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DRUG–PROTEIN CONJUGATES—XII

A STUDY OF THE DISPOSITION, IRREVERSIBLE BINDING AND IMMUNOGENICITY OF PENICILLIN IN THE RAT

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Abstract—The disposition, irreversible binding and immunogenicity of benzylpenicillin (BP†) were studied in male Wistar rats. [^{3}H]BP, administered i.v. to anaesthetized rats at two doses (27 μmol/kg, 2.7 mmol/kg), showed dose-dependent kinetics: plasma and tissue concentrations of total BP were disproportionately increased at the higher dose. BP was rapidly cleared from the plasma at both doses (<0.05% of administered dose/ml plasma after 3 hr). In spite of the disproportionately elevated levels of total BP after the higher dose, covalent binding to plasma proteins was quantitatively similar as a percentage of the dose at both doses. Three hours after i.v. injection of 27 μmol/kg and 2.7 mmol/kg of the drug, 5.6% ± 1.7% and 3.3% ± 1.1% respectively of circulating BP was covalently bound, representing <0.004% of the administered dose bound per ml of plasma in each case. Covalent binding of BP to rat plasma proteins *in vitro* was of a similar magnitude to that observed *in vivo*: 1.6% ± 0.4% of BP was bound to 25% rat plasma after 3 hr incubation at 37°. In a separate series of experiments the immunogenicity of BP was studied by chronic administration of the drug to rats. Following daily i.v. or i.m. administration of BP (27 μmol/kg, 270 μmol/kg, 2.7 mmol/kg) for 4 consecutive days at 4-week intervals (three series of injections) neither IgG nor IgM anti-benzylpenicilloyl (BPO) antibodies were detected by enzyme-linked immunosorbent assay (ELISA). Intravenous administration of the high dose of BP was discontinued after the first series of injections due to local necrosis. In contrast to free BP, BPO–keyhole limpet haemocyanin (BPO–KLH, 42 nmol BP bound/mg KLH) administered by single i.v. injection at 4-week intervals at two doses (20 and 200 μg conjugate/kg, corresponding to 0.84 and 8.4 nmol BPO/kg) readily induced IgG and IgM anti-BPO antibody responses (median IgG titres were 872 and 5470 one week after the third injection of the low and high dose of conjugate respectively; corresponding IgM titres were 4513 and 22,866). The specificity of the IgG and IgM antibodies for the BPO determinant was confirmed by ELISA inhibition with BPO–aminocaproate. These experiments show that BP binds irreversibly, but to a limited extent, to plasma proteins *in vivo*, and that such a degree of conjugation appears to be insufficient to elicit a detectable anti-BPO antibody response.

Penicillin is one of the least toxic antibiotics and can be tolerated in man at doses as high as 100–1000 mg/kg/day. However, in a minority (between 1 and 10%) of patients, adverse reactions do occur, ranging in severity from mild skin rashes to much rarer conditions such as anaphylactic shock and blood cytopenias [1, 2]. A large proportion of adverse reactions to penicillins are mediated by the immune system, as evidenced by circulating and cell-bound, penicillin-specific antibodies in susceptible individuals. In common with other low molecular weight compounds, penicillin is thought to conjugate to a macromolecular carrier in order to be recognized by the immune system, and hence to act as immunogen for induction of antibody synthesis [3]. The major antigenic determinant derived from penicillin is known to be the protein-conjugated penicilloyl group [4], but there is still controversy over whether penicilloyl–protein conjugates are generated *in vivo* or are present as contaminants in drug preparations. De Weck and colleagues claim that penicilloylation of autologous proteins *in vivo* leads to generation of an immunogen [2, 5, 6], whereas Ahlstedt and coworkers have failed to demonstrate protein conjugation and generation of immunogen by pure penicillin *in vivo* [1, 7, 8].

