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DRUG–PROTEIN CONJUGATES—VIII

THE METABOLIC FATE OF THE DINITROPHENYL HAPTEN CONJUGATED TO ALBUMIN*

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Abstract—Dinitrofluorobenzene has been used as a model chemically reactive metabolite to investigate factors which determine the fate of drug–protein conjugates formed *in vivo*.

The disposition of homologous and heterologous albumin conjugated with ^{3}H-dinitrophenyl groups (^{3}H-DNP) has been investigated in the male Wistar rat. After intravenous administration, conjugates were cleared from plasma, predominantly through the liver, and the hapten was excreted into bile and urine as the novel amino acid derivative N^2-acetyl-N^6-DNP-lysine. It is assumed that the product arises from lysosomal hydrolysis of the conjugate, followed by *N*-acetylation, since its biliary excretion was significantly reduced in animals pretreated with suramin, an inhibitor of lysosomal proteolysis. The clearance of DNP–albumin conjugates was dependent upon the degree of conjugation; conjugates with an epitope density of >20 had a short (*ca.* 1 hr) half-life.

In a second study, the disposition of DNP-autologous protein conjugates was monitored in the rabbit over 21 days. The plasma concentration–time profile of the serum conjugates indicated that clearance was dependent upon non-immune mechanisms and that intravenous administration of DNP–serum protein conjugates did not elicit an anti-DNP response.

Allergic drug reactions represent a major clinical problem, and it has been estimated that between 35 and 50% of adverse drug reactions have an immunological basis [1]. This is surprising since most drugs are low-molecular-weight organic compounds and are therefore unlikely to be immunogenic *per se*. To account for this apparent discrepancy, it has been suggested that drugs become covalently attached to macromolecular carriers, such as proteins, and are thereby able to act as haptens, antigens and immunogens. This concept is the basis of the "hapten hypothesis" of drug hypersensitivity, which was derived from classical immunochemical studies using compounds such as dinitrofluorobenzene (DNFB) and penicillin, both of which react readily with nucleophilic groups on proteins [2, 3]. However, since most drugs do not react directly with proteins, it is necessary to assume that a chemically reactive metabolite is formed *in vivo*, which is able to form immunogenic protein conjugates. In the last twenty years it has been clearly established that chemically reactive metabolites are mediators of chemical carcinogenesis [4] and necrosis [5, 6]. In contrast, the role of such metabolites in immunotoxicity is less well-defined.

It is thought that conjugation of drugs to either autologous macromolecules or cells produces a loss of tolerance to the macromolecule and hence an immune response. However, little is known about the relationship between either the nature or extent of conjugation and antibody response during chronic drug administration, and even less about the effect of epitope density on the disposition of conjugates. Therefore, the purpose of this study was to investigate the effect of epitope density on the disposition and metabolic fate of hapten–protein conjugates prepared from heterologous, homologous and autologous proteins.§ The hapten used was the dinitrophenyl group derived from DNFB, which is a mild arylating agent widely employed as a hapten in immunochemical studies. Such monohalogenated dinitrobenzenes are used in drug metabolism studies as substrates for glutathione transferase [7]. Thus, in the context of drug-induced hypersensitivity reactions, DNFB may be regarded as a model chemically reactive metabolite.

MATERIALS AND METHODS

2,4-[3,5-^{3}H]Dinitrofluorobenzene ([^{3}H]DNFB), sp. act. 16.6 Ci/mmole, was obtained from Amersham International plc (Amersham, U.K.). Human serum albumin (HSA, fraction V), unlabelled DNFB, N^6-2,4-dinitrophenyl-L-lysine (DNP-lys) and acylase 1 (*N*-acylamino acid amidohydrolase, Grades

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§ Glossary of terms: heterologous protein, a protein obtained from an animal of one species and administered to an animal of a different species; homologous protein, a protein obtained from an animal of one species and administered to a second animal of the same species; autologous protein, a protein obtained from and administered to the same individual animal; epitope density, the number of groups of a low molecular weight compound covalently linked to a protein molecule.

I and II from porcine kidney) 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). Scintillation fluid (Scintillator 299) was from Packard-Becker B.V. (Groningen, The Netherlands). Suramin, the symmetrical 3″ urea of sodium 8-(3-benzamido-4-methylbenzamido)naphthalene-1,3,5-trisulphonate, was a gift from Bayer A.G. (Leverkusen, F.R.G.). All other reagents were of analytical grade. Dinitrophenyl derivatives of *N*-acetylcysteine, *N*-acetyl lysine, glutathione and cysteine (*S*-linked), were prepared and characterised as described previously [8–11].

Synthesis of [^{3}H]DNP–protein conjugates. HSA conjugates with three different [^{3}H]DNP:protein molar ratios were prepared. HSA (100 mg) dissolved in 10 ml 0.1 M sodium phosphate buffer, pH 7.4, was mixed with 0.16, 5 or 10 mg of [^{3}H]DNFB dissolved in 0.5 ml dioxan (100 μCi/ml) and incubated overnight at 37° in a shaking waterbath. The solutions were dialysed against flowing tap water for 72 hr to remove any unreacted [^{3}H]DNFB, and were then dialysed against distilled water (2 × 5 l.) for 24 hr. The dialysed solutions were lyophilized. The degrees of conjugation (DNP:HSA) were calculated by determining the specific activities of the lyophilized protein conjugates, and were found to be 0.5:1 (HSA-[^{3}H]DNP$_{0.5}$), 10:1 (HSA-[^{3}H]DNP$_{10}$) and 20:1 (HSA-[^{3}H]DNP$_{20}$) for the three conjugates. In order to estimate the amounts of covalently bound ^{3}H-labelled material, 1 mg of conjugate was dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.4, containing 2% (w/v) sodium dodecyl sulphate (SDS) and boiled for 15 min. Thereafter the solution was dialysed overnight against 1 l of 0.01 M phosphate buffer, pH 7.0, containing 0.1% (w/v) SDS. The loss of radioactivity, representing non-covalently bound material, was calculated after correction for protein dilution. For all three conjugates, covalent binding was >90%. This was confirmed by chromatography on silica plates (0.2 mm thick Merck, Darmstadt, F.R.G.) in propanol:water:acetic acid (90:10:5, v/v) which resolved 2,4-dinitrophenol (R_f 0.76) and DNFB (R_f 0.88) but left >90% of the radioactivity of the protein conjugate preparations at the origin. The purity of the protein conjugates was assessed by HPLC: samples were eluted from a gel filtration column (packed with TSK G3000 SW, Toyo Soda Manufacturing Company, Japan) with sodium phosphate buffer (0.1 M; pH 7.4) at 1 ml/min. More than 90% of the radioactivity was associated with a single peak of u.v. absorbance (280 nm) which had the same retention time (18 min) as unmodified HSA.

