilized during the drying of the paper chromatogram. The Raney nickel test revealed sulphur in the thiophenol conjugate but not in the phenol conjugate or in controls. These results are consistent with the conjugate being linked through the sulphur atom of thiophenol.

Large-scale ($\times 3$) incubations employing phenol or thiophenol as acceptor substrate were performed and conjugate areas located, eluted and treated with β -glucuronidase as described for previous substrates. Although results from a preliminary experiment indicated that conjugates from both substrates were hydrolysable by β -glucuronidase of the preputial gland of rat (Illing & Dutton, 1970), in subsequent experiments done under apparently identical conditions (Table 3) no hydrolysis of the presumed thiophenol conjugate was observed. Similar negative results were obtained with the enzyme from rat liver, in agreement with the results of Hétu & Gianetto (1970). This characteristic resistance to hydrolysis by β -glucuronidase strengthens the evidence above that the conjugate is the thio- β -glucuronide of thiophenol.

Production of uridine 5'-pyrophosphate

If UDP-glucuronyltransferase is responsible for the above UDP-glucuronic acid-dependent glucuronidation of aglycones, then UDP should be detected in the reaction mixture after incubation.

Guinea-pig liver microsomal preparations [0.2 ml of suspension, prepared from 10% (w/v) homogenate] were incubated with (a) 0.85 mM-UDP-glucuronic acid, 32 mM-tris-HCl buffer, pH 7.4, and 0.20 mM-*p*-nitrothiophenol in a total volume of 0.6 ml for 10 min at 37°C; to a control (b), *p*-nitrothiophenol was added only after incubation. Ethanol (0.6 ml) was then added to mixtures (a) and (b), protein was spun down and the supernatants were treated with 2 drops of 20% (w/v) BaCl_2 at 0°C. After centrifugation the precipitates were dissolved in the minimum amount of 0.1 M-HCl; excess of 10% $(\text{NH}_4)_2\text{SO}_4$ solution was then added and the resulting BaSO_4

spun down. Samples (50 μ l) of supernatant were chromatographed against known uridine nucleotides on thin-layer cellulose (with fluorescent background) in solvent system G (Randerath, 1962). At 254 nm chromatograms from mixture (a) absorbed markedly at the R_F value (0.26) of UDP, whereas those from mixture (b) absorbed very little at that value. Little absorption was noted at the R_F value (0.45) of UMP. UDP-glucuronic acid remained at the origin.

UDP is therefore produced when the thioglucuronide of *p*-nitrothiophenol is synthesized, and the enzyme is presumably a UDP-glucuronyltransferase.

Correlation of radioactive with colorimetric assays

To confirm that glucuronide assays based on the amounts of radioactivity derived from UDP-[U- 14 C]-glucuronic acid appearing in the areas of paper chromatograms containing the presumed conjugates were as quantitatively significant as the more convenient colorimetric methods, and could be correlated with them, *o*-aminophenol and *p*-nitrothiophenol were incubated as described previously with UDP-[U- 14 C]glucuronic acid and conjugation was estimated both colorimetrically and by radioactivity. A satisfactory correspondence (Fig. 1) was obtained between the two methods for both these substrates. Thus for each mol of aglycone conjugated approximately one mol of labelled glucuronic acid appeared in the conjugate.

Thiol compounds not behaving as substrates

Two endogenous compounds, L-cysteine and glutathione, containing a thiol group were examined but no evidence of thioglucuronide formation was obtained under the exploratory conditions employed.

L-Cysteine (0.53 mM) was incubated with 2.3 mM-UDP-[U- 14 C]glucuronic acid (93 μ Ci/mmol) and buffered mouse liver microsomal preparations; L-[35 S]cysteine (36 mCi/mmol) was incubated with unlabelled UDP-glucuronic acid in another series of experiments. Subsequent chromatography in solvent systems C or H failed to reveal new radioactive peaks, ninhydrin-positive or sulphur-containing areas when compared with chromatograms derived from control incubation mixtures where labelled UDP-glucuronic acid or L-cysteine had been added after incubation.

When 0.42 mM-GSH replaced cysteine in the above incubation mixture, again no new radioactive peaks, ninhydrin-positive or sulphur-containing areas were detected under the conditions described for cysteine.

