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DRUG–PROTEIN CONJUGATES—XIII

THE DISPOSITION OF THE BENZYLPENICILLOYL HAPTEN CONJUGATED TO ALBUMIN

G. CHRISTIE, N. R. KITTERINGHAM and B. K. PARK*
Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX, U.K.

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Abstract—The disposition and metabolic fate of benzylpenicillin conjugated to a protein, human serum albumin (HSA), were compared with those of free penicillin in the rat. The conjugate was prepared by *in vitro* incubation of [^{3}H]-benzylpenicillin and HSA at pH 10.8 for 24 hr at 37°, conditions which favour the formation of penicilloyl-lysine residues.

The synthetic conjugate was cleared more slowly from plasma than free penicillin after intravenous administration; thus at 3 hr, concentrations of 5.08 ± 0.50% dose/ml of the conjugate (0.31 μCi; 2.92 mg protein) was obtained. In an earlier study a concentration of 0.03 ± 0.01% dose/ml was obtained after administration of free BP (2.7 mmol kg^{-1}). During this time, 1.41 ± 0.50% of the conjugate dose was excreted in urine while 5.0 ± 0.2% of the dose was excreted in bile. Tissue analysis indicated that the liver contained 15.3 ± 0.9% of the dose, while other tissues contained <6% of the dose. In long term metabolism studies it was found that 39.5 ± 1.0% and 46.5 ± 0.9% of the dose (0.43 μCi; 6.33 mg protein) was excreted in the urine after 3 and 7 days respectively.

The principal metabolite (63–68%) excreted in both bile and urine was identified on the basis of co-chromatography and fast atom bombardment mass spectrometry as benzylpenicilloic acid, indicating that the conjugate undergoes specific cleavage at the bond between the benzylpenicilloyl moiety and the protein. *In vitro* degradation studies indicate that the metabolism occurs primarily in the liver. Therefore benzylpenicilloic acid excreted in urine, after administration of free BP, may be formed either by direct hydrolysis of the β-lactam ring, and/or result from catabolism of protein conjugates formed *in vivo*.

The penicillin group of drugs is prescribed widely for the treatment of various bacterial infections, being active against most of the common pathogenic bacteria [1]. The penicillins are remarkably nontoxic compared with other antibiotics; in man daily doses as high as 1 g/kg can be tolerated for several months. However, it has been estimated that between 1 and 10% of treated patients exhibit some form of allergic reaction, including haemolytic anaemia [2], thrombocytopenia and IgE mediated anaphylactic reactions [3]. The majority of these adverse reactions are believed to have an immunological aetiology [4].

In accordance with the hapten hypothesis of drug hypersensitivity it is accepted that penicillins, being low molecular weight compounds, must first become irreversibly bound to a macromolecular carrier in order to be recognised by the immune system as foreign and act as an immunogen for the induction of antibody synthesis [5, 6]. In fact this group of antibiotics provides the best example of a group of drugs for which fully characterised anti-drug antibodies are formed. Thus, the penicillins have become accepted as the classical model of drug-induced hypersensitivity. The major antigenic determinant derived from penicillin has been identified as the protein-conjugated penicilloyl group [7, 8]. However, little is known of the ultimate fate of drugs after conjugation to proteins.

Therefore, the aim of this study was to determine the disposition and metabolism of [^{3}H]-benzylpenicilloyl human serum albumin ([^{3}H]-BPO-HSA), with particular reference to the excretion of metabolites which might be used to monitor the irreversible protein binding of benzylpenicillin, *in vivo*.

MATERIALS AND METHODS

Chemicals

[Phenyl-4(*n*)-^{3}H]benzylpenicillin ([^{3}H]BP; 15 Ci/mmol) was obtained from Amersham International plc (Bucks, U.K.). Benzylpenicillin (BP, sodium salt)† and human serum albumin (HSA) were obtained from Sigma (London) Chemical Co. (Poole, Dorset). HPLC grade solvents were products of Fisons plc (Loughborough, Leics.) and all other reagents were of analytical grade. NCS tissue solubilizer was from Amersham Corp. (Arlington Heights, IL). Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA). Silica gel thin-layer chromatographic plates (Merck no. 5735 20 × 20 × 0.2 cm) were obtained from British Drug Houses (Poole, U.K.).

