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DRUG–PROTEIN CONJUGATES—X

THE ROLE OF PROTEIN CONJUGATION IN THE DISPOSITION OF DINITROFLUOROBENZENE*

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Abstract—The metabolism and irreversible protein binding of 2,4-[3,5-^{3}H]dinitrofluorobenzene (^{3}H-DNFB), a model chemically reactive compound, were studied in the rat. ^{3}H-DNFB given intravenously (5 μg, 5 mg or 25 mg per kg) to anaesthetized cannulated rats was rapidly metabolized via the mercapturic acid pathway. The metabolites were extensively eliminated in bile and urine: predominantly as the glutathione conjugate and mercapturate in bile, and as the mercapturate in urine. Only *ca.* 3–10% of the doses remained in the liver, kidneys, spleen, heart and lungs at 3 hr. Dinitrophenyl mercapturate was the principal urinary metabolite in conscious rats dosed i.v. (5 mg or 25 mg per kg). Only 15–25% of the radiolabelled material in liver and kidney at 3 hr was irreversibly bound to protein, but 45–99% of that in the other organs and 49–88% in plasma was irreversibly bound. Preliminary evidence for the metabolism of ^{3}H-DNFB (5 mg/kg and 25 mg/kg doses) to N^2-acetyl-N^6-DNP-lysine, a novel conjugate and metabolite of dinitrophenylated proteins *in vivo*, is presented.

Many drugs and other chemicals undergo metabolism *in vivo* to metabolites that react irreversibly with endogenous macromolecules [1], thereby causing potentially toxic structural changes.

For an estimation of the risks associated with irreversible binding, it is necessary to have a measure of the degree of binding *in vivo*. In experimental animals this may be determined with radiolabelled compounds [2–4]. Estimating the formation of drug-macromolecule adducts in man clearly requires other methods. Thus alkylated products of haemoglobin have been used for monitoring *in vivo* binding of unlabelled alkenes in both animals and man [4]. However, short-lived metabolites, which never leave the tissues in which they are formed [5], will give rise to adducts that can be directly measured only by invasive means.

Recently, Kitteringham *et al.* [6, 7] have suggested that excreted derivatives of amino acids derived from covalently modified proteins might be used for assessing the formation of drug–protein conjugates *in vivo*. It was found that highly ^{3}H-dinitrophenylated serum albumins given intravenously to anaesthetized rats were rapidly cleared by the liver, and that the dinitrophenyl (DNP) moiety was extensively eliminated in bile and urine as N^2-acetyl-N^6-dinitrophenyl-lysine (acetyl-DNP-lys) [8]. Acetyl-DNP-lys was also a urinary metabolite of DNP-albumin conjugates (16–27% ^{3}H activity in 0–24 hr urine) in conscious rats (unpublished observations). The modified albumins were produced by reacting the proteins with ^{3}H-2,4-dinitrofluorobenzene (^{3}H-DNFB) under mild conditions, when DNFB reacts with C-6 amino groups of lysine residues [9].

In this study, the metabolism and irreversible protein binding of ^{3}H-DNFB *in vivo* were investigated with reference to the excretion of DNP-amino acid conjugates. Parts of this work have been published in abstract form [10].

MATERIALS AND METHODS

Chemicals and enzymes. 2,4-[3,5-^{3}H]Dinitrofluorobenzene (^{3}H-DNFB) (16.6 Ci/mmol) was purchased from Amersham International plc (Bucks, U.K.). Unlabelled DNFB, N^6-2,4-dinitrophenyl-L-lysine (DNP-lys) and Diazald were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). HPLC grade solvents were products of Fisons plc (Loughborough, Leics., U.K.). NCS solubilizer was from Amersham Corp. (Arlington Heights, IL, U.S.A.). Scintillation fluid (Scintillator 299) was from Packard-Becker B.V. (Groningen, The Netherlands).

Dinitrophenyl derivatives of N^2-acetyl lysine, glutathione and cysteine (S-linked) were prepared and characterized by methods cited previously [7]. Methyl esters of N^2-acetyl-DNP-lys and DNP-mercapturate were prepared using diazomethane and characterized by HPLC and chemical ionization mass spectrometry (base peaks at M + 1 were observed).

Acylase 1 (*N*-acylamino acid amidohydrolase, Grades I and II from porcine kidney) was from Sigma Chemical Co.

Synthesis of N-acetyl-S-dinitrophenylcysteine. *N*-Acetyl-*S*-dinitrophenylcysteine (DNP-mercapturate) was prepared by an improvement of a published method [11]. An emulsion of DNFB in ethanol (0.93 g/3 ml) was added to *N*-acetylcysteine (1 g) in

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1 M $KHCO_3$ (20 ml) and the mixture stirred at room temperature for 1 hr. The reaction mixture was extracted with ether (2 × 40 ml); the extracts were discarded. Acidification of the aqueous phase with 1 M HCl (20 ml) yielded a precipitate which was removed. The DNP-mercapturate was extracted into ethyl acetate (2 × 40 ml). The combined organic phases were washed with saturated NaCl solution (30 ml), dried over Na_2SO_4 and evaporated to dryness *in vacuo*. Crystallization from ether–ethanol and recrystallization from water yielded 1.2 g of product; the yield from DNFB was 67%. The product (R_T 13 min) was homogeneous on an ODS-2 HPLC column eluted with a linear gradient of methanol (30–60% at 2%/min) in 43 mM $NH_4H_2PO_4$ buffer, pH 2.3. Its λ_{max} in 0.1 M sodium phosphate buffer, pH 7.5, was 345 nm. A fast-atom bombardment spectrum obtained in the negative ion mode contained peaks at m/z 328($[M-1]^-$) and m/z 199($[DNPS]^-$; base peak).