Rat serum albumin (RSA) conjugates were prepared from freshly purified RSA. Rats were anaesthetized with ether and exsanguinated by cardiac puncture. The serum was chromatographed on an affinity chromatography column (packed with Cibacron Blue-agarose equilibrated with Tris–HCl) and RSA was eluted with Tris–HCl (0.02 M; pH 8.0) containing 0.2 M sodium isothiocyanate at 4°. It was dialysed against flowing tap water for 48 hr and then against distilled water (2 × 5 l) for 24 hr. RSA-[^{3}H]-DNP conjugates were prepared from the lyophilized purified albumin as described above, producing [^{3}H]-DNP:RSA ratios of 0.6:1 (RSA-[^{3}H]DNP$_{0.6}$) and 24:1 (RSA-[^{3}H]DNP$_{24}$).

Rabbit autologous protein (RAP) conjugates were prepared from the serum of male New Zealand White rabbits (2–3 kg); blood (10 ml) was obtained from the marginal ear vein two weeks before administration of the conjugates. The protein concentration of serum from individual rabbits was estimated by the method of Lowry *et al.* [12], and serum containing 250 mg of protein was diluted to 25 ml with phosphate buffer, pH 7.4. [^{3}H]DNFB, 0.4, 12.5 and 25 mg (sp. act. 1.0, 30 and 60 μCi/mg respectively) dissolved in dioxan (0.75 ml), was added to each serum solution and the mixture was incubated at 37° overnight in a shaking water bath. The conjugates were dialysed and lyophilized as above, and the degrees of conjugation were found to be 0.5:1 (RAP-[^{3}H]DNP$_{0.5}$), 15:1 (RAP-[^{3}H]DNP$_{15}$) and 30:1 (RAP-[^{3}H]DNP$_{30}$).

Disposition of [^{3}H]DNP–protein conjugates in rats. Male Wistar rats (200–300 g) were anaesthetized with urethane (1.4 g/kg body weight in 0.15 M NaCl; 10 ml/kg i.p.), and their tracheae, carotid arteries, external jugular veins and bile ducts were cannulated. Following heparinization (400 i.u./kg), the animals received [^{3}H]DNP–protein conjugate (5 mg) via the jugular vein. Blood samples (0.4 ml) were removed at 5, 30, 60, 120 and 180 min and immediately centrifuged to obtain plasma. Fluid loss was made good with 0.15 M NaCl after each sampling. Bile was collected in 30 min fractions for 3 hr. After 3 hr the animals were killed and their livers, kidneys, hearts, lungs and spleens removed. Urine was removed from the bladder by aspiration.

Duplicate samples of plasma, bile and urine (50 μl) were assayed for radioactivity by liquid scintillation spectrometry in 4 ml of scintillant. The irreversible binding of ^{3}H-labelled material to plasma protein was determined by dialysis in the presence of SDS as described above. Samples (*ca.* 50 mg) of the organs were solubilized in NCS solubilizer (1 ml) overnight at 50°. They were decolorized with hydrogen peroxide (250 μl), neutralized with glacial acetic acid (30 μl) and dissolved in scintillation fluid (12 ml) for the determination of radioactive content.

The effect of suramin on the disposition of HSA-[^{3}H]DNP$_{20}$ in rats. Male Wistar rats (320–370 g) were given suramin (250 mg/kg i.p.), an inhibitor of lysosomal proteolysis [13], in saline (0.15 M; 1 ml/100 g) 24 hr prior to anaesthetization and administration of HSA-[^{3}H]DNP$_{20}$ (5 mg) as described above. Control animals were injected with saline alone 24 hr before administration of the conjugate.

Disposition of [^{3}H]DNP–autologous protein conjugates in rabbits. [^{3}H]DNP conjugates were dissolved in sterile 0.15 M NaCl, centrifuged at 50,000 *g* for 2 hr to sediment aggregates and finally passed through 0.2 μm filters (Minisart, Sartorius GmbH, Göttingen, F.R.G.). Conjugates (200 mg in 3 ml) were injected into the marginal ear vein of the same male New Zealand White rabbit from which the original serum had been obtained. Blood samples (5–10 ml) obtained by venipuncture of the contralateral ear were collected into heparinized tubes; collections were made at particular times after con-

jugate administration for up to 22 days, depending upon the conjugate given. Aliquots (50–200 μl) of plasma were assayed for radioactivity. The criterion for the occurrence of an immunological response to the conjugates (through anti-DNP antibody formation) was the detection of a precipitous fall in plasma radioactivity occurring between days 10 and 14 following conjugate administration. Such an effect has been documented previously following administration of foreign proteins to rabbits [14, 15].