Benzylpenicilloic acid was synthesised from benzylpenicillin according to the method described

* Wellcome Trust Senior Lecturer. To whom correspondence should be addressed.

† *Abbreviations used:* BP, benzylpenicillin; BPO, benzylpenicilloyl; HSA, human serum albumin; HPLC, high performance liquid chromatography; RT, retention time; HPIEC, high performance ion-exchange chromatography; TLC, thin layer chromatography.

by Cole *et al.* [9]; *N*-(α-D-benzylpenicilloyl)-*N*-acetyl-lysine by the method of Levine [10] and, benzylpenicilloic acid by the method described by Mozingo and Folkers [11].

The radioactive content of the samples was determined in 4 ml scintillation fluid (Aqualuma plus, May & Baker Chemical Division, Manchester, U.K.) unless otherwise stated, in a Packard Tricarb 4640 liquid scintillation spectrometer.

Preparation of benzylpenicilloyl–protein conjugate

Human serum albumin (HSA, 150 mg) and [^{3}H]-BP (264 μCi; 58.4 mg) were dissolved in carbonate buffer (9 ml, pH 10.8, 0.1 M) and incubated at 37° for 24 hr. The incubation mixture was then dialysed for 5 days against phosphate buffer (5 l, pH 8.4, 0.07 M), the buffer being replaced every 24 hr. The radioactive content of duplicate 2 ml aliquots of the dialysis buffer was measured in 20 ml of scintillant until less than 1% of the incubated radioactivity was present in the buffer. The dialysate was chromatographed using a Sephadex G-25 column (30 × 2.5 cm) and eluted with distilled water, the eluate being monitored at 278 nm with a u.v. spectrophotometer (LKB Uvicord). The peak corresponding to the retention time (RT 25 min) of the protein was collected and lyophilized. The purity of the conjugate was determined using high performance ion-exchange chromatography (HPIEC). HPIEC was performed on a Gilson gradient HPLC system, using a TSK DEAE 5-PW chromatographic column (Toyo, Soda Mfg. Co., Tokyo, Japan), equilibrated with acetate buffer (pH 5.0, 20 mM). A gradient profile of increasing ionic strength (300 mM–1 M) of the buffer was used. The protein conjugate was eluted first (RT 15 min), with acetate buffer (pH 5.0, 300 mM), followed by free BP and benzylpenicilloic acid (RT 26 min) which were eluted together with acetate buffer (pH 5.0, 1 M); the two steps were separated by a 10 min washing period with the equilibrating buffer. The flow rate was 1 ml/min throughout and fractions were collected every 0.5 min. Each fraction was assayed for radioactivity and the protein concentration in each sample was determined using the method of Lowry *et al.* [12]. The degree of benzylpenicilloylation was determined by penamaldate assay [10], and by determining the amount of radioactivity irreversibly bound to protein.

Thin layer chromatography and high performance liquid chromatography

The urinary and biliary metabolites of [^{3}H]-BPO-HSA were analysed by TLC on silica plates using a solvent system of ethyl acetate : glacial acetic acid : water (8 : 1 : 1 v/v). The metabolites were identified by co-chromatography with authentic standards of BP (R_f 0.71), benzylpenilloic acid (R_f 0.41), *N*-(α-D-benzylpenicilloyl)-*N*-acetyl-lysine (R_f 0.29) and benzylpenicilloic acid (R_f 0.17).

These metabolites were also analysed by reversed phase HPLC using a radially compressed (Z-module, Waters) reversed phase C_{18} column (5 μm packing) protected by a guard-column of Co-Pell ODS (Whatman Inc., Clifton, NJ). The solvent system employed a linear gradient of methanol (10–100% at 2% per min) in ammonium phosphate buffer (pH 2.3, 43 mM) at a flow rate of 1.5 ml/min. Under these conditions the retention times of the standards, measured by u.v. absorbance at 254 nm (LKB 2151), were 14 min for benzylpenilloic acid (produced by decarboxylation of benzylpenicilloic acid), 19 min for both benzylpenicilloic acid and *N*-(α-D-benzylpenicilloyl)-N-acetyl-lysine and 23 min for BP. Bile and urine samples were analysed without prior treatment, the eluate being collected in 30-sec intervals and assayed for radioactivity.