Experiments with cannulated rats. Male Wistar rats (190–260 g body wt) were anaesthetized with urethane (1.4 g/kg in 0.15 M saline given i.p.), and their jugular veins cannulated. Thereafter, either their carotid arteries or common bile ducts were cannulated. ^{3}H-DNFB (*ca.* 5 or 15 μCi) dissolved in polyethylene glycol 200 (PEG)-isotonic saline (1:1, v/v; 1 ml/kg) was administered via the jugular vein at a dose of either 5 μg/kg, 5 mg/kg or 25 mg/kg body wt. Bile was collected for either three or five hours. Urine was taken from bladders after three hours. Blood samples (0.5 ml) obtained from the carotid artery were collected into lysine solution (1 mg in 0.1 ml saline) to remove any unreacted ^{3}H-DNFB. After three hours, the animals were killed by exsanguination and organs were removed for determination of total radioactivity and irreversibly bound radiolabelled material. The organs were frozen in liquid N_2 and stored at −70° until analysed.

Experiments with conscious rats. Male Wistar rats (270–350 g body wt) were used for studies with conscious animals. ^{3}H-DNFB (*ca.* 10 μCi; 5 mg/kg or 25 mg/kg body wt) was administered by injection into the tail vein (in 1 ml/kg PEG). The rats were housed in individual metabolism cages that allowed separate collection of urine and faeces (faeces were not analysed). Food and water were available *ad libitum*. Urine collections were removed every 24 hr for four days.

Measurement of radioactivity and irreversibly bound radiolabelled material. Samples of plasma, bile and urine were assayed for radioactivity as described previously [7].

Radiolabelled material irreversibly bound to plasma and tissue protein was determined by equilibrium dialysis in the presence of sodium dodecyl sulphate (SDS) [12]. This method gave the same results for plasma as dialysis in the presence of either tetrahydrofuran (THF; 30%, v/v) or propan-2-ol (30%, v/v) but was preferable because the organic solvents precipitated some of the protein. The same degree of irreversible binding was measured by exhaustive extraction of plasma with THF, propan-2-ol or acetone. Ethyl acetate, methanol and ethanol were less effective in removing radiolabelled material than was SDS-dialysis (unpublished observations). For determination of total radioactivity and irreversibly bound radiolabelled material in tissues, whole organs were homogenized in 0.1 M sodium phosphate buffer (1:9, w/v), pH 7.4, containing lysine (1 mg/ml). Portions of homogenate (4 ml) were mixed with SDS (80 mg) and heated in a boiling water bath for 10 min. After cooling, duplicate aliquots (1 ml) were dialysed against 1 litre of 0.01 M phosphate buffer, pH 7.4, containing 0.1% (w/v) SDS at 37° for 18 hr. Aliquots (0.4 ml) of pre-dialysis homogenate, post-dialysis homogenate and dialysate were assayed for radioactivity in NE295 scintillant (12 ml). The pre- and post-dialysis homogenates (20–50 μl aliquots) were assayed for protein by the method of Lowry *et al.* [13]. Plasma samples were diluted with 0.1 M phosphate buffer, pH 7.4, and dialysed as above.

High-performance liquid chromatography. The urinary and biliary metabolites of ^{3}H-DNFB were analysed by reversed-phase HPLC using a Partisil ODS-2 column (25 cm × 0.46 cm i.d., 10 μm; Technicol Ltd., Stockport, Cheshire, U.K.) protected by a guard-column of Co:Pell ODS (Whatman Inc., Clifton, NJ, U.S.A.). An Aerograph 8500 (Varian Instruments Division, Palo Alto, CA, U.S.A.) or Gilson 302-802 (Gilson, Villiers-Le-Bel, France) chromatograph was used. Urine (10–200 μl, 10–100 × 10^3 d.p.m.) and bile (5–75 μl, 15–60 × 10^3 d.p.m.) were chromatographed on a column eluted with a linear gradient of methanol (30–60% at 2%/min) in 43 mM $NH_4H_2PO_4$, pH 2.3. The flow rate was 2 ml/min. Urine from conscious rats was filtered (Minisart 0.2 μm, Sartorious GmbH, Göttingen, F.R.G.) before analysis. Bile and bladder urine were analysed without prior treatment. Eluate was monitored at 360 nm with an LC3 absorbance detector (Pye Unicam Ltd., Cambridge, U.K.), collected in 1 ml fractions and dissolved in Scintillator 299 for measurement of radioactivity. The peaks of radioactivity were chromatographically identified by comparing their retention times with those of co-injected authentic unlabelled compounds. Recoveries from the column of radiolabelled metabolites were 101 ± 9% (mean ± S.D., N = 30) and 97 ± 10% (N = 14) for urine and bile respectively.

Isolation and identification of metabolites. DNP-mercapturate and a component (component A) which was chromatographically similar to N^2-acetyl-N^6-dinitrophenyl-lysine (acetyl-DNP-lys) (Fig. 4b) were isolated from the 0–24 hr urines of rats given ^{3}H-DNFB (5 mg/kg or 25 mg/kg). Urine (14 ml, 19 ml and 24 ml vol., total 57 ml; total ^{3}H activity 50 × 10^6 d.p.m.) was acidified to pH 2.0 with HCl and twice extracted with an equal volume of redistilled ethyl acetate; the recovery of ^{3}H in ethyl acetate was *ca.* 75%. Pooled extracts were evaporated to dryness under N_2 at 40°. The residues were dissolved in 500 μl or 600 μl of methanol. Aliquots (40–100 μl) were chromatographed on an ODS-2 column using methanol-phosphate buffer as described above. Eluate fractions (1 ml) corresponding to the absorbance peaks of DNP-mercapturate (R_T 13 min) and component A (R_T 15 min), and representing *ca.* 60% and *ca.* 5% of injected ^{3}H respectively, were combined. They were concentrated *ca.* twofold under N_2 at 40° and extracted with

ethyl acetate. The two components were further purified by re-chromatographing them on the methanol–phosphate buffer gradient. DNP-mercapturate was also isolated from the bladder urine (7 ml) and bile (5–6 ml) of anaesthetized rats (dosed i.v., 5 mg/kg or 25 mg/kg) by solvent extraction and reversed-phase HPLC.