Analysis of biliary and urinary metabolites. The urinary and biliary metabolites of serum albumin-[^{3}H]DNP conjugates from bile duct-cannulated rats were analysed by reversed-phase HPLC. Metabolites were routinely separated with an Aerograph Model 8500 liquid chromatograph (Varian Instruments Division, Palo Alto, CA) linked to a Partisil® ODS-2 column (10 μm, 25 cm × 0.46 cm i.d., Technicol Ltd., Stockport, Cheshire, U.K.) protected by a guard-column of Co: Pell ODS (Whatman Inc., Clifton, NJ). Samples (5–200 μl) were eluted from the column with a linear gradient of methanol (30% to 60% at 2%/min) in ammonium dihydrogen phosphate buffer (0.043 M; pH 2.3) at a flow rate of 2 ml/min. The eluate was monitored at 360 nm with a Model LC3 u.v. absorbance detector (Pye Unicam Ltd., Cambridge, U.K.). Eluate fractions were collected at 30-sec intervals into scintillation vial inserts and scintillant (4 ml) added for measurement of radioactivity. Proportions of metabolites were expressed as a percentage of the eluate radioactivity. The ^{3}H-labelled metabolites were co-injected with solutions of authentic unlabelled compounds for identification by co-chromatography.

Samples (50–150 μl, 15–25 × 10^3 dpm) of bile from rats given either HSA-[^{3}H]DNP_{20} or RSA-[^{3}H]-DNP_{20} were analysed without prior treatment. Bile from rats given either HSA-[^{3}H]$DNP_{0.5}$ or RSA-[^{3}H]$DNP_{0.6}$ (0.3–0.7 ml, 10–35 × 10^3 dpm) was mixed with methanol (1:3, v/v) at 5° and centrifuged to sediment precipitated material. The supernatant was evaporated to *ca.* 100 μl under a stream of nitrogen at 40° and an aliquot (40 or 50 μl) chromatographed. Urine obtained by bladder puncture was either chromatographed (50 μl, 25–55 × 10^3 dpm) without prior treatment or first concentrated in the same manner as bile: up to 0.5 ml urine was concentrated to *ca.* 100 μl and 50 μl (30–40 × 10^3 dpm) analysed. Recoveries from the column of ^{3}H-labelled metabolites were 99 ± 9% (mean ± S.D., N = 14).

Purification and identification of N^2*-acetyl-* N^6*-dinitrophenyl-lysine.* Samples of the principal ^{3}H-labelled biliary and urinary metabolite of extensively conjugated serum albumin-[^{3}H]DNP were obtained by HPLC. Preliminary chromatographic analyses showed it to be identical to N^2-acetyl-N^6-dinitrophenyl-lysine (acetyl-DNP-lys). In keeping with its postulated structure, the metabolite was readily extracted into ethyl acetate following acidification of bile and urine to pH 2.0. To prepare the metabolite from bile, 26 ml (8.5 × 10^6 dpm) were collected over 3–10 hr from eight anaesthetized rats each given 5 mg of HSA-[^{3}H]DNP_{20}. The pH of bile aliquots (1–21 ml) was adjusted from 7.7–9.3 to 2.0 with HCl, and the resulting precipitate separated by centrifugation. The supernatant was saturated with sodium chloride and twice extracted with redistilled ethyl acetate (3:2, v/v); the recovery of ^{3}H in ethyl acetate was 68–81%. Pooled extracts were evaporated to *ca.* 5 ml under nitrogen at 40°, extracted with water (0.2 ml) and finally evaporated to dryness. Residues, containing 0.4–4.0 × 10^6 dpm ^{3}H, were dissolved in methanol (200 or 400 μl) and aliquots (20–50 μl) injected onto the reversed-phase column. Eluate fractions (1 ml) corresponding to the principal peak of u.v. absorbance (retention time 14 min) were combined, and the methanol removed under nitrogen at 40°. The recovery of ^{3}H in the isolated fractions was 70–80%. Aqueous residues of the pooled eluate fractions (pH 2.3) were extracted with ethyl acetate to recover the metabolite. Prior to mass spectrometric analysis, the metabolite was further purified by normal-phase HPLC using a Lichrosorb diol column (25 cm × 0.4 cm i.d., 10 μm, HPLC Technology, Macclesfield, U.K.). Methanolic solutions of the metabolite (20–50 μl, 200–500 × 10^3 dpm) were injected onto the column, and eluted with a linear gradient of propan-2-ol in hexane–acetic acid (9:1, v/v) (5–35% at 2%/min) flowing at 2 ml/min. The fractions (1 ml) comprising the peak of u.v. absorbance (monitored at 360 nm, retention time 11 min) were pooled and evaporated to dryness under nitrogen at 40°; the recovery of ^{3}H in them was 80%. The residue (equivalent to *ca.* 0.65 μmol) was analysed by positive ion fast-atom bombardment (FAB) mass spectrometry. It was re-isolated by reversed phase HPLC before negative ion FAB analysis.

Confirmatory evidence of the metabolite's identity was obtained by u.v. spectroscopy, acid hydrolysis and enzymic hydrolysis. Samples of metabolite isolated from bile by reversed-phase HPLC, and from urine by sequential reversed-phase and normal-phase HPLC, were dissolved in 0.1 M sodium phosphate buffer, pH 7.5, and their spectra recorded on a Pye Unicam SP-8 100 spectrophotometer. Urinary metabolite (200 × 10^3 dpm), concentrated by extraction into ethyl acetate, was refluxed with carrier acetyl-DNP-lys (20 mg) in 5 ml of HCl (20%, v/v). Metabolite isolated from bile and urine by reversed-phase HPLC was incubated with acylase 1 [16] (*ca.* 200–1400 U/0.1 μmol) in 0.1 M sodium phosphate buffer, pH 7.5, at 37° for 16 hr. A portion of the material analysed by mass spectrometry was incubated with 1200 U/0.1 μmol under the same conditions. Complete hydrolysis was achieved with *ca.* 1200–1400 U. Acid and enzymic hydrolysis were monitored by reverse-phase HPLC.

Mass spectrometry. Low-resolution FAB spectra of the isolated metabolite and reference compounds were obtained by either the negative or positive ion mode of instrument operation. A VG-Micromass 70-70F coupled to a Finigan Incos Data System and fitted with an Ion-Tech fast atom gun (xenon beam) was used for both positive and negative ion spectrometry.