Disposition of [^{3}H]*-benzylpenicilloyl-protein conjugate in the rat*

Short term experiments (3 hr). Male Wistar rats (200–250 g body wt) were anaesthetised with urethane (1.4 g/kg in 0.15 M saline) i.p. and their trachea, carotid artery, jugular vein and bile ducts were cannulated. The rats' penises were ligated to prevent urine loss. After heparinization of the animals (400 I.U./kg), [^{3}H]-BPO-HSA (0.31 μCi; 2.92 mg protein) was administered via the jugular vein in 1 ml/kg of 0.15 M NaCl; the protein conjugate was dissolved immediately prior to administration. Blood samples (*ca.* 0.25 ml) were withdrawn from the carotid artery at 5, 30, 60, 90, 120, 150 and 180 min, and replaced with an equal volume of isotonic saline. The blood samples were centrifuged immediately (2000 *g*, 10 min) to obtain plasma. Bile was collected into preweighed microcentrifuge tubes in 0.5 hr fractions. After 3 hr the animals were killed by exsanguination and the brain, heart, kidney, liver, lung and spleen were removed and immediately frozen (−78°). These tissues were stored frozen until assayed for total radioactivity as described previously [13]. Urine was obtained by terminal aspiration of the bladder. The radioactive content of duplicate 50 μl samples of the plasma, 20 μl of the bile and 50 μl of the urine was determined. The biliary and urinary metabolite profiles were determined by HPLC as described above.

Long term study (7 days). Male Wistar rats (200–250 g body wt) 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*. [^{3}H]-BPO-HSA (0.43 μCi; 6.33 mg protein) was administered by injection into the tail vein (in 1 ml/kg saline) of the rat, under ether anaesthesia. Urine collections were removed every 24 hr for 7 days. The volume of urine collected in each 24 hr sample was determined and duplicate 100 μl aliquots were assayed for radioactivity. The urine samples were stored frozen until assayed as described below.

Isolation and identification of urinary metabolites of [^{3}H]*-BPO-HSA*

The 24-hr urine collections were acidified to pH 2.0 with HCl and extracted three times with an equal volume of redistilled ethyl acetate; the recovery of radioactivity in the ethyl acetate was *ca.* 70%. Pooled extracts were evaporated to dryness under a stream of nitrogen at 40°. The residues were dissolved in 500 μl of methanol. Aliquots (100–150 μl) were then chromatographed by HPLC as described earlier. Eluate fractions (3 ml), corresponding to the absorbance peak at 19 min, and representing *ca.* 70% of

the injected radioactivity were combined. The radioactive metabolites were concentrated using Sep-Pak C_{18} cartridges under the following conditions; the Sep-Pak C_{18} cartridges were pre-conditioned with methanol (20 ml) and distilled water (20 ml) prior to use. The combined eluate from the urines was passed through the Sep-Pak C_{18} cartridge at a rate of 2 ml/min, then washed through with distilled water (5 ml) and the radioactivity eluted with methanol (5 ml). The methanol containing *ca.* 60% of the radioactivity, was evaporated to dryness under a stream of N_2 at 40°. The residue was reconstituted in methanol and again chromatographed, after Sep-Pak concentration of the eluate; the sample was analysed by mass spectrometry as described below. In addition, synthetically prepared benzylpenicilloic acid was mixed with blank rat urine, extracted under identical conditions to the unknown metabolite and subjected to mass spectrometry.

Mass spectrometry

Low resolution fast atom (xenon) bombardment (FAB) mass spectra were obtained with a V.G. Micromass 70-70F mass spectrometer coupled to a Finnigan Incos Data System. An Ion-Tech Ltd. fast atom gun was used. The conditions used for FAB in the positive-ion mode were: discharge, 1 mA; mass spectrometer accelerating voltage, 4×10^3 V; scanning range, 40–700 m/z. The solvent matrix was glycerol and the solvent background spectrum was removed by the data system.