There is little quantitative information on the covalent binding of penicillin to proteins either *in vitro* or *in vivo*. The penamaldate assay, as used by several groups [9–12] for measurement of pencilloyl groups on proteins after dialysis of penicillin-treated serum, is subject to interference, for example by penicilloic acid, and dialysis alone may not be sufficient to dissociate totally penicillin and its non-covalently bound metabolites from proteins. Lapresle and Wal [13] measured very small amounts of penicilloylated albumin in man by radioimmunoassay, however accurate quantification was not possible as only a fraction of the bound penicilloyl groups were accessible to the antibody. Recently, caution has been advised in the use of radioimmunoassay to determine the degree of protein penicilloylation since differences in hapten density can alter the results obtained [12]. Several studies (e.g. [4, 5]) claiming that free penicillin induces anti-penicillin antibodies by generating *in vivo* an immunogenic conjugate

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† Abbreviations: BP, benzylpenicillin; BPO, benzylpenicilloyl; OVA, ovalbumin; KLH, keyhole limpet haemocyanin; ELISA, enzyme-linked immunosorbent assay.

with autologous proteins, can generally be discounted on the grounds that a foreign adjuvant (e.g. complete Freund's) was invariably used, thus providing a source of highly immunogenic heterologous carrier to which the penicillin may also bind.

The purpose of this study was, therefore, twofold: first, to develop sensitive methods for quantification of covalent binding between penicillin and rat plasma proteins, both *in vitro* and *in vivo*, at doses equivalent to those used therapeutically; second, to investigate the immunogenicity of free benzylpenicillin at the same doses administered chronically i.v. and i.m. without adjuvant to rats.

MATERIALS AND METHODS

Chemicals. [phenyl-4(*n*)-^{3}H] Benzylpenicillin ([^{3}H]BP; 11.4 Ci/mmol) was obtained from Amersham International (Bucks, U.K.). Benzylpenicillin (BP, sodium salt), ovalbumin (OVA, grade V), keyhole limpet haemocyanin (KLH), *o*-phenylenediamine dihydrochloride and Tween 20 were all obtained from Sigma (London) Chemical Co. (Poole, Dorset). HPLC grade solvents were products of Fisons (Loughborough, Leics) and all other reagents were of analytical grade. NCS tissue solubilizer was from Amersham Corp. (Arlington Heights, IL, U.S.A.). Scintillation fluid (scintillator 299) was from Packard-Becker B.V. (Groningen, Holland).

Microtitre plates (Falcon 3912-Microtest III) were obtained from Becton Dickinson Labware (Oxford, U.K.). Rabbit anti-rat IgG (Fc-specific), rabbit anti-rat IgM (Fc-specific) and horseradish peroxidase-labelled goat anti-rabbit IgG were obtained from Nordic Immunological Laboratories (Maidenhead, U.K.).

Penicilloic acid was synthesized from benzylpenicillin according to the method described by Cole *et al.* [14].

Synthesis of benzylpenicilloyl–protein conjugates. BP (229 mg) was mixed with 100 mg of protein (OVA or KLH) in 6 ml carbonate buffer (pH 10.8, 0.1 M) and incubated for 18 hr at 37°. Incubation mixtures were dialysed for 5 days against 5 l of phosphate buffer (pH 8.4, 0.07 M), with replacement of the buffer every 24 hr. The conjugates were then dialysed against 5 l of distilled water for 24 hr (replaced every 6 hr), to remove buffer salts, and were lyophilized. The degree of benzylpenicilloylation was determined by penamaldate assay [15] and purity of the conjugate verified by ion-exchange chromatography. The epitope densities (molar ratio of hapten to protein) of the synthesized conjugates were found to be 5:1 for benzylpenicilloyl (BPO)–OVA and 40:1 for BPO–KLH (assuming a molecular weight for KLH of 10^6).

N-(α-D-Benzylpenicilloyl) aminocaproic acid (BPO–aminocaproate) was synthesized according to the method of Levine [15].

Analytical methods. The following HPLC method was developed for separation of penicillin from its metabolites. Standard solutions of BP and benzylpenicilloic acid were separated on a radially compressed (Z-module, Waters) reverse-phase C_{18} column (5 μm packing) protected by a guard column of Co-Pell ODS (Whatman Inc., Clifton, NJ, U.S.A.). The solvent system was 50% methanol:50% ammonium phosphate buffer (pH 2.3, 0.05 M) at a flow rate of 2 ml/min (LKB 2152 solvent delivery system). Under these conditions the retention times of the standards, measured by UV absorbance at 285 nm (LKB 2151), were 3.8 min for benzylpenicilloic acid and 6.6 min for BP. Extracts of plasma from *in vivo* and *in vitro* experiments were analysed in the same way as the standards except that fractions of the eluant were collected every 30 sec and dissolved in scintillant (4 ml) for measurement of radioactivity (Beckman LS1801).