Dinitrophenyl-*S*-glutathione (DNP-GT) was isolated from the bile of cannulated rats dosed i.v. with 5 mg/kg or 25 mg/kg ^{3}H-DNFB. Bile (5–6 ml 0–3 hr collections) that had been acidified and extracted to obtain DNP-mercapturate was evaporated to dryness *in vacuo* at *ca.* 40°. The residue was dissolved in water (600 μl), and aliquots (40–70 μl) were chromatographed on an ODS-2 column with a gradient of methanol in 1% (v/v) aqueous acetic acid (30% for 2 min, 30–60% at 2%/min). Fractions containing the metabolite (R_T 15 min) were combined, evaporated to low volume, diluted with methanol (final volume 500 μl) and re-chromatographed on a methanol–1% (v/v) aqueous acetic acid gradient (30% for 7 min, 30–60% at 2%/min). The metabolite was recovered by evaporation of the eluate solvent.

DNP-mercapturate and component A isolated from urine, and metabolites extracted into ethyl acetate from bile (5.5 ml 0–5 hr collections, 25 mg/kg dose) acidified to pH 2.0 with HCl, were dissolved in methanol and reacted with excess diazomethane (from Diazald [14]) at room temperature for 15–30 min. The derivatives were isolated by reversed-phase HPLC using either the methanol–phosphate buffer gradient or a methanol–water (or 1% acetic acid) gradient (30%–60% at 2%/min). Methylated component A was resolved into three ^{3}H-labelled derivatives (R_T 18, 22 and 26 min; approximate ^{3}H-activity ratio 14:17:15, respectively) on an ODS-2 column eluted with methanol–acetic acid. The principal 360 nm-absorbing derivative, representing *ca.* 35% of the radiolabelled material in the mixture, had the same R_T (22 min) as the synthetic methyl ester of acetyl DNP-lys. It was isolated using the methanol–acetic acid gradient: the eluate fractions were combined and the solvent removed *in vacuo*. The isolated material (*ca.* 20 μg, 36×10^3 d.p.m. ^{3}H) was chromatographically (absorbance monitored at 360 nm) and radiochromatographically homogeneous. Methylated biliary metabolites having the same R_T as the synthetic methyl esters of DNP-mercapturate and acetyl-DNP-lys were isolated using two and three elutions, respectively. All the isolated derivatives were analysed by direct-insertion CI mass spectrometry.

Samples of the isolated metabolites (*ca.* 75–1000 μg) and derivatives (*ca.* 10–250 μg) were variously analysed by mass spectrometry, nuclear magnetic resonance spectrometry and u.v.–visible spectroscopy (samples dissolved in 0.1 M sodium phosphate buffer, pH 7.5, and spectra recorded on a Pye Unicam SP-8100).

Enzymic hydrolysis of acetyl-DNP-lys. Confirmatory evidence for the excretion of acetyl-DNP-lys was sought using enzymic hydrolysis. Radiolabelled material co-chromatographing with added acetyl-DNP-lys was isolated from first, second or third day urine and incubated with acylase 1. This enzyme selectively hydrolyses N^2-acetyl derivatives of amino acids with alkyl side chains [15, 16]. It rapidly hydrolysed authentic N^2-acetyl-N^6-DNP-lysine (acetyl-DNP-lys) but, contrary to a previous report [17], it did not hydrolyse DNP-mercapturate. Urine (0.5–13 ml) was acidified and extracted as above. The combined extracts were evaporated, redissolved in methanol (150 μl or 175 μl) containing tracer quantities of unlabelled acetyl-DNP-lys, and aliquots chromatographed on an ODS-2 column eluted with the methanol-phosphate buffer gradient. The acetyl-DNP-lys (R_T 15 min) was extracted from concentrated eluate into ethyl acetate. The residue obtained after evaporation of the solvent (25–100×10^3 dpm ^{3}H, *ca.* 0.03–0.1 μmol acetyl-[^{3}H] DNP-lys) was dissolved in 150–250 μl of 0.1 M sodium phosphate buffer, pH 7.5, containing 300–1400 Units of acylase 1 (200–600 Units/100 μl) and incubated at 37° for 15–22 hr. Aliquots were analysed by HPLC.

Mass spectrometry. Low-resolution fast-atom (xenon) bombardment (FAB) and chemical ionization (CI) mass spectra of reference compounds and metabolites were obtained with a V.G. Micromass 70-70F instrument. The spectrometer was interfaced to a Finnigan Incos Data System. An Ion-Tech Ltd. fast atom gun was used. For FAB in the negative-ion mode the conditions were: discharge, 2 mA, 7×10^3 eV; mass spectrometer accelerating voltage, 2×10^3 V; scanning range, 80–1400 m/z; source pressure, *ca.* 5×10^{-6} Torr. Samples were solubilized in either glycerol or polyethylene glycol 200. For CI (isobutane reagent gas) the conditions were: electron energy, 50 eV; filament current, 500 μA; accelerating voltage, 4×10^3 V; scanning range, 70–550 m/z; source temperature, 200°; source pressure, 5×10^{-5} Torr.