In vitro *degradation of* [^{3}H]-*benzylpenicilloyl-protein conjugate in rat plasma and rat liver homogenate.*

Male Wistar rats (200–350 g body wt) were anaesthetized with ether. Blood was collected into heparinized tubes by cardiac puncture and immediately centrifuged (2000 *g*, 10 min) to obtain plasma. After cervical dislocation, the livers were excised into ice-cold Krebs–Henseleit buffer (pH 7.4). After weighing, the livers were roughly chopped and a 30% homogenate in ice-cold Krebs–Henseleit buffer (pH 7.4) was prepared using a mechanical homogenizer (Ultra Turax) for 10 sec, followed by homogenisation in a glass homogenising vessel with a loose fitting motor-driven Teflon pestle. The homogenate was kept at 4° until used. The protein concentration of an aliquot of the homogenate was determined using the method of Lowry *et al.* [12], and the homogenate diluted to give a protein concentration of 100 mg/ml.

Aliquots of the homogenate (2 ml) or plasma (2 ml) were incubated for 0 or 90 min in the presence of the [^{3}H]-BPO-HSA conjugate (0.11 μCi; 0.54 mg protein) at 37°. After this time any reaction was terminated by the addition of trichloroacetic acid (6 M, 200 μl), followed by thorough mixing, to precipitate protein, and the samples were centrifuged (3500 *g*, 15 min). The supernatant was removed and assayed for radioactivity in 20 ml scintillant. The extent of degradation of the conjugate was determined as the increase in the amount of radioactivity present in the supernatant after the incubation period minus that present at time zero.

Pharmacokinetic calculations

Plasma radioactivity data, expressed as percentages of the dose, were analysed by log-linear regression according to a one compartment model. The apparent volume of distribution was calculated by dividing the dose administered by the extrapolated zero-time plasma concentration. The elimination half-life of the conjugate was determined using the cumulative urine data from the long term experiment using the sigma-minus method [14]. For statistical analysis a non-paired Student's *t*-test was used. All values quoted in text and on figures are mean ± SEM.

RESULTS

Characterization of [^{3}H]-*BPO-HSA conjugate*

After purification of the conjugate by chromatography and exhaustive dialysis more than 96% of the [^{3}H]-activity was assessed to be covalently bound; 53.2% yield of protein was obtained. The protein conjugate eluted as a single homogeneous peak on both Sephadex and HPIEC as measured by both u.v. and radiometric detection.

The penamaldate assay was used to quantify the extent of irreversible binding of BP to the protein. It is known that penicilloic acids and their derivatives with modified α-carboxyl groups (i.e. those with an intact thiazolidine ring) can be converted to penamaldic acid derivatives by mercuric salts. The products exhibit large spectral absorbance bands under u.v. light. The absorbance obtained with the conjugate did not decline in intensity after 10 min, indicating that the absorbance was due to penicilloyl derivatives and not free penicilloic acid [10, 15]. The epitope density (molar ratio of hapten to protein) of the conjugate was found to be 7.6, and the specific activity was 0.2 μCi/mg protein.

Disposition of [^{3}H]-*BPO-HSA conjugate in rats*

Short term experiments. The plasma disappearance of radioactivity following administration of the conjugate is shown in Fig. 1; the plasma concentration–time curve of free [^{3}H]-BP (2.7 mmol/kg, i.v.) obtained in a previous study with rats [16, 17] is shown for comparison. Total plasma radioactivity declined monoexponentially with an apparent half-life of 177 ± 18 min (N = 3), and an apparent volume of distribution of 40.0 ± 0.4 ml/kg (N = 3). After 3 hr, 5.08 ± 0.50% dose/ml (N = 3) was present in the plasma, whereas with free BP, 0.03 ± 0.01% dose/ml (N = 7) was present [16]. The cumulative biliary excretion of radioactivity after administration of the conjugate is shown in Fig. 2. After 3 hr biliary excretion accounted for 4.98 ± 0.24% (N = 4) of the administered dose.

Figure 3 shows the total amount of radioactivity present in the organs examined and that excreted in the urine, expressed as the percent dose. The liver was the principal site of accumulation, containing 15.3 ± 0.9% (N = 4) of the dose, no other organ contained more than 6% of the dose. However, the kidney and lung contained more radioactivity than the heart, spleen or brain. The urinary excretion of radioactivity was variable and accounted for 1.4 ± 0.5% (N = 4) of the dose.