Pharmacokinetic calculations and statistical analysis. Plasma radioactivity data relating to the rats, expressed as percentages of administered dose, were analysed by log-linear regression according to a one-compartment model. The volume of distribution (V_d) was calculated by dividing the extrapolated zero-

time plasma concentration (C_0) by the dose. The area under the concentration–time curve (AUC) from zero to infinite time was calculated from the AUC to 3 hr (estimated by the trapezoidal rule) extrapolated to infinity by dividing the 3 hr plasma concentration by the elimination rate constant. Clearance was estimated as dose/AUC. Rabbit plasma concentration (C_p) data were analysed according to a two-compartment model according to the equation $C_p = Ae^{-\alpha t} + Be^{-\beta t}$ (where A and B are the extrapolated zero time concentrations and α and β the distribution and elimination rate constants respectively), using the method of residuals [17]. The volumes of distribution ($V_d(\alpha)$) of RAP conjugates were calculated from dose/$A + B$. Other calculations were analogous to those applied to data from rats.

All statistical analyses were performed using Student's t-test for non-paired data.

RESULTS

Disposition of [^{3}H]DNP–protein conjugates in rats

The plasma disappearance of radioactivity following administration of HSA-[^{3}H]DNP$_{20}$ was considerably faster than that following administration of either HSA-[^{3}H]DNP$_{10}$ or HSA-[^{3}H]DNP$_{0.5}$ (Fig. 1). The volumes of distribution of all three conjugates were similar (Table 1). Homologous protein conjugates (RSA-[^{3}H]DNP$_{24}$ and RSA-[^{3}H]DNP$_{0.6}$) also displayed a significant difference in elimination rate (Fig. 2), though this difference was less than that seen with the HSA conjugates. The dissimilarities in plasma clearance and similarity of the V_d are reflected in the large differences between the values of the elimination rate of extensively and poorly conjugated proteins (Table 1). This is due to the far greater plasma clearance of the conjugates with high hapten density. Estimation of the degree of covalent binding of [^{3}H]DNP in plasma showed that >90% remained bound at all times of collection.

Biliary excretion of radioactivity (0–3 hr) after administration of HSA-[^{3}H]DNP$_{20}$ (27.58 ± 3.84% of dose, mean ± S.D.) greatly exceeded that which followed administration of HSA-[^{3}H]DNP$_{0.5}$ (3.57 ± 0.21%) (Fig. 3). A similar pattern of excretion was seen with the RSA conjugates, although the cumulative elimination of radioactivity by rats given RSA-[^{3}H]DNP$_{24}$ was only about half that by rats given HSA-[^{3}H]DNP$_{20}$.

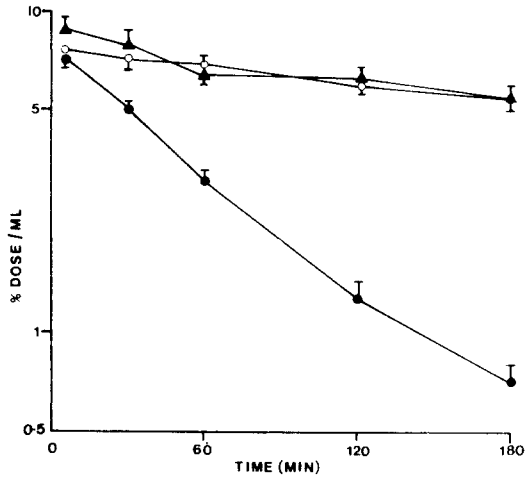

Fig. 1. Plasma disappearance of total radioactivity (expressed as percentage of dose/ml) following i.v. administration of HSA-[^{3}H]DNP$_{0.5}$ (○, N = 8), HSA-[^{3}H]-DNP$_{10}$ (▲, N = 4) and HSA-[^{3}H]DNP$_{20}$ (●, N = 8) to male Wistar rats. Each point represents the mean ± S.E.M.

The liver was the principal site of accumulation of radioactivity upon injection of all the conjugates (Fig. 4). Hepatic radioactivity at 3 hr was significantly greater after HSA-[^{3}H]DNP$_{20}$ administration (*ca.* 31%) as compared with either HSA-[^{3}H]DNP$_{10}$ (*ca.* 8%) or HSA-[^{3}H]DNP$_{0.5}$ (*ca.* 5%) administration. Although no other organ contained more than 6% of the radioactive dose, the spleen accumulated a significantly greater proportion from HSA-[^{3}H]-DNP$_{20}$ than from HSA-[^{3}H]DNP$_{0.5}$; the reverse occurred in the heart. A similar tissue distribution of radioactivity was obtained with the RSA conjugates (Fig. 5).

Table 1. Pharmacokinetic values for dinitrophenyl-heterologous protein (HSA) and dinitrophenyl-homologous protein (RSA) conjugates after i.v. administration to rats at a dose of 5 mg

	Volume of distribution (ml/kg)	Plasma clearance (ml/hr)	Plasma half-life (hr)
Human serum albumin			
HSA-DNP$_{0.5}$ (7)	54.4 ± 4.0	1.4 ± 0.1	6.8 ± 0.1
HSA-DNP$_{10}$ (4)	61.0 ± 6.0	1.9 ± 0.2	4.6 ± 0.5
HSA-DNP$_{20}$ (8)	60.0 ± 3.3	11.4† ± 0.6	0.9† ± 0.1
Rat serum albumin			
RSA-DNP$_{0.6}$ (4)	50.0 ± 2.1	2.3 ± 0.2	3.5 ± 0.2
RSA-DNP$_{24}$ (5)	72.9* ± 7.1	7.2† ± 0.8	1.7† ± 0.1

Numbers in parentheses are the numbers of rats used for each conjugate. Each value is the mean ± S.E.M. Statistical comparison of values from extensively conjugated albumin with values from the least conjugated albumin were performed by Student's t-test for unpaired data (*$P < 0.05$; †$P < 0.001$).