Nuclear magnetic resonance (NMR) spectroscopy. ^{1}H-NMR spectra were recorded on a Brüker Model 360 instrument at 360 MHz. Samples were dissolved in d_4-methanol.

RESULTS

Excretion and tissue distribution of radioactivity

Following the i.v. administration of 5 μg/kg, 5 mg/kg and 25 mg/kg ^{3}H-DNFB to bile-duct cannulated rats, 26.2 ± 4.9% (mean ± S.D., N = 4), 24.0 ± 4.6% (N = 6) and 26.0 ± 4.9% (N = 4), respectively, of the radioactive dose was excreted in bile over three hours. After three hours, 31.1 ± 10.0%, 22.6 ± 5.7% and 9.7 ± 2.5%, respectively, was recovered in their bladder urine. The corresponding urinary recoveries from i.v.-dosed rats without bile-duct cannulae were 27.3 ± 2.6% (5 μg/kg; N = 4), 27.0 ± 4.9% (5 mg/kg; N = 5) and 5.7 ± 4.0% (25 mg/kg; N = 5).

Conscious rats dosed i.v. with 5 mg/kg ^{3}H-DNFB eliminated 70.9 ± 14.2% of the radioactive dose in urine over four days; rats dosed with 25 mg/kg excreted 50.8 ± 6.8% (Table 1).

The rapid clearance of radioactivity (5 μg–25 mg/kg doses) from the plasma of anaesthetized rats without bile-duct cannulae is shown in Fig. 1. After 5 min, only 1.0–3.2 %dose/ml remained; and this fell to 0.3–1.0 %dose/ml at 3 hr.

Table 1. Urinary excretion of radioactivity after intravenous administration of ^{3}H-dinitrofluorobenzene to conscious rats

Dose (per kg)	Collection period (hr) 0–24	24–48	48–72	72–96	0–96
5 mg (N = 9)	63.6 ± 14.0	3.9 ± 0.6	2.2 ± 0.6	1.2 ± 0.2	70.9 ± 14.2
25 mg (N = 4)	43.3 ± 7.4	5.2 ± 0.8	1.6 ± 0.6	0.7 ± 0.2	50.8 ± 6.8

Values are mean % administered dose of radioactivity ± S.D. (N = number of animals per experiment).

The tissue distribution of radioactivity (5 μg–25 mg/kg doses) in anaesthetized rats with and without bile-duct cannulae three hours after dosing is shown in Fig. 2. In individual animals, the radioactivity in the organs examined amounted to 2.5–5.5% (5 μg/kg), 3.2–6.7% (5 mg/kg) and 4.3–7.8% (25 mg/kg) of the doses; the liver and kidneys contained the greatest quantities: 1.2–3.8% and 0.7–4.1%, respectively (combined data for three doses). No radioactivity was detected in brain. The kidneys contained the highest concentrations (% dose/g) of radiolabelled material, but the greatest proportional increases in concentrations with dose occurred in the spleen, heart and lungs.

Irreversible binding of ^{3}H-labelled material to plasma and tissue protein

Three hours after the i.v. administration of 5 μg–25 mg/kg ^{3}H-DNFB to anaesthetized rats, small amounts of radiolabelled material were irreversibly bound to plasma protein and tissue protein (Fig. 2). Only 15–25% of the radiolabelled material in the liver and kidney was irreversibly bound (representing 0.2–1.3% and 0.1–0.6% of the doses, respectively), but 45–99% of that in spleen, heart and lung was bound. The values for plasma were 49–88%.

Biliary metabolites

DNP-GT and DNP-mercapturate, identified by co-chromatography on an ODS-2 column, were the principal biliary metabolites of i.v.-administered ^{3}H-DNFB over the dose range 5 μg–25 mg/kg (Fig. 3, Table 2). Analysis of serial 30-min fractions revealed a rapid metabolism of DNP-GT to DNP-mercapturate: the proportions of mercapturate in 0–30 min and 4.5–5 hr fractions were 26–42% and 35–54% respectively (combined data for 5 mg/kg and 25 mg/kg doses). One of the minor ^{3}H-activity peaks (R_T 10 min, 4–6% 0–3 hr ^{3}H) co-chromatographed with N^6-DNP-lysine; in conformity with this, most of the radioactivity was not extracted into ethyl acetate at pH 2.0. Another minor component of bile from rats

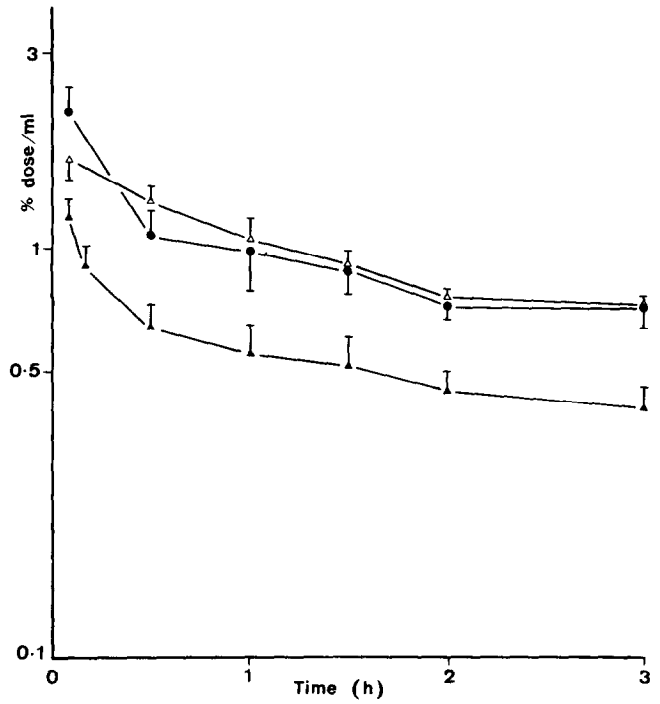

Fig. 1. Clearance of radioactivity from the plasma of anaesthetized male rats after i.v. administration of 5 μg/kg (▲, N = 8), 5 mg/kg (●, N = 10) and 25 mg/kg (△, N = 5) ^{3}H-DNFB. Points represent means, bars indicate S.E.M.