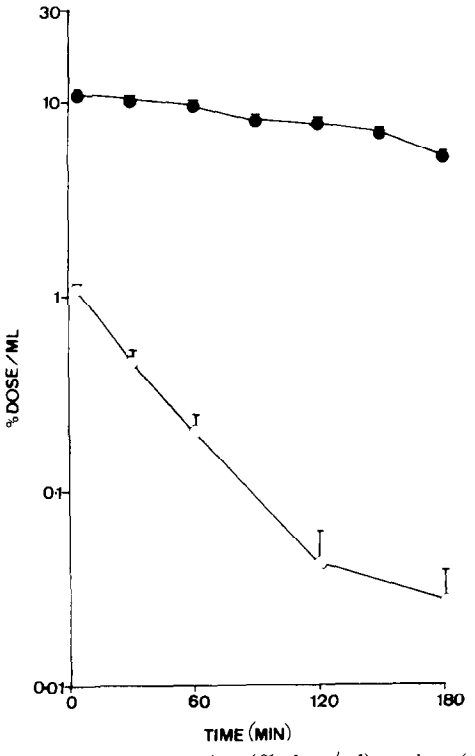

Fig. 1. Plasma concentration (% dose/ml) vs time (min) curve after i.v. administration of [^{3}H]-BPO-HSA (●), 0.31 μCi; 2.92 mg protein (N = 3) to anaesthetised male Wistar rats. The plasma concentration of free [^{3}H]-penicillin (○) 2.7 mmol/kg (N = 7) is shown for comparison. Each point represents the mean ± SEM.

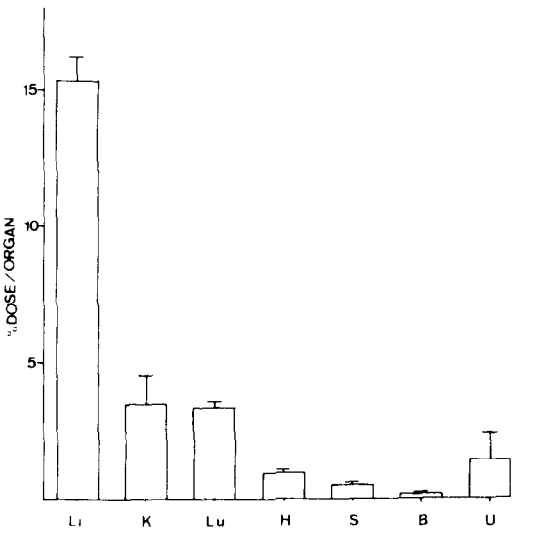

Fig. 3. Tissue distribution into liver (Li), kidney (K), lung (Lu), heart (H), spleen (S), brain (B) and urinary excretion (U) of radioactivity 3 hr after i.v. administration of [^{3}H]-BPO-HSA, 0.31 μCi; 2.92 mg protein, to anaesthetised male Wistar rats (N = 4). Each column represents the mean ± SEM.

Long term urinary excretion. The cumulative urinary excretion of radioactivity following i.v. administration of the conjugate to the rats kept in metabolism cages is shown in Fig. 4. After 3 days,

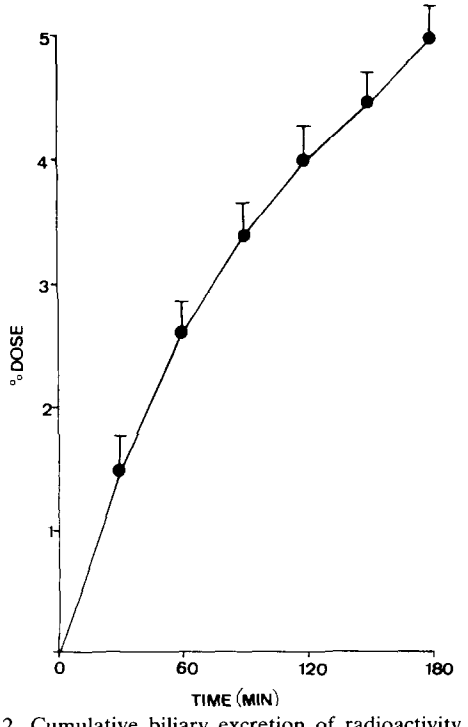

Fig. 2. Cumulative biliary excretion of radioactivity following i.v. administration of [^{3}H]-BPO-HSA, 0.31 μCi, 2.92 mg protein (N = 4) to anaesthetised male Wistar rats. Each point represents the mean ± SEM.