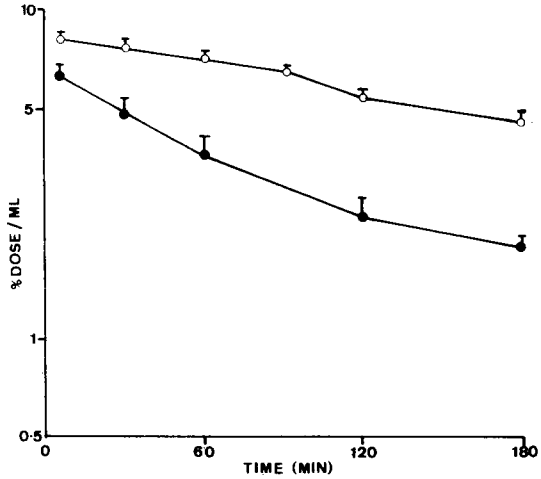

Fig. 2. Plasma disappearance of total radioactivity following i.v. administration of RSA-[^{3}H]DNP$_{0.6}$ (○, N = 4) and RSA-[^{3}H]DNP$_{24}$ (●, N = 5) to male Wistar rats. Each point represents the mean ± S.E.M.

The effect of suramin on [^{3}H]DNP–protein conjugate disposition

Pretreatment of rats with suramin resulted in a marked and statistically significant ($P < 0.05$, N = 4) decrease in the excretion of radioactivity in bile of 58% over the 3 hr experimentation period.

Disposition of RAP conjugates in rabbits

The clearance of radioactivity from the plasma of rabbits administered DNP-serum conjugates was biphasic in all animals (Fig. 6) and was dependent upon the degree of conjugation of the serum protein with DNP (Table 2), being significantly increased as the degree of dinitrophenylation increased. The initial volumes of distribution ($V_d(\alpha)$) of the conjugates were virtually identical and, as observed in

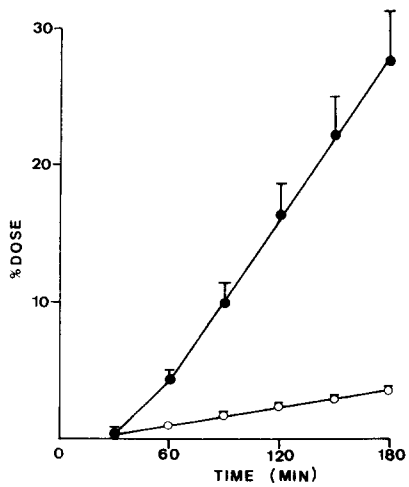

Fig. 3. Cumulative biliary excretion of radioactivity following i.v. administration of HSA-[^{3}H]DNP$_{0.5}$ (○, N = 6) and HSA-[^{3}H]DNP$_{20}$ (●, N = 7) to male Wistar rats. Each point represents the mean ± S.E.M.

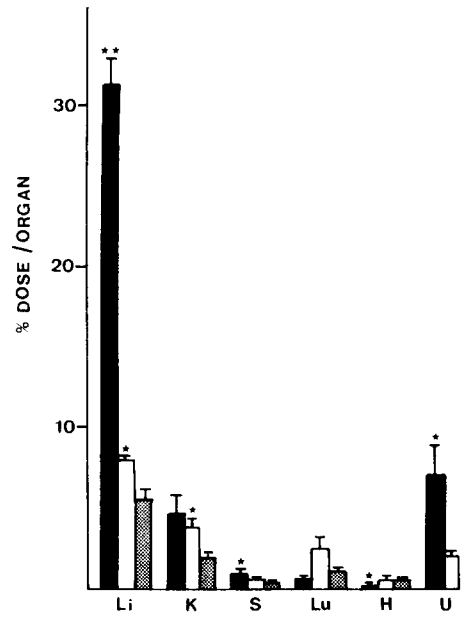

Fig. 4. Tissue distribution into liver (Li), kidney (K), spleen (S), lung (Lu), heart (H) and urinary excretion (U) of radioactivity 3 hr after i.v. administration of HSA-[^{3}H]-DNP$_{20}$ (■, N = 7), HSA-[^{3}H]DNP$_{10}$ (□, N = 4) and HSA-[^{3}H]DNP$_{0.5}$ (▒, N = 6). Columns represent the mean ± S.E.M. Starred columns were statistically different from HSA-[^{3}H]DNP$_{0.5}$ (*$P < 0.05$; **$P < 0.001$).

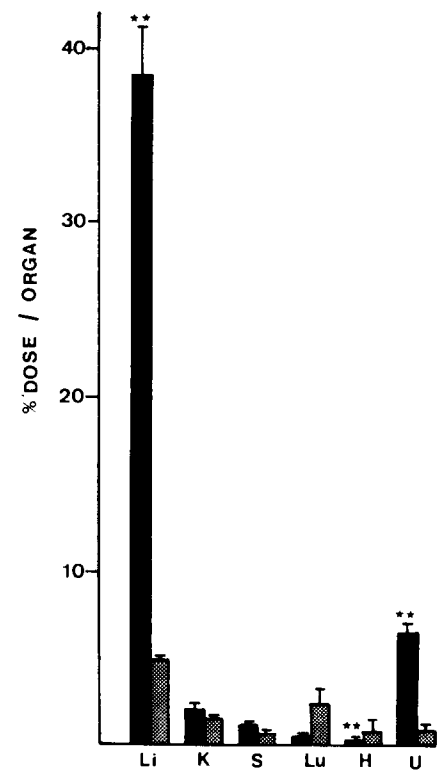

Fig. 5. Tissue distribution and urinary excretion of radioactivity 3 hr after i.v. administration of RSA-[^{3}H]DNP$_{24}$ (■, N = 5) and RSA-[^{3}H]DNP$_{0.6}$ (▒, N = 4). Each column represents the mean ± S.E.M. (**$P < 0.001$). See legend to Fig. 4 for explanation of abbreviations.