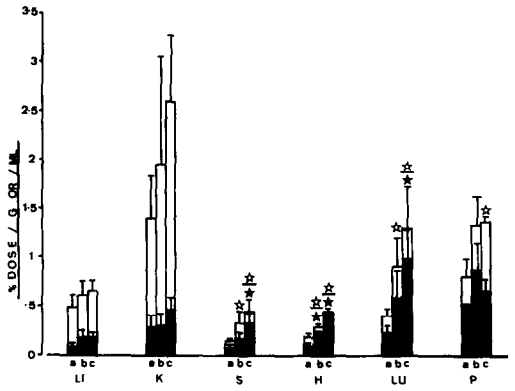

Fig. 2. Tissue distribution (open columns) and irreversible binding (closed columns) of radiolabelled material in anaesthetized rats 3 hr after administration of ^{3}H-DNFB (5 μg (a), 5 mg (b) or 25 mg/kg (c) body wt; data for rats with and without bile-duct cannulae combined). Data expressed as % dose ^{3}H-DNFB per gram of tissue or per ml of plasma. LI, liver; K, kidney; S, spleen; H, heart; Lu, lung; P, plasma. Stars (open for total radioactivity, closed for irreversibly bound radioactivity) indicate statistical difference ($P < 0.001$, Student's t-test) from 5 μg/kg dose.

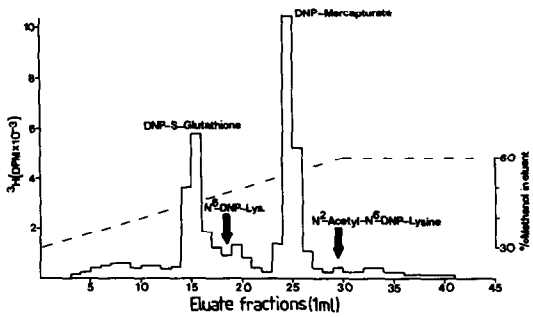

Fig. 3. High-performance liquid chromatogram of the biliary metabolites of ^{3}H-DNFB in the rat. ^{3}H-DNFB (5 μg/kg) was administered i.v. to an anaesthetized male rat with a bile-duct cannula, and bile collected for three hours. Bile was analysed on a reversed-phase column eluted with a gradient (broken line) of methanol in phosphate buffer. Dinitrophenyl (DNP)-*S*-glutathione was identified by FAB mass spectrometry; DNP-mercapturate by FABms and by CIms of its methyl ester.

given 25 mg/kg ^{3}H-DNFB was chromatographically similar to acetyl-DNP-lys. However, attempts to isolate this material by HPLC were frustrated by its instability.

Urinary metabolites

DNP-mercapturate was the principal urinary metabolite of ^{3}H-DNFB given to anaesthetized (5 μg/kg, 5 mg/kg and 25 mg/kg) and conscious (5 mg/kg and 25 mg/kg) rats (Fig. 4). The data for bile-duct cannulated animals are recorded in Table 2. In anaesthetized animals without bile-duct cannulae, its proportions were 36–37% (N = 2), 75 ± 4% (mean ± S.D., N = 4) and 59 ± 7% (N = 5) at 5 μg, 5 mg and 25 mg/kg respectively. DNP-mercapturate represented 61 ± 2% (N = 4) and 18 ± 2% (N = 4) of 0–24 hr and 24–48 hr urinary ^{3}H-activity, respectively, in conscious rats given 5 mg/kg ^{3}H-DNFB i.v. At an i.v. dose of 25 mg/kg, the corresponding data were 39 ± 10% (N = 4) and 18 ± 7%. A minor metabolite co-chromatographed with DNP-*S*-cysteine. One of the other minor urinary components (A) in conscious rats attracted particular attention because its chromatographic characteristics were similar to those of acetyl-DNP-lys (Fig. 4b). Component A's proportions in 1st and 2nd-day urine were 3–5% at 5 mg/kg and 3–11% at 25 mg/kg. Another component (R_T 10 min) was chromatographically similar to DNP-lys, but could not have been this conjugate since the latter was extractable into ethyl acetate at pH 2.0.

Analysis of metabolites

The FAB spectrum of DNP-GT (*ca.* 400 μg) contained diagnostic peaks at m/z 472([M-1]$^-$; relative intensity (RI) 29%) and m/z 199 ([DNPS]$^-$; RI 61%). It closely resembled the FAB spectra of other thioethers [18, 19].