Biosynthesis of S-glucuronides in liver slices

o-Aminothiophenol, *NN*-diethyldithiocarbamic acid, *p*-nitrothiophenol or thiophenol (all 1.8 mM) were incubated with 50–80 mg wet wt. of mouse liver slices and 2.0 mM-D-glucose in 2.0 ml of sulphate-free

Fig. 1. Correlation of colorimetric and radioactive assay of glucuronide formation

o-Aminophenol (○) or *p*-nitrothiophenol (●) was incubated with liver microsomal preparation from rat or mouse respectively and UDP-[U-¹⁴C]glucuronic acid for various periods of time, and assayed colorimetrically for conjugate formation as described in the text. Parallel incubation samples in each experiment were chromatographed and scanned for radioactivity; the conjugate areas were eluted and their radioactivity was measured. The amounts of substrate conjugated as estimated in the colorimetric assay are plotted in the graph against amount of ¹⁴C calculated as glucuronic acid appearing in the conjugate. Each point represents the mean values obtained from the duplicate incubations of a single experiment.

Ringer bicarbonate (Krebs & Henseleit, 1932). After incubation slices were removed, 0.5 ml of ethanol was added and samples of the centrifuged supernatant were chromatographed on thin-layer cellulose in solvent system C or D. In control incubations substrate was added after incubation. Thio- β -glucuronides of each of the substrates were identified on the chromatograms by the methods described previously. The *S*-glucuronides, like the *O*-glucuronides (Karunairatnam *et al.*, 1949), can therefore be formed in and presumably excreted from intact liver cells.

Biosynthesis of thioglucosides

The invertebrate tissue chosen was the fore- and hind-gut of *Arion*, including the hepatic caeca; it contains UDP-glucosyltransferase activity towards

phenols (Dutton, 1966b). Experiments were similar to those described above, with UDP-[U-¹⁴C]glucose replacing UDP-glucuronic acid.

Diethyldithiocarbamic acid as substrate. The reaction mixture contained 0.14 mM-diethyldithiocarbamic acid, 97 mM-tris-HCl buffer, pH 8.5, 10 mM-Mg²⁺, 1.0 mM-UDP-[U-¹⁴C]glucose (93 μ Ci/mmol) and 0.1 ml of 30% (w/v) sonicated tissue preparation. Incubation was terminated by adding 0.3 ml of ethanol, and samples of the supernatant were chromatographed in solvent system C or D (Table 1). When donor and acceptor substrates have been present together during incubation a new radioactive area could be located on the chromatogram.

In solvent D, this area contained sulphur, detected by the NaN₃-I₂ and Raney nickel tests. Free diethyldithiocarbamic acid could not be detected in solvent system D, presumably because of instability (Martin, 1953; Bode, 1954); in system C it occupied the conjugate area (Table 1), although observations suggested increased amounts of sulphur at this area when the full reaction mixture had been incubated.

Areas of the paper chromatograms containing presumed conjugate were eluted into 1.0 ml of water, 1.0 ml of 2M-HCl was added and the solution was heated at 100°C for 20 min. The pH was then adjusted to 6-7 with NaOH and 1.0 ml of glucose oxidase reagent added. Glucose was detectable, but only in eluates derived from full incubation mixtures. β -Glucosidase of known activity (see below) failed to hydrolyse the conjugate.

Thiophenol as substrate. The procedure was as for diethyldithiocarbamic acid. The radioactive conjugate had a lower *R_F* value than the free substrate in both solvents (Table 1), but appeared also when phenol replaced thiophenol. The presence of the phenolic ring was detected in the conjugates by Lieberman's test only after standard incubation with either thiophenol or phenol present. Sulphur, judged by the Raney nickel test, was only apparent in the conjugate when thiophenol had been present in the full incubation mixture. The NaN₃-I₂ test, as expected, was positive only at the *R_F* of free thiophenol. The presence of glucose in the thiophenol conjugate could only be deduced from the radioactivity derived from UDP-[U-¹⁴C]glucose, for neither 1M-HCl at 100°C for 1 h nor β -glucosidase released glucose detectable by the glucose oxidase test. The conjugate formed when phenol was the substrate, however, readily gave glucose by either method. The hydrolysis by β -glucosidase could be inhibited by the specific inhibitor glucono-(1 $\rightarrow$ 5)-lactone (Conchie & Levvy, 1957). The difference observed in the susceptibility of the conjugates to hydrolysis clearly distinguished the unknown conjugate from phenyl β -glucoside and, being characteristic of the thio- β -glucoside of thiophenol (Pigman, 1941), supports the conclusion that this conjugate had been formed.