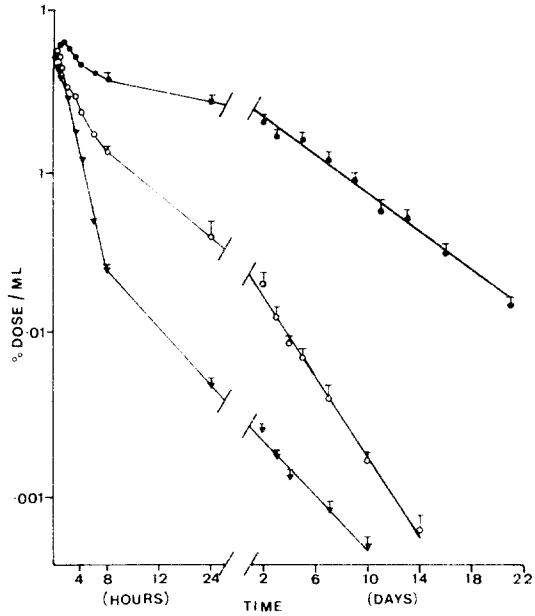

Fig. 6. Plasma disappearance of radioactivity following administration of RAP-[^{3}H]DNP$_{0.5}$ (●, N = 4), RAP-[^{3}H]-DNP$_{15}$ (○, N = 4) and RAP-[^{3}H]DNP$_{30}$ (▼, N = 4), via the marginal ear vein, to male New Zealand White rabbits. The 0–24 hr values are shown on an expanded scale to display the biphasic clearance. The lines connecting values from 24 hr onwards are the best fit obtained by log-linear regression analysis. Each point represents the mean ± S.E.M.

the experiments with rats (in which values of V_d were very similar), suggested intravascular distribution. In the experiments with medium and slightly conjugated serum it was possible to detect radioactivity for 14 and 22 days respectively. In every animal there was no change in the elimination rate between 10 and 14 days. In previous studies it has been demonstrated that the formation of antibodies to foreign proteins, with the associated complement fixation, results in a dramatic clearance of the radiolabelled antigen 10–14 days after administration [14, 15]. Since no such phenomenon was observed in the present study it appears that the administration of DNP–autologous protein conjugates does not evoke an immunological response.

Biliary and urinary metabolites of serum albumin-[^{3}H]DNP conjugates

The ^{3}H-labelled biliary metabolites of HSA-[^{3}H]-DNP$_{20}$ and RSA-[^{3}H]DNP$_{20}$ were resolved into one major and a number of minor components by HPLC (Fig. 7a). The principal metabolite co-eluted with N^2-acetyl-N^6-dinitrophenyl-lysine (acetyl-DNP-lys). In various proportions, it was also observed in bile from rats given slightly conjugated albumin (Fig. 7b) and in urine from cannulated rats (Fig. 7c). Acetyl-DNP-lys represented 52–59% (N = 4) and 36–48% (mean, 42%; N = 4) of the 0–3 hr biliary metabolites of extensively conjugated HSA and RSA, respectively. Urinary metabolites taken from the bladders of three of the animals given HSA-[^{3}H]DNP$_{20}$ at 3 hr comprised 39–47% acetyl-DNP-lys; but the corresponding value for the fourth rat was only 14%. The metabolite was also a minor product in urine from rats given HSA-[^{3}H]DNP$_{0.5}$ (16–20%, N = 3) and in bile following administration of either HSA-[^{3}H]DNP$_{0.5}$ (18–25%, N = 4) or RSA-[^{3}H]DNP$_{0.6}$ (14–16%, N = 3). Although no attempt was made to analyse the other biliary and urinary ^{3}H-labelled metabolites in detail, three minor biliary metabolites of HSA-[^{3}H]DNP$_{20}$ were chromatographically similar to DNP-glutathione (DNP-GT), DNP-lys and DNP-mercapturate, respectively (Fig. 7a). Additionally, DNP-GT was a major biliary metabolite of HSA-[^{3}H]DNP$_{0.5}$ (19–25%) and RSA-[^{3}H]DNP$_{0.6}$ (26–31%) (Fig. 7b).

Identification of N^2-acetyl-N^6-dinitrophenyl-lysine as a metabolite of dinitrophenyl-conjugated albumins

The metabolite isolated from bile and urine had the same λ_{max} (365 nm) as authentic acetyl-DNP-lys. After acid hydrolysis (1 hr) the carrier acetyl-DNP-lys and metabolite were converted to one major ^{3}H-labelled product, which co-chromatographed with unlabelled DNP-lys. Acylase I completely hydrolysed the metabolite, and ^{3}H-labelled DNP-lys, identified by co-chromatography and u.v. spectroscopy, was the sole product. The metabolite's FAB spectra contained the expected pseudo-molecular ions m/z

Table 2. Pharmacokinetic values for dinitrophenyl-autologous protein (RAP) conjugates after i.v. administration to rabbits at a dose of 200 mg

	Initial volume of distribution ($V_d(\alpha)$) (ml/kg)	Plasma clearance (ml/hr/kg)	Plasma half-lives‡ $t_{1/2(\alpha)}$ (hr)	$t_{1/2\beta}$ (hr)
Rabbit serum protein				
RAP-DNP$_{0.5}$ (4)	64.7 ± 3.2	2.0 ± 0.2	4.1 ± 0.6	125 ± 7
RAP-DNP$_{15}$ (4)	54.1 ± 8.8	16.6† ± 0.2	3.4 ± 0.2	59† ± 5
RAP-DNP$_{30}$ (4)	65.0 ± 1.9	52.7† ± 6.4	1.6* ± 0.1	86† ± 6

Numbers in parentheses are the numbers of rabbits used for each experiment. Each value is the mean ± S.E.M. Statistical comparison was made between the extensively conjugated (RAP-DNP$_{15}$ and -DNP$_{30}$) and the slightly conjugated (RAP-DNP$_{0.5}$) proteins using Student's *t*-test for unpaired data (*$P < 0.05$; †$P < 0.001$).