DNP-mercapturate (*ca.* 400–1000 μg) from the urine of conscious and anaesthetized rats and from bile yielded an FAB spectrum with diagnostic peaks at m/z 328 ([M-1]$^-$; RI 5–18%) and m/z 199 ([DNPS]$^-$; RI 100%). The ^{1}H-NMR spectrum of metabolite (*ca.* 400 μg) isolated from 0–24 hr urine contained chemical shifts characteristic of the *N*-acetylcysteine moiety and aromatic (Ar) protons: δ 8.99 (s,1H,ArH), 8.46 (d,1H,ArH), 7.95 (d,1H,ArH), 4.74 (double doublet, CH), 3.76 and 3.45 (double doublets, 2H, CH_2) and 1.98

Table 2. HPLC analyses of biliary and urinary thioether metabolites of ^{3}H-DNFB in anaesthetized rats

Metabolite	% Radioactivity in column eluate		
	5 μg/kg (N = 4)	5 mg/kg (N = 6)	25 mg/kg (N = 4)
DNP-Glutathione (Bile)	25 ± 2	27 ± 5	33 ± 3
DNP-Mercapturate (Bile)	40 ± 9	37 ± 7	43 ± 4
DNP-Mercapturate (Urine)	41 (19–50)	61 ± 6	69 ± 7

Values are mean % ± S.D. except for DNP-mercapturate (urine) at 5 μg/kg (N = number of animals). ^{3}H-DNFB was administered i.v. to anaesthetized rats with bile-duct cannulae. Bile was collected over 3 hr. Urine was removed from the bladder after 3 hr. The metabolites were analysed by reversed-phase HPLC.

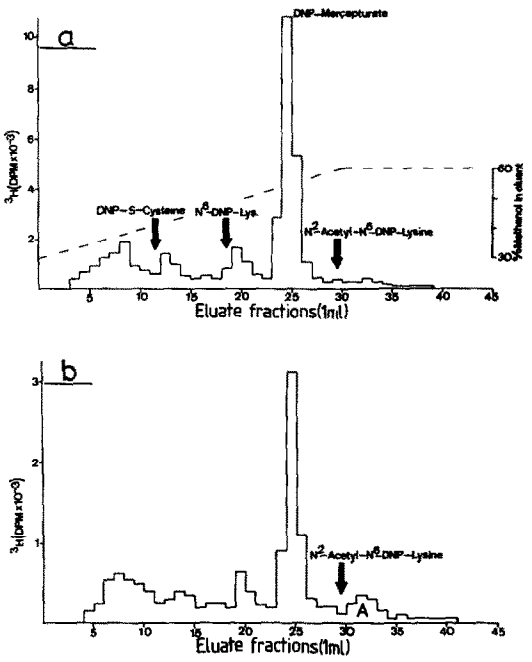

Fig. 4. High-performance liquid chromatograms of the urinary metabolites of ^{3}H-DNFB in the rat. (a) ^{3}H-DNFB (5 μg/kg) was administered i.v. to an anaesthetized male rat with a bile-duct cannula, and bladder urine removed at three hours. (b) ^{3}H-DNFB (25 mg/kg) was administered i.v. to a conscious male rat, and urine collected for 24 hr. Urine was analysed on a reversed-phase column eluted with a gradient (broken line) of methanol in phosphate buffer. DNP-mercapturate was identified by mass and NMR spectrometry. Component A in the urine of conscious rats appeared to be heterogeneous, but enzymic hydrolysis with acylase 1, FABms and CIms of its principal methylated derivative indicated the presence of N^2-acetyl-N^6-DNP-lysine.

(s,3H,$COCH_3$)ppm (cf. [20, 21]). The metabolite's λ_{max} (aqueous buffer, pH 7.5) was 345 nm, the same as that of authentic DNP-mercapturate. Its methyl ester co-chromatographed with the authentic methyl ester of DNP-mercapturate (R_T 16 min) on an ODS-2 column and gave a CI spectrum with the base peak at m/z 344 ($[M + 1]^+$) and the major fragment ion at m/z 144 ($[M + 1 - DNPSH]^+$).

Component A's FAB spectrum (enhanced fivefold) contained a peak at m/z 353 (RI 1.6%) corresponding to the pseudo-molecular $[M - 1]^-$ ion of acetyl-DNP-lys. The CI spectrum of the principal methylated derivative included all of the major ions in the CI spectrum of the authentic methyl ester of acetyl-DNP-lys (Fig. 5): a protonated molecule was present at m/z 369 ($[M + 1]^+$, base peak), an adduct ion at m/z 409 ($[M + C_3H_5]^+$) and fragment ions at m/z 339 ($[M + 1 - 30]^{+\cdot}$, $M + 1 - NO^{\cdot}$) and m/z 202 ($[M + 1 - 167]^{+\cdot}$, $M + 1 - DNP^{\cdot}$). The methyl ester of the principal biliary metabolite of dinitrophenylated human serum albumin also yielded this CI spectrum [8]. An $[M + 1]^+$ base peak, allylated ion ($[M + C_3H_5]^+$) and $[M + 1 - 30]^{+\cdot}$ ion formed by loss of $NO^{\cdot}$ from a protonated nitro group are characteristic of the alkane CI spectra of nitroarenes [22]. A number of ions in the methylated metabolite's spectrum were absent from that of the authentic compound and could not be assigned. Since the sample of methylated metabolite was radio-chromatographically homogeneous, it is probable that these derived from a co-eluting endogenous urinary compound(s). The CI spectrum of the material isolated from the methylated extract of bile, although clearly contaminated, contained a peak at m/z 369 (RI 30%). Enzymic hydrolysis of authentic acetyl-DNP-lys and co-chromatographing ^{3}H-labelled material from first-third day urine provided additional evidence that acetyl-DNP-lys was a metabolite of DNFB. Extensive or very nearly complete hydrolysis of the isolated material to ^{3}H-labelled DNP-lys, identified by co-chromatography with authentic DNP-lys, was obtained, if necessary by incubation with additional enzyme for up to 43 hr.