Biosynthesis of thioglucosides in Arion gut strips. The two substrates, diethyldithiocarbamic acid and thiophenol, were incubated with *Arion* gut in Ringer solution in the presence of [U - ^{14}C]glucose (760 μ Ci/mmol). The two conjugates presumed to have been formed were located by radioactivity scans of appropriate paper chromatograms and identified as described above. No hexuronic acid was detected. Thio- β -glucosides like the *O*- β -glucosides (Dutton, 1966b) can therefore be synthesized by strips of *Arion* gut, probably from carbohydrate precursors.

Donor substrates

Conjugates identified above as thio- β -glucuronides were only formed when UDP-glucuronic acid was present in the full reaction mixture during biosynthetic incubation. They were absent if it was replaced by similar concentrations of glucuronolactone, glucuronate and UDP (present singly or together), or of UDP-glucose. Similarly, conjugates identified above as thio- β -glucosides required UDP-glucose in the full incubation mixture and were not formed if this nucleotide was replaced by glucose and UDP or by UDP-glucuronic acid.

Discussion

Results presented above indicate that thio- β -D-glucuronides of *o*-aminothiophenol, diethyldithiocarbamic acid, *p*-nitrothiophenol and probably thiophenol are formed in broken- or intact-cell preparations of mammalian liver. Biosyntheses of the thio- β -glucuronides of *o*-aminothiophenol and *p*-nitrothiophenol do not seem to have been reported before; thioglucuronides of diethyldithiocarbamic acid (Kaslander, 1966; Strömme, 1965) and thiophenol (Parke, 1952) have been found in urine or tissue fluids, and Gessner & Jakubowski (1972) noted the probable formation of diethylthiocarbamoyl thioglucuronide in UDP-glucuronic acid-fortified mouse liver homogenates.

The enzyme responsible requires UDP-glucuronic acid and appears to be related to, if not identical with (Illing & Dutton, 1972), UDP-glucuronyl-transferase (EC 2.4.1.17). In the biosynthesis, the glucuronyl moiety of the nucleotide is transferred to the sulphur atom of either a thiol group (as in *o*-aminothiophenol, *p*-nitrothiophenol and thiophenol) or a carbodithioic group (as in diethyldithiocarbamic acid). It is transferred to the oxygen atom in acyl, alkyl and aryl hydroxyl or carboxyl groups, to the nitrogen atom in carbamino and possibly amino groups (see Dutton, 1966a), and to various sugar residues during polysaccharide biosynthesis (see Silbert, 1966). UDP-glucuronic acid thus appears to be concerned in forming all known types of glucuronic acid-containing compounds.

UDP-glucose is the glycosyl donor for the ana-

logous formation of thio- β -D-glucosides in *Arion*, and the mechanism appears identical with that forming *O*- β -D-glucosides (Dutton, 1966b).

Administered phenols are predominantly glucuronidated by mammals and glucosylated by plants and invertebrates; the above results extend this broad taxonomic guide to the aromatic thiols. The respective glucosides and glucuronides were clearly distinguishable by their R_F values, sugar tests and demonstration of radioactivity from separate sugar nucleotides. Our conditions gave no evidence of significant glucoside formation in mouse liver preparations nor of glucuronide in those from *Arion*.

Although all the thio- β -glucuronides studied were of xenobiotics and preliminary observations with cysteine and glutathione proved negative, further work may show that thioglucuronides of suitable endogenous substrates are formed by the same mechanism.

Some but not all thio- β -glucuronides are hydrolysed by β -glucuronidase. β -Glucuronidase from *E. coli* failed to hydrolyse thio- β -glucuronides of aliphatic thiols and thiophenol (Stoeber, 1957). Bovine liver β -glucuronidase appeared to hydrolyse the thioglucuronide of 2-benzothiazole (Colucci & Buyske, 1965) and diethylthiocarbamoyl thio- β -glucuronide was hydrolysed by specific (i.e. glucarolactone-inhibited) β -glucuronidase activity from rat preputial gland (Dutton & Illing, 1969). Héty & Gianetto (1970), using rat liver β -glucuronidase, have clearly demonstrated that the thio- β -glucuronides of 2-benzothiazole or *p*-nitrothiophenol are hydrolysed whereas that of thiophenol is not; they suggest an induced shortening of the C-S bond towards C-O dimensions in the hydrolysable conjugates. Such shortening could account for the hydrolysis of the thioglucuronide of *o*-aminothiophenol. The thio- β -glucosides studied above are, like many others, difficult to hydrolyse, either enzymically (Wagner & Metzner, 1965; but see Goodman *et al.*, 1959) or with acid (Pigman, 1941; Wagner & Metzner, 1965). In view of the different susceptibilities of *O*- and *S*-glycosides to hydrolysis the similarity of their biosynthetic mechanism is noteworthy.

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