‡ The concentration–time curves for individual rabbits were resolved into two exponential components by the method of residuals.

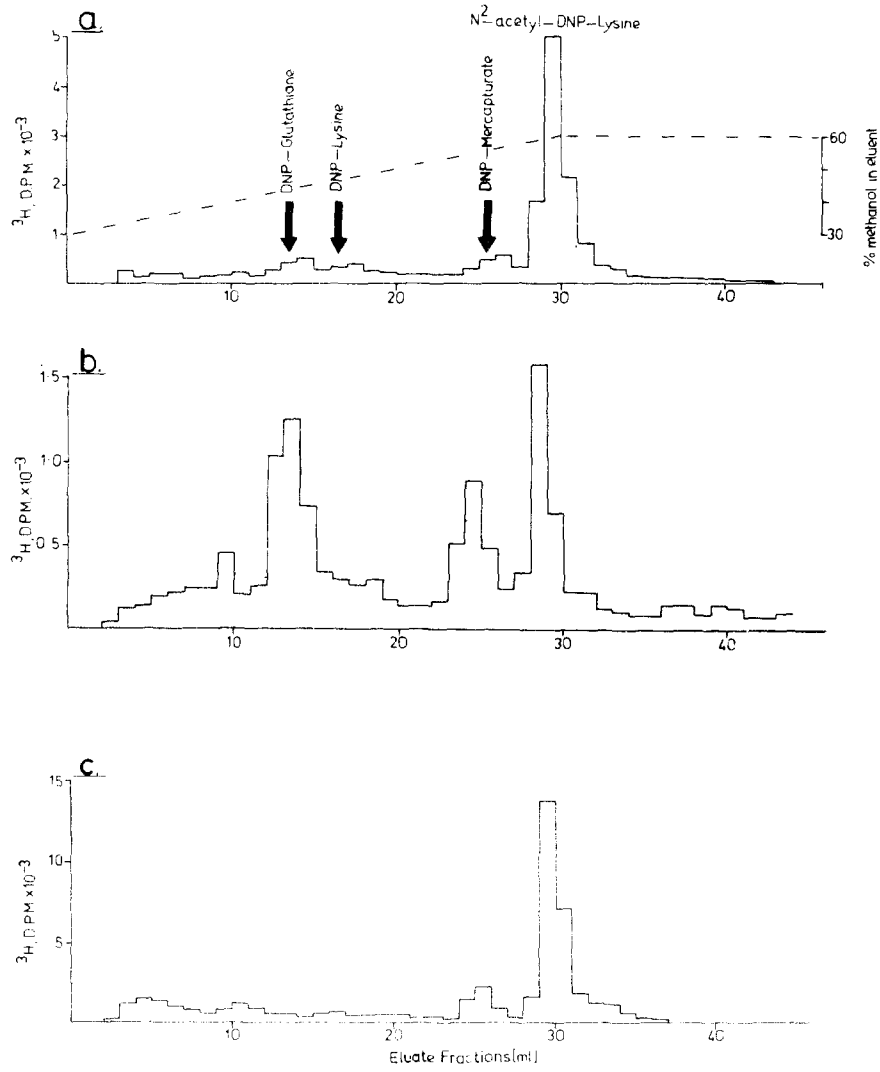

Fig. 7. Reversed-phase high-performance liquid chromatograms of the ^{3}H-labelled biliary and urinary metabolites of (a) HSA-[^{3}H]DNP_{20} (bile); (b) HSA-[^{3}H]$DNP_{0.5}$ (bile); (c) HSA-[^{3}H]DNP_{20} (urine). The chromatographic methods and peak assignments are described in the text. The solid lines show profiles of radioactivity; the broken line the gradient of methanol in phosphate buffer.

353 (M − 1; relative intensity 10.4%) and m/z 355 (M + 1; relative intensity 11.9%) when they were recorded in the negative- and positive-ion modes, respectively.

DISCUSSION

According to current concepts, the formation of drug–protein conjugates is a central event in drug hypersensitivity since the conjugate must (1) act as an immunogen and stimulate antibody production, and (2) function as an antigen which, in combination with specific antibody, can precipitate a hypersensitivity reaction. Thus, the disposition of the conjugate may influence not only the initial sensitization to a drug but also the subsequent allergic reaction. We have investigated the metabolic fate of a simple model hapten, the dinitrophenyl residue, conjugated to homologous as well as heterologous albumin. The conjugates were administered intravenously in order to simulate drug–protein conjugation *in vivo*, and thus allow the development of an experimental system for the quantitative assessment of the role of chemically reactive metabolites in drug immunotoxicity.

Epitope density was a crucial determinant of the rate of non-immune clearance from plasma of DNP–albumin conjugates derived from either homologous (rat) or heterologous (human) serum albumin. Indeed, the nature of the albumin carrier appeared to have little influence on either the plasma half-life or the tissue distribution of the low density conjugates. However, when an epitope density of 20 or greater was achieved the plasma clearance of both types of albumin conjugate increased markedly (Figs. 1 and 2). Tissue distribution studies also indi-

cated a corresponding increase in the rate of uptake of radioactivity into the liver and in the excretion of radioactivity into bile with the higher density conjugates (Figs. 4 and 5). Thus it would appear that there is no simple relationship between the rate of conjugate uptake and epitope density.

Furthermore, there was no evidence for dissociation of the hapten group from the protein in plasma, indicating that the conjugates were taken up by the liver intact. This finding is consistent with previous studies in the mouse and rat, which have shown that albumin that is heavily substituted with a variety of chemical groups (epitope densities of 35–55) is rapidly cleared from plasma and taken up by Kupffer cells [18–20], where it accumulates in lysosomes [18].