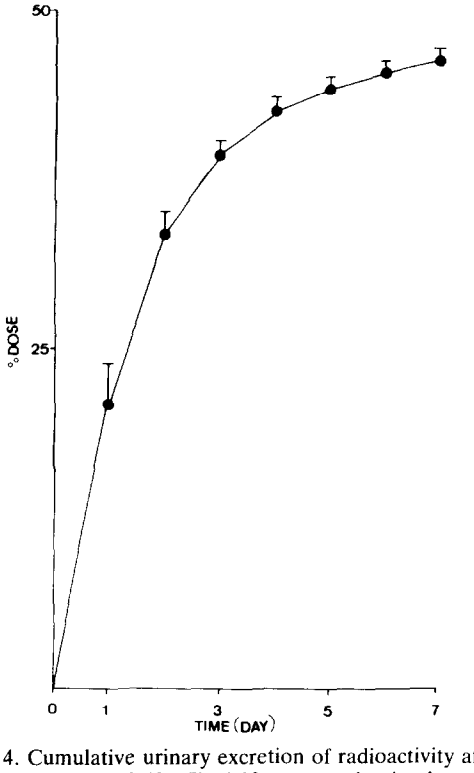

Fig. 4. Cumulative urinary excretion of radioactivity after [^{3}H]-BPO-HSA, 0.43 μCi; 6.33 mg protein, in the male Wistar rat (N = 4). Each point represents the mean ± SEM.

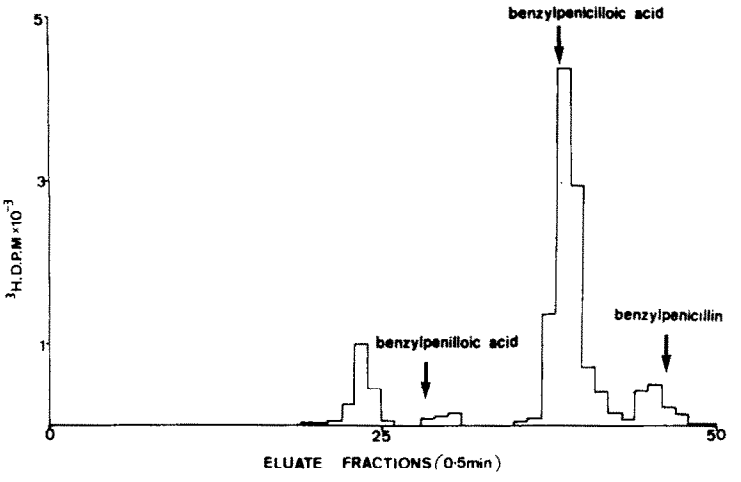

Fig. 5. Reversed-phase high performance chromatograph of the [^{3}H]-labelled urinary metabolites of [^{3}H]-BPO-HSA in the rat (each fraction 30 sec). [^{3}H]-BPO-HSA (0.43 μCi; 6.33 mg protein) was administered i.v. to the male Wistar rats housed in metabolism cages and urine collected at 24 hr intervals. Benzylpenicilloic acid was identified by FAB mass spectrometry.

39.5 ± 1.0% (N = 4) of the dose had been excreted, this increased to 46.5 ± 0.9% (N = 4) of the dose after 7 days. The elimination half life was 26.4 ± 1.9 hr (N = 4).

Identification of the biliary and urinary metabolites of [^{3}H]-*BPO-HSA*

The majority (60–65%) of the tritiated material present in both bile and urine ran as a single band on the TLC system. This polar metabolite (R_f 0.17) co-chromatographed with authentic benzylpenicilloic acid. However, the remaining radioactivity did not co-chromatograph with any of the authentic standards including *N*-(α-D-benzylpenicilloyl)-*N*-acetyllysine (R_f 0.29).

The [^{3}H]-labelled urinary metabolites of [^{3}H]-BPO-HSA from the long term experiment were resolved into one major and a number of minor components by HPLC (Fig. 5). Between 63–68% of the excreted radioactivity eluted as a single homogeneous peak (RT 19 min). This peak co-eluted with that due to benzylpenicilloic acid as identified by the TLC system. The majority (60–70%) of radioactivity excreted in both the bile and urine from the cannulated rat experiments also co-chromatographed with this peak. Although no attempt was made to identify the other minor biliary and urinary metabolites, the minor polar metabolite (RT 12 min) was still present (13% of total radioactivity) when the peak with a retention time of 19 min was isolated and reanalysed on the same system. Thus, suggesting that this minor polar compound was in some way formed from the major metabolite.