In vivo *binding of* [^{3}H]*BP to rat plasma proteins.* Male Wistar rats (180–250 g) were anaesthetized with urethane (1.4 g/kg) and the trachea, carotid artery and jugular vein were cannulated. The rats' penises were ligated to prevent urine loss. [^{3}H]BP (27 μmol or 2.7 mmol/kg, 100 μCi/kg) was administered i.v. in 1 ml/kg of 0.15 M NaCl. The drug was dissolved immediately before dosing. Blood samples (~0.5 ml) were withdrawn at 5, 30, 60, 120 and 180 min and immediately centrifuged (1000 *g*) to obtain plasma. An aliquot of plasma (300 μl) was removed, diluted to 1 ml with phosphate buffer (pH 7.4; 0.05 M), and immediately extracted with 3 ml ice-cold acetone and centrifuged for 5 min at 2000 r.p.m. The acetone extract was removed and an aliquot (50 μl) was taken for liquid scintillation spectrometry. The remainder was evaporated under a stream of nitrogen and analysed by HPLC. Covalent binding of [^{3}H]BP to plasma proteins was then determined as described below. Aliquots of the plasma (20 μl) and acetone extracts (50 μl) were assayed for radioactivity to determine the efficiency of extraction at each time point. After 3 hr the animals were killed by exsanguination and the major organs were removed. Urine was removed from the bladder by aspiration and aliquots (50 μl) assayed for radioactivity. Urine was also analysed for parent drug and metabolites by radiometric HPLC.

In vitro *binding of* [^{3}H]*BP to rat serum proteins.* Rat serum (250 μl) was added to [^{3}H]BP (0.015 mg, 0.35 μCi) in phosphate buffer (50 μl, pH 7.4, 0.05 M) in 10 ml glass tubes, diluted to 1 ml with phosphate buffer and incubated for 0, 0.25, 0.5, 1, 2, 6, 24 and 48 hours in a water bath at 37°. At the end of the incubation period the protein was precipitated by addition of 3 ml ice-cold acetone and treated as above.

The covalent binding of [^{3}H]BP to serum proteins was determined as described below.

Determination of irreversible binding of [^{3}H]*BP.* The amount of [^{3}H]BP irreversibly bound to plasma proteins was determined after acetone extraction by the method of Sun and Dent [16] with some modifications as previously described [17]. Briefly, the acetone precipitated proteins were dissolved in 0.1 M phosphate buffer (pH 7.4) containing 2% sodium dodecyl sulphate (SDS), then dialysed against 1 l of 0.01 M phosphate buffer (pH 7.0) containing 0.1% SDS [16] for 24 hr. The radioactive content of an aliquot of the dialysed sample and an aliquot (1 ml) of the dialysis buffer were then

determined by liquid scintillation spectrometry. The amount of covalently bound radioactivity remaining in the dialysis bag was determined by subtracting the amount of radioactivity in the same volume of dialysis buffer. The protein concentration before and after dialysis was determined [18] for correction of dilution. The possibility that free BP, or its non-covalently bound degradation products, were not dissociating completely from the protein during dialysis, thereby resulting in spuriously high values for covalent binding, was investigated with the following controls.

i [^{3}H]BP was added to protein and extracted immediately.
ii [^{3}H]BP was incubated in buffer for varying periods of time and protein was added immediately prior to extraction.
iii [^{3}H]Benzylpenicilloic acid, the major breakdown product of [^{3}H]BP, was incubated with serum proteins for 2 hr before extraction. The apparent covalent binding to all the precipitated protein samples was determined as described above.

Distribution of [^{3}H]benzylpenicillin in anaesthetized rats. After the 3-hr blood sample was taken, rats were killed by exsanguination and the major organs were removed; the tissues were immediately frozen in dry ice and stored frozen until assayed for radioactivity. Total radioactivity in brain, kidney, heart, liver, lung and spleen was determined as described previously [19]. No attempt was made to determine covalently bound radioactivity in individual tissues.

Immunization of rats. Male Wistar rats (150–250 g) were treated as follows:

Experiment 1. BPO–KLH (1.0 mg/kg in 1.0 ml/kg 0.15 M NaCl) was injected i.p. into three rats. The injection was repeated 14 days later and blood samples were removed by cardiac puncture 7 days after the second injection. Blood was allowed to clot overnight at room temperature and sera were separated and stored at −50° for subsequent use as positive reference anti-BPO antisera.