DISCUSSION

During the present study, dinitrofluorobenzene (DNFB), because of its high chemical reactivity [23], was used as a model of a chemically reactive metabolite formed *in vivo*. Its use in this respect was supported by its display of two of the characteristic reactions of electrophilic reactive metabolites, viz. irreversible binding to proteins [1] and formation of glutathione conjugates [24]. Such reactive metabolites as those formed *in vivo* are, in most cases, likely to be too reactive for a study such as this. A probable exception is *N*-acetyl-p-benzoquinoneimine, the putative reactive metabolite of paracetamol [25]. The specific reason for using DNFB was the finding that the products of its reaction with serum albumins *in vitro* were degraded to the novel amino acid conjugate N^2-acetyl-N^6-dinitrophenyllysine (acetyl-DNP-lys) *in vivo* [6–8]. This clearly suggested that DNFB might react with lysine residues *in vivo* and be eliminated as acetyl-DNP-lys; and hence presented the possibility that similar amino acid conjugates might be used as indices of irreversible protein modification by drugs *in vivo*. DNFB arylates the C-2 amino groups of proteins and several of their side-chain functional groups, viz. C-6 amino groups, imidazoles, sulphydryls and aliphatic and phenolic hydroxyls [26]. Under mild conditions (pH 8.5, 25°) it selectively arylates two of the C-6 amino groups of lysine residues in serum albumin [9].

Following i.v. administration, small proportions of the DNFB became irreversibly bound to plasma and tissue protein. A notable feature of the binding was the considerable difference between its proportions in liver and kidney and those in other tissues and plasma. This disparity can be partly rationalized in terms of the route of administration and competition between protein and reduced glutathione (GSH). The large proportions of bound material in heart and lung conform with the i.v. administration and the low concentration of GSH in plasma [27]: little of the DNFB would be conjugated before it reached these organs. The much lower proportions in liver and kidney reflect the tissue's high concentrations of GSH [28, 29] and high activities of glutathione-*S*-transferases (GTase) [30].

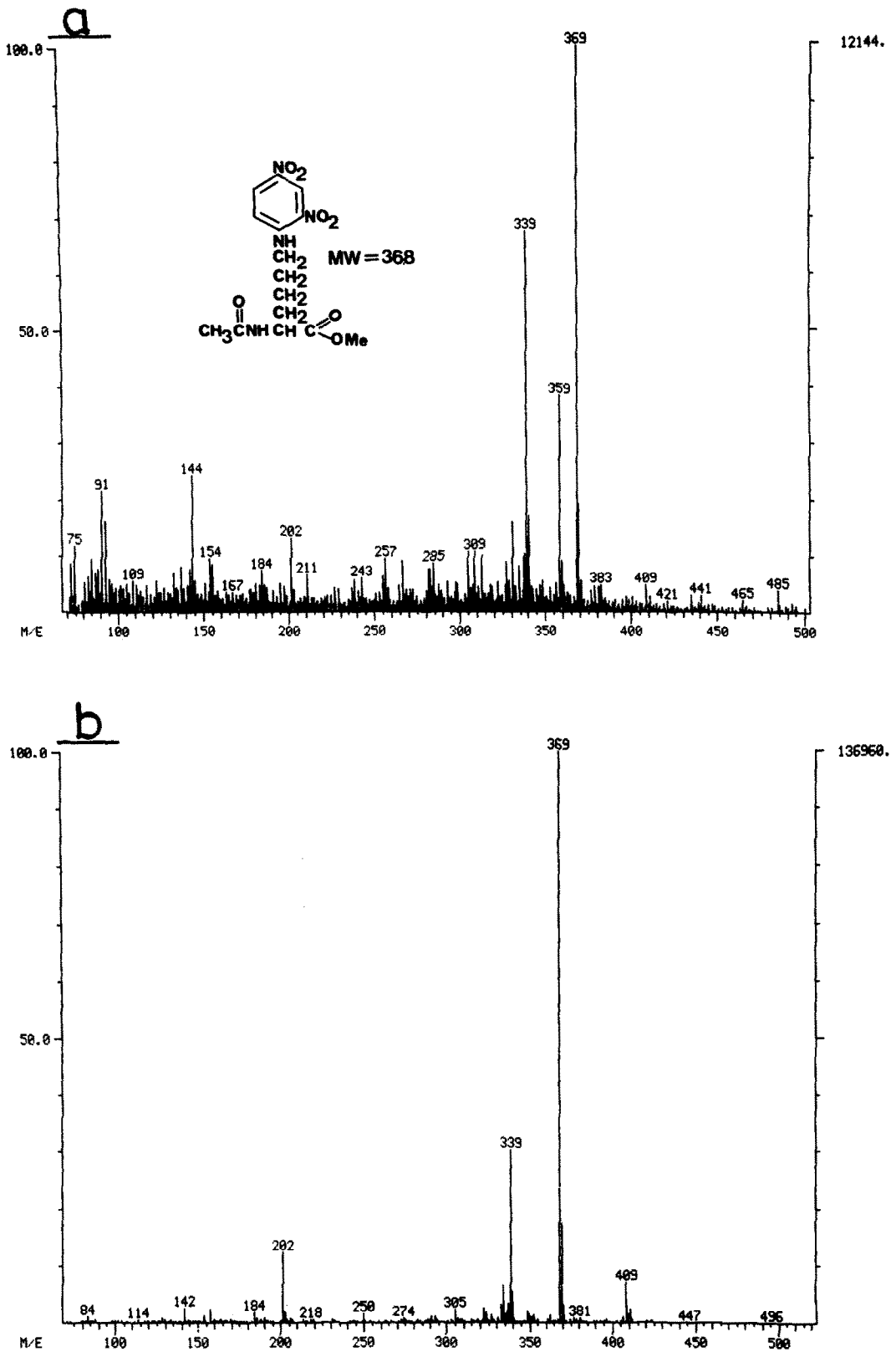

Fig. 5. CI spectra of (a) the principal methylated derivative of component A isolated from the 0–24 hr urine of conscious rats, (b) authentic methyl ester of N^2-acetyl-N^6-dinitrophenyl-lysine. The derivatives were prepared using diazomethane. The reagent gas was isobutane. See text for peak assignments.

Kitteringham *et al.* [6, 7] have shown that the degree of dinitrophenylation of synthetic DNP-albumin conjugates affects their clearance from rat blood: conjugates bearing >20 moieties are cleared considerably faster than those with 10 or 0.5. However, it is not anticipated that the extent of conjugation following DNFB administration will alter the clearance of plasma proteins. The stability of the DNP-protein linkage will depend upon the amino acid moiety involved: DNP-side chain derivatives of histidine, tyrosine and cysteine readily undergo thiolysis whilst derivatives involving C-2 and C-6 (lys) amino groups are much more stable [31].

Conjugation with GSH and subsequent metabolism to mercapturates is a major route of biotransformation of electrophilic compounds and metabolites [32, 33]. In accordance with this, ^{3}H-DNFB given i.v. to rats was rapidly and considerably metabolized via the mercapturic acid pathway and depleted hepatic GSH [34]. Reaction with GSH is catalyzed by GTase, and DNFB is an excellent substrate for mouse hepatic GTase [23]. The chloro congener of DNFB, 2,4-dinitrochlorobenzene, is avidly conjugated by three out of five rat GTase isoenzymes [35] and by GTase in several rat tissues [30]. Compounds with highly electrophilic centres, such as DNFB [23] and 2,4-dinitrobromobenzene (DNBB) [17], also undergo non-enzymic conjugation with GSH. Hence DNFB conjugation *in vivo* may involve both types of reaction. The early appearance of substantial proportions of DNP-mercapturate in bile revealed rapid hydrolysis of DNP-GT and N-acetylation of DNP-cysteine; cysteine *N*-acetyltransferase from rat has a high affinity for DNP-cysteine [36]. DNP-mercapturate is in addition the principal urinary metabolite of DNBB in rats [17]. Thus the major route of dinitrohalobenzene biotransformation exemplifies that of many arenes with strong electron withdrawing groups, e.g. mono- and dihalonitrobenzenes [37], pentachloronitrobenzene and hexachlorobenzene [38]. DNP–mercapturate (identified by HPLC) is a minor metabolite of DNP–protein conjugates [7]. Although it is probably derived from DNP–cysteine residues, it might in part be formed by thiolysis of other DNP–protein linkages [31]. The formation of DNP-*S*-glutathione from DNP–protein conjugates [7] can be explained by thiolytic transfer of the DNP group from the conjugate to glutathione. In preliminary studies, it was found that some of the DNP groups were released from conjugates incubated with glutathione (unpublished observations).

Taken collectively, the HPLC, mass spectrometric and enzymic hydrolysis data indicated that acetyl-DNP-lys was a minor urinary metabolite of DNFB. Additionally, there was tentative evidence for its excretion in bile. Its urinary excretion was characterized by a delay of a few hours: component A was absent from 0–3 hr urine but present in 0–24 hr collections. Estimating the formation of acetyl-DNP-lys was complicated by the finding that the chromatographically resolved material (component A) appeared to be heterogeneous: it yielded three radiolabelled derivatives in approximately equal proportions. In which case not more than *ca.* 1–3% of the radiolabelled material excreted in 0–48 hr urine following administration of 5 mg/kg–25 mg/kg ^{3}H-DNFB would have been acetyl-DNP-lys. An N^2-acetyl-lysine conjugate of a xenobiotic has not been reported previously, although an N^2-acetylornithine conjugate of 3-phenoxybenzoic acid has been described [39]. The non-specific reactivity of DNFB [26] and the excretion of several minor metabolites in addition to acetyl-DNP-lys (as revealed by HPLC) indicate that other novel amino acid conjugates might be formed.

The acetyl-DNP-lys was presumably formed in one or more of three ways: (1) by N^2-acetylation of N^6-DNP-lys liberated upon degradation of DNP-protein adducts, (2) by N^2-acetylation of N^6-DNP-lys formed by the reaction of lysine with DNFB, (3) by the reaction of N^2-acetyl-lys with DNFB. Enzymic acetylation of lysine occurs *in vitro* [40] and N^2-acetyl-lys is excreted in human urine [41]. N^6-DNP-lys undergoes extensive metabolism and urinary elimination in rats but the metabolites have not been characterized [42]. The delayed excretion of acetyl-DNP-lys conforms with the first mechanism. A precedence for the urinary excretion of modified amino acid residues is provided by the elimination of *N*-methylated amino acids, e.g. N^6-trimethyl-lys, upon degradation of endogenous methylated protein [43]. In addition, N^6-trimethyl-lys residues are metabolized to N^2-acetyl-N^6-trimethyl-lys and N-acetyl-carnitine [44]. An alternative mechanism for the elimination of modified amino acid residues is indicated by the suggestion that the urinary metabolites of 1-(2-chloroethyl)-3-alkyl-1-nitrosoureas include pepetide conjugates derived from carbamylated proteins [45].

These findings suggest that other compounds reacting with the C-6 amino group of lysine residues, e.g. chloramphenicol [46], thioacetamide [47], 2,5-hexanedione [48], and 16α-hydroxyesterone [49], may also be excreted as N^2-acetyl-lys conjugates.

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