The positive ion FAB spectrum provided additional evidence that benzylpenicilloic acid was a metabolite of the [^{3}H]-BPO-HSA. The positive ion FAB spectrum of the isolated metabolite included all of the major ions in the FAB spectrum of authentic benzylpenicilloic acid. Intense ions were observed at m/z 309 $[(M + 1)\text{-}CO_2]^+$ due to loss of carbon dioxide from m/z 353, $[MH]^+$. The base peak in the spectrum was the benzyl cation at m/z 91 with other fragment ions being observed at m/z 174 (the thiazolidine cation) and 128.

In vitro *degradation of* [^{3}H]-*BPO-HSA in rat plasma and rat liver homogenate*

The amount of radioactivity present in the supernatant after centrifugation of the incubation mixture was significantly greater ($P < 0.001$) for the liver homogenate (7.46 ± 1.30% total incubated radioactivity, N = 5), compared to that obtained for the plasma (0.11 ± 0.05% total incubated radioactivity, N = 5).

DISCUSSION

It is thought that conjugation of penicillin, or a rearrangement product, to either autologous or heterologous proteins may be the initial step in the sequence of events which lead to a hypersensitivity reaction. The penicillin molecule is a chemically reactive structure and may form covalent bonds with carbohydrates and proteins by reaction with amino, hydroxy, mercapto, histidine or disulphide groups. A large number of antigenic determinants may be formed, the major one being the protein bound benzylpenicilloyl (BPO) group [7, 18]. This moiety is a bifunctional structure consisting of a polar thiazolidine carboxylic acid nucleus and a non-polar phenylacetamide side-chain. We have previously shown that BP binds irreversibly to rat plasma proteins *in vitro* (5% after 24 hr) and *in vivo* in the rat (6×10^{-8}% dose bound/mg protein, 3 hr after 2.7 mmol/kg i.v.) [16, 17].

The method used to produce the protein conjugate, *in vitro*, involved incubation of BP and protein at an alkaline pH; under these conditions the BP reacts with protein amino groups in lysine residues, forming the BPO determinant, and the formation of penicillenate does not occur [19]. The drug–protein conjugate was administered intravenously to rats in order to parallel drug–protein conjugation which we have previously shown occurs in plasma *in vivo* [16, 17]. After intravenous infusion the conjugate

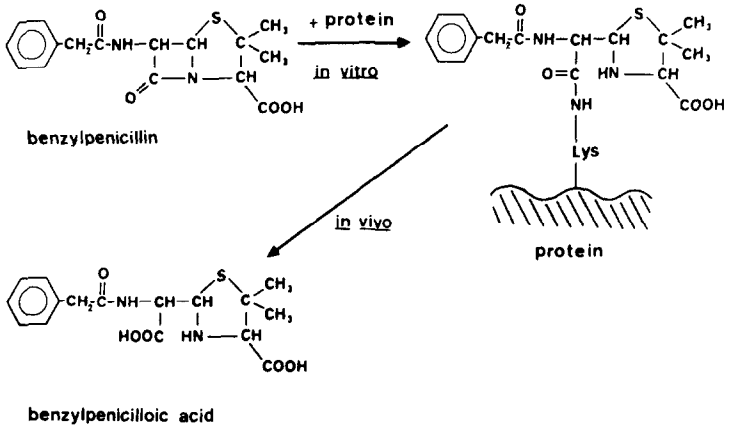

Fig. 6. Scheme summarizing the formation and breakdown of benzylpenicilloyl–albumin conjugates. BP reacts with lysine groups on albumin *in vitro* to form covalently bound conjugates. *In vivo* the conjugate undergoes hydrolysis resulting in the formation of benzylpenicilloic acid, which is then excreted in both bile and urine.