Experiment 2. BPO–KLH in 0.15 M NaCl was injected i.p. via the tail vein into two groups of seven rats. The rats received a single injection on day 0, and repeat single injections 4 weeks and 8 weeks later. The first group received 20 μg/kg and the second group received 200 μg/kg of conjugate. Blood samples (~1 ml) were removed from the tail vein 2 weeks after the first injection, and 1 week and 2 weeks after the second and third injections.

Experiment 3. BP was injected in 0.15 M NaCl into six groups of seven rats. Three groups received 27 μmol, 270 μmol and 2.7 mmol/kg of BP i.v. via the tail vein; three groups received the same doses intramuscularly into one hind limb. The injections were performed daily for four consecutive days, and each series of injections was repeated 4 weeks and 8 weeks after the first. Blood samples (~1 ml) were removed from the tail vein 2 weeks after completion of the first series of injections and 1 week and 2 weeks after completion of the second and third series of injections. Following the first series of i.v. injections of 2.7 mmol of BP, severe tissue necrosis of the tail developed and further i.v. injections were discontinued in this group of rats.

Enzyme-linked immunosorbent assay (ELISA) for detection of IgG and IgM anti-BPO antibodies. Microtitre plates were coated with 50 μg/ml BPO–OVA (epitope density 5:1) or OVA in 0.05 M phosphate buffer, pH 7.2, overnight at 4° (125 μl/well). The plates were then washed three times with 0.15 M phosphate-buffered saline containing 0.05% Tween 20 (PBS–Tween; pH 7.2) and shaken dry. All subsequent washes were performed in the same way. Each well was then successively incubated for 1 hr at room temperature with the following, in a moist box, with washing between each step: 100 μl of rat serum serially diluted 3-fold down columns in duplicate in PBS–Tween (starting dilution 1/50); 100 μl of rabbit anti-rat IgG or rabbit anti-rat IgM (diluted 1000:1 in PBS–Tween); 100 μl of peroxidase-labelled goat anti-rabbit IgG (diluted 4000:1 in PBS–Tween); 100 μl of substrate solution containing 0.1% hydrogen peroxide (30% w/v) and 400 μg/ml of *o*-phenylenediamine in 0.15 M citrate–phosphate buffer (pH 5.0). The enzyme–substrate reaction was terminated after 10 min by addition of 50 μl of 25% sulphuric acid. Absorbances were read at 490 nm by a dual wavelength automated plate reader (Dynatech MR600) with the reference wavelength set at 630 nm. IgG and IgM anti-BPO activities were expressed as antibody titre following coating of wells with BPO–OVA in the absence of activity against OVA alone. Titres were calculated as dilution of rat serum giving an optical density five times the background reading (~0.2), and also as dilution of serum giving an optical density equal to that obtained for a standard reference serum at a dilution of 12,150:1 incorporated in each assay. There was no significant difference in the titres obtained by either method.

ELISA inhibition. ELISAs for IgG and IgM anti-BPO activities were performed as above, except that at the second step of the assay a fixed amount of BPO–aminocaproate (final concentration 100 μg/ml or 1.0 mg/ml) was added to each dilution of the rat sera.

Statistical analysis. In all cases statistical comparisons between groups were performed using Student's *t*-test for unpaired data. A difference was deemed significant when the P value was less than 0.05. All values quoted in text and tables are mean ± SD, those in figures mean ± SEM.

RESULTS

Disposition and irreversible binding of [^{3}H]BP in vivo

The disposition of [^{3}H]BP showed clear dose dependency following i.v. administration to anaesthetized rats at two doses (27 μmol/kg and 2.7 mmol/kg; Fig. 1). Both the total radioactivity and concentrations of free BP were higher, as percentage of the dose, after the higher dose. However, the most marked effect was seen with the availability of BP (AUC_{0-3h}) which was increased 530-fold for a 100-fold increase in dose (Table 1). Degradation of

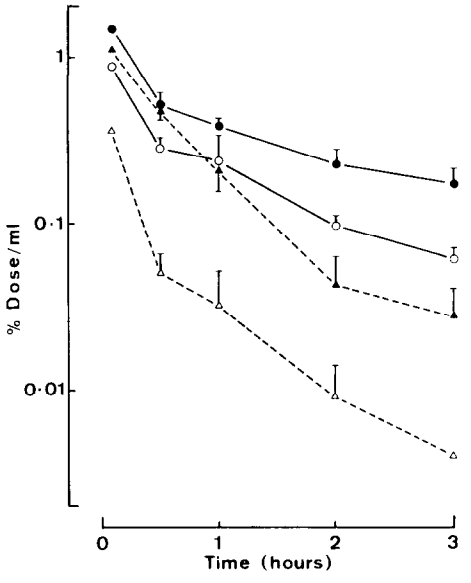

Fig. 1. Total radioactivity (solid lines) and concentration of [^{3}H]BP (broken lines) in plasma of rats administered i.v. [^{3}H]BP at doses of 27 μmol/kg (open symbols; N = 11*) and 2.7 mmol/kg (closed symbols; N = 7). Error bars represent SEM. *[^{3}H]BP value for 3 hr in rats given 27 μmol/kg represents the mean of two points; concentrations in other animals being below the limit of sensitivity of the assay.

BP occurred very rapidly *in vivo* as shown by the radiochromatograms in Fig. 2, which were obtained from a rat given 2.7 mmol/kg of [^{3}H]BP. After 5 min ~80% of the radioactivity extracted from plasma co-chromatographed with authentic BP (Fig. 2a) whereas after 3 hr only ~10% ran with BP (Fig. 2b). Most of the radioactivity not associated with BP had a retention time equal to that of penicilloic acid, though at later time points radioactivity associated with more polar metabolites was discernable as a shoulder on the penicilloic acid peak. No attempt was made to characterize low molecular weight metabolites further and consequently results are expressed in terms of total metabolites. The plasma clearance of BP was more rapid after the lower dose of 27 μmol/kg and BP was virtually undetectable at 3 hr (Fig. 1).

Although the availability of BP was not directly proportional to the dose, the covalent binding of BP appeared to relate more closely to the dose administered than to the AUC of free BP. Thus the AUC_{0-3h} for covalently bound BP was only 140 times greater at the 100-fold higher dose, despite a 530-fold increase in free penicillin AUC (Table 1). The dose-dependent disposition of BP is demonstrated more clearly when the data are expressed as percentage of the total radioactivity per ml plasma (Fig. 3). After a dose of 27 μmol/kg, free BP accounted for less than 50% of plasma radioactivity only 5 min

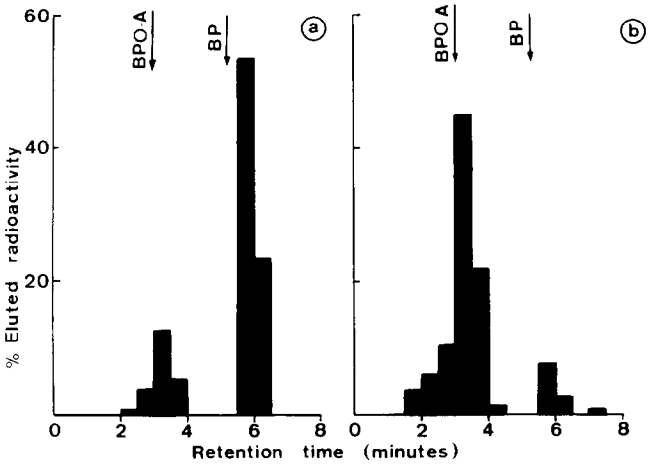

Fig. 2. Radiochromatograms of extracts of plasma samples taken 5 min (a) and 3 hr (b) after administration of ^{3}[H]BP (2.7 mmol/kg) to an anaesthetized rat. Radioactivity, quantified in 30-sec fractions, is expressed as the percentage of the total radioactivity eluted off the column. The retention times of benzylpenicilloic acid (BPO-A) and BP, as measured by UV absorbance, are indicated by the arrows.

Table 1. Total radioactivity, free [^{3}H]BP and covalently bound [^{3}H]BP, expressed as area under the curve from 0–3 hr (AUC) 3 hr after the administration of [^{3}H]BP to anaesthetized rats

Dose	N	Total radioactivity AUC (nmol/ml h)	Free BP AUC (nmol/ml h)	Covalent binding (pmol/mg protein)
27 μmol/kg	4	56.5 ± 27.5	8.4 ± 3.3	3.3 ± 1.9
2.7 mmol/kg	7	7851 ± 2899	4438 ± 1756	521 ± 263

Values are means of N determinations ± SD.

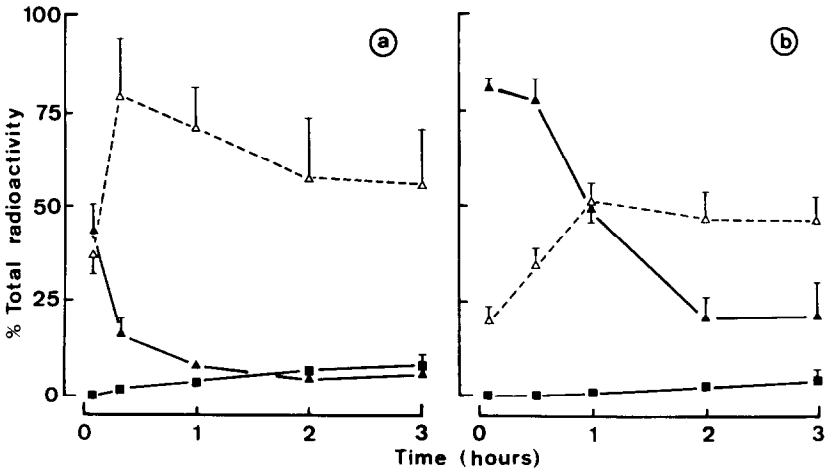

Fig. 3. Proportion of total radioactivity present as [^{3}H]BP (▲), polar acetone-extractable metabolites (△) and irreversibly bound to proteins (■) in plasma of rats administered [^{3}H]BP (20 μCi) i.v. at doses of 27 μmol/kg (graph a) and 2.7 mmol/kg (graph b). Each point represents the mean of 4–7 animals ± SEM.

after dosing (Fig. 3a). With the higher dose (2.7 mmol/kg) free BP represented more than 50% of total radioactivity 1 hr post dosing (Fig. 3b). It should be noted that during the early time points all the radioactivity in plasma can be accounted for by the sum of extractable BP, metabolites and covalently bound radioactivity; at later time points this is not the case. Thus, BP must be converted to other, highly polar, metabolites which are not extracted with acetone, but which dissociate from the proteins under the conditions of the dialysis.

The distribution of radioactivity into the major organs is shown in Fig. 4. In all tissues there was numerically more radioactivity following the higher dose of BP, however, this was only statistically significant in the liver, lung and plasma. The highest concentrations of radioactivity were found in the kidney; however, there was extremely high inter-individual variation in the amounts accumulated and no significant difference was observed. The excretion of radioactivity in urine was also highly variable, being 46 ± 16% and 37 ± 19% for doses of 27 μmol and 2.7 mmol/kg, respectively.

Degradation and irreversible binding of [^{3}H]*BP* in vitro

The degradation of [^{3}H]BP to its more polar metabolites in rat serum (Fig. 5) occurred at a much slower rate *in vitro* than *in vivo*. The concentration of free BP did not fall below 50% of total radio-

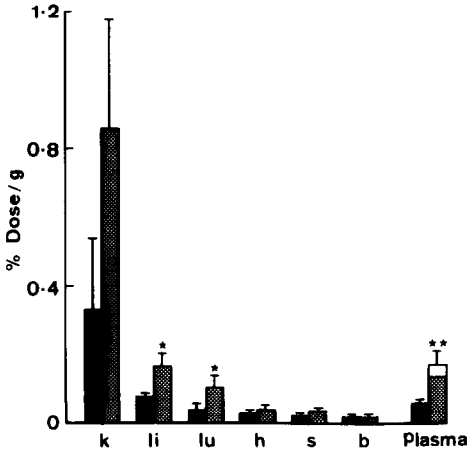

Fig. 4. Distribution of radioactivity into kidney (k), liver (li), lung (lu), heart (h), spleen (s), brain (b) and plasma 3 hr after administration of [^{3}H]BP to anaesthetized rats at doses of 27 μmol/kg (■; N = 11) or 2.7 mmol/kg (▨; n = 7). The fraction of radioactivity present in plasma as unchanged [^{3}H]BP is represented by the unshaded area. Each column represents the mean ± SEM. Starred columns were statistically different (*$P < 0.05$, **$P < 0.005$).

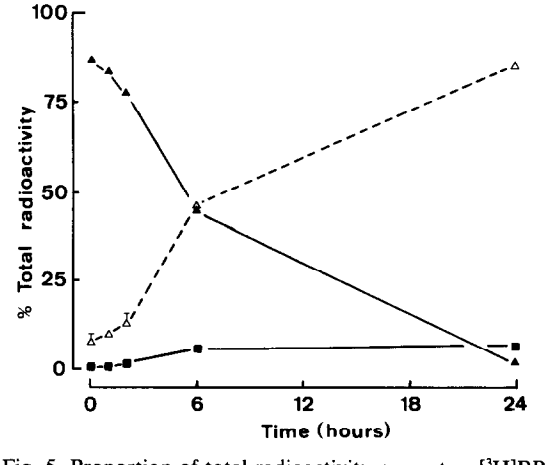

Fig. 5. Proportion of total radioactivity present as [^{3}H]BP (▲), extractable polar metabolites (△) and covalently bound to serum proteins (■) after incubation of [^{3}H]BP (11 μmol, 0.13 μCi) with rat serum. Each point represents the mean of four duplicate determinations. Standard errors, unless shown, were <10%.

Table 2. IgG and IgM anti-BPO responses in rats administered BPO–KLH i.v. on days 0, 28 and 56

		Day				
		14	35	42	63	70
		IgG anti-BPO responses				
BPO–KLH	Responders*	2/7	4/7	3/7	6/6	5/6
20 μg/kg	Titre†	—	262	150	872	149
BPO–KLH	Responders	4/7	6/6	5/6	5/5	5/5
200 μg/kg	Titre	92	812	1315	5470	3707
		IgM anti-BPO responses				
BPO–KLH	Responders	0/7	4/7	6/7	6/6	5/6
20 μg/kg	Titre	—	614	214	4513	560
BPO–KLH	Responders	0/7	6/6	4/6	5/5	5/5
200 μg/kg	Titre	—	2053	892	22866	3926

* Number of rats per group with positive serum anti-BPO activity (titre >50 for IgG; >100 for IgM). Antibody titres were calculated as the reciprocal of the serum dilution giving an optical density of five times background.

† Median antibody titre where number of responders >3.

activity until 6 hr incubation at 37° (Fig. 5). At this time 4.0% ± 1.0% of radioactivity was covalently bound and this increased to a maximum value of 4.5% ± 0.5% after 24 hr incubation. After 24 hr virtually all the free BP had disappeared and covalent binding remained constant up to 48 hr (value not shown). It was not possible to compare directly the *in vitro* results with those obtained *in vivo* since the plasma was diluted 4-fold in the *in vitro* incubations. Nevertheless, at 3 hr *in vitro* the covalent binding was 1.6% ± 0.4% which, allowing for the dilution, most closely resembles the lower dose value *in vivo* (5.6% ± 1.7%).

IgG and IgM anti-BPO antibodies

Following the preliminary immunization schedule in which BPO–KLH was administered i.p. to three rats (1.0 mg/kg) without adjuvant, high titre IgG and IgM anti-BPO antibody activity was detected in the sera obtained one week after the second injection (IgG titres 11,000–36,000; IgM titres 5000–32,000: titres calculated as reciprocal of serum dilution giving five times background optical density). One of the antisera thus obtained was incorporated as reference standard in all subsequent antibody assays.

Following intravenous administration of BPO–KLH (20 μg/kg) by a single injection at 4-week intervals, serum IgG and IgM anti-BPO antibody activities were readily detected after the second and third injections. IgG and IgM anti-BPO antibody activities reached a maximum one week after the third injection (median titres 872 and 4513, respectively; Table 2). A 10-fold higher dose of BPO–KLH (200 μg/kg) induced stronger IgG and IgM anti-BPO responses which again reached a maximum one week after the third injection (median titres 5470 and 22,866, respectively; Table 2). The specificity of the IgG and IgM antibodies for the BPO determinant was confirmed in ELISA inhibition experiments: IgG titres were reduced ~20-fold by 100 μg/ml BPO–aminocaproate; IgM titres were reduced ~10-fold by 1.0 mg/ml BPO–aminocaproate.

In the final experiment rats were administered four daily injections of BP either i.v. or i.m. at 4-week intervals. Following administration of doses of 27 μmol and 270 μmol/kg of the drug by both routes, neither IgG nor IgM anti-BPO activities were detected after each series of injections. Following administration