The mechanism by which the conjugates are recognized for endocytosis by macrophages in the liver has not been defined. There is no evidence from either the present study or from studies with peritoneal macrophages [19] that aggregate formation is responsible for the rapid uptake of high-density conjugates. It has also been shown that complement is not involved in the rapid clearance of DNP–HSA conjugates from blood [21]. It has been suggested that changes in the physicochemical properties of albumin, such as an increase in hydrophobicity and loss of charged lysine residues, lead to increased adherence of the protein to cell membranes, thereby accelerating its endocytosis [22]. Dinitrophenylation of lysine residues would lead to a decrease in the net positive charge on the albumin molecule and an increase in hydrophobicity.

Following uptake by the liver, the highly conjugated HSA and RSA conjugates were hydrolysed, and the dinitrophenyl group was excreted into bile and urine as a simple and novel amino acid conjugate: N^2-acetyl-N^6-dinitrophenyl-lysine (Fig. 8). The conjugates are presumably hydrolysed by the lysosomal proteases of Kupffer cells since suramin, a potent and selective inhibitor of lysosomal proteolysis in these cells [13, 23, 24], markedly reduced the release of radioactivity into bile.

It is notable that the α-amino group of the N^6-DNP-lysine released by proteolysis was acetylated before excretion in bile and urine. We have not identified the *N*-acetyl transferase responsible for this process, but a number of such enzymes are able to convert xenobiotic cysteine conjugates to the corresponding mercapturic acids [25], and a variety of other amino acid *N*-acetyl transferases are known [26–28]. N^5-acetylornithine and N^2-acetyl-lysine have been detected in human blood [29] and urine [30] respectively.

The disposition of DNP–protein conjugates was studied in rabbits in order to study the effect of chemical modifications of autologous proteins, since serum from individual rabbits was conjugated and returned to its donor. Radioactivity was cleared from plasma biphasically, the early half-life being dependent upon the epitope density. However, the disappearance rate of all the conjugates decreased after 1–2 days and then declined linearly at a rate consistent with the normal turnover rate of albumin [31]. Similar experiments using ^{131}I-labelled bovine serum albumin (BSA) showed a triphasic excretion of radio-

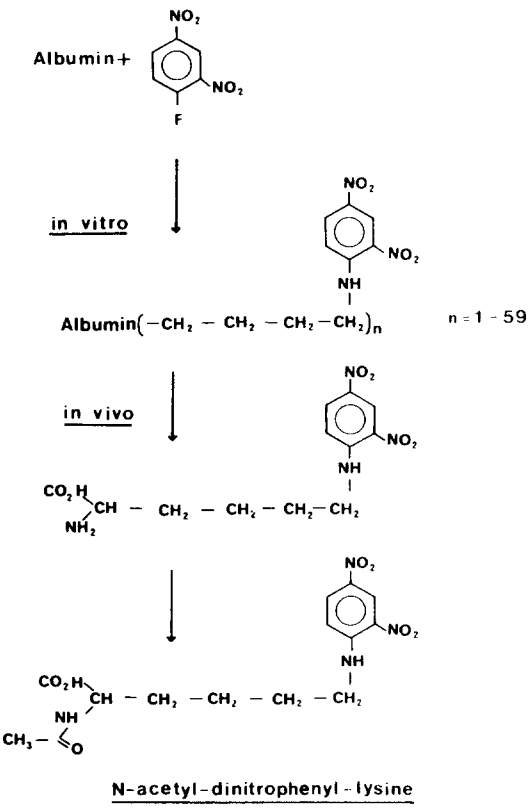

Fig. 8. Scheme summarizing the formation and breakdown of DNP-albumin conjugates. DNFB reacts with lysine groups on albumin, *in vitro*, to form covalently bound conjugates with varying epitope densities. *In vivo* the conjugates undergo proteolytic hydrolysis resulting in the formation of DNP-lysine. This undergoes acetylation prior to its excretion in bile and urine as *N*-acetyl-dinitrophenyl-lysine.

activity with the third phase, occurring between days 10 and 14, involving a precipitous drop in plasma radioactivity [14]. The third phase was due to an immune response involving specific anti-BSA antibody formation and complement fixation. In contrast, there was no evidence for an immune clearance during our experiments, irrespective of the degree of dinitrophenylation. The early elimination phase was influenced by the epitope density and this presumably involved hepatic uptake. This is supported by the fact that the half lives ($t_{\frac{1}{2}(\alpha)}$) calculated for this early phase (Table 2) were similar to the half lives obtained in rats when homologous protein conjugates with similar epitope densities were administered (Table 1). This suggests that a similar mechanism for removal of the conjugates obtained in both species and that this mechanism is markedly influenced by the degree of substitution of the proteins. Since whole serum, rather than purified albumin, was conjugated, it is possible that the second phase in our experiment represents the normal clearance of proteins other than albumin, which have been radiolabelled but not sufficiently modified to result in enhanced endocytosis. The short duration of the rat experiments did not permit the detection of a second elimination phase.

In conclusion, the results indicate that a chemically reactive compound, such as DNFB, which binds covalently to plasma proteins can alter the non-immune clearance of those proteins at a rate dependent on the epitope density. Uptake of the extensively dinitrophenylated conjugates by rat liver resulted in rapid hydrolysis of the protein carrier and excretion, principally in bile, of a simple acetylated amino acid derivative. It appears that the rat liver is able to deal with chemically modified proteins without recourse to immune clearance through antibody formation. Indeed, dinitrophenylated whole serum which was returned to the donor rabbit, in a procedure designed to resemble more closely the formation of protein conjugates *in vivo*, did not produce an antibody response even though conjugated proteins were detectable in plasma for three weeks.

These results suggest that DNFB can be used as a model reactive metabolite and that measurement of the acetyl-DNP-lysine metabolite may provide a simple means of quantifying DNP covalent binding *in vivo*. Such an approach may have wider application.

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