was mainly distributed intravascularly, as indicated by the low apparent volume of distribution. The apparent mean plasma half-life of the conjugate was considerably longer than the 29 min reported for free BP [20], which undergoes active renal tubular secretion. The BPO–HSA conjugate was cleared slower than captopril human plasma protein conjugates in the rat [21]. However, for [^{14}C]-captopril-protein conjugates the low half-life has been attributed to dissociation of the conjugate *in vivo*. The clearance of BPO–HSA was also lower than those observed for dinitrophenylated-HSA conjugates in the rat. Dinitrophenylated conjugates provide a useful comparison with benzylpenicilloylated conjugates in that the dinitrophenyl group is bound to the protein via lysine residues. We have previously shown the clearance of drug-modified proteins to be dependent upon the degree of conjugation, and that conjugates with high epitope densities have shorter half-lives. However, there is no simple relationship between conjugate turnover and epitope density [13]. The elimination half-life of the conjugate (26 hr) may be accurately determined from the long term urinary excretion data. This value is considerably lower than the 45–89 hr reported for non-conjugated plasma albumin in the rat [22]. The plasma half-life was considerably shorter than the elimination half life. However, this may reflect uptake of the conjugate into the tissues.

Examination of the tissue distribution of radioactivity, 3 hr after administration of the conjugate, revealed the largest accumulation of radioactivity in the liver, which contained a mean of 15% of the dose (Fig. 3), whereas with free BP the greatest accumulation occurred in the kidney. The accumulation of radioactivity in the liver, was presumably due to uptake of the conjugate from the blood. However, a small proportion of this radioactivity may be due to the blood content of the liver. Uptake by the liver is expected since it is known that exposure of hydrophobic residues or the masking of cationic lysine residues is responsible for increasing the affinity of the albumin for protein present on cell membranes of the liver, and this may result in increased endocytosis [23].

Benzylpenicilloic acid, in which the β-lactam ring is cleaved, was identified as the major metabolite derived from the drug–protein conjugate, being excreted in both bile and urine. This product could be produced by specific cleavage of the amine bond linking the BPO moiety to the protein (Fig. 6) by an esterase enzyme. Thus, benzylpenicilloic acid could be produced from the drug–protein conjugate by two routes: (1) uptake into the liver followed by subsequent hydrolysis, or (2) direct hydrolysis in the plasma, since it is known that enzymes capable of breaking such a bond are located in the liver and plasma [24]. Although it is not possible to compare directly the *in vitro* degradation results obtained with rat plasma and whole rat liver homogenate, the results obtained suggest that turnover of the conjugate may occur primarily in the liver. This corresponds with the fate of dinitrophenylated-proteins, which are taken up by the liver and after lysosomal metabolism, the simple amino acid conjugate N^2-acetyl-N^6-DNP lysine was excreted principally in the bile [13]. In this study the metabolic fate of BP bound to human serum albumin was studied, but we have shown previously that the fate of dinitrophenylated proteins was the same for heterologous and autologous albumin conjugates [13].

In conclusion, these results indicate that the benzylpenicilloylated protein conjugate was taken up by the liver resulting in hydrolysis of the conjugate and excretion of benzylpenicilloic acid. We have shown previously that small amounts of BP bound to self proteins, *in vivo*, do not produce a detectable anti-BPO antibody response in the rat [16, 17]. Thus, it is possible that benzylpenicilloylation of albumin and subsequent metabolism of the conjugates may act as a protective mechanism, preventing binding to other proteins or cell surface macromolecules which might produce more immunogenic conjugates. In addition, it has been proposed that the rat liver is able to deal with drug-modified proteins without utilising immune clearance through antibody for-

mation [13]. Benzylpenicilloic acid has also been identified as a urinary metabolite after administration of free BP to both man [9] and rats [25, 26]. Thus, it is possible that at least a small proportion of the excreted benzylpenicilloic acid observed in such cases may be derived from the metabolism of drug–protein conjugates formed *in vivo*. Therefore, with benzylpenicilloylated proteins, in contrast to dinitrophenylated proteins, measurement of urinary metabolites will not prove useful for monitoring protein-conjugation of BP *in vivo*. Other minor metabolites, for example the higher retention time material (RT 23 min), were detected after administration of the conjugate. However, it is unlikely that such metabolites will prove useful to monitor covalent binding *in vivo*, due to the low level of covalent binding observed *in vivo* after administration of free BP [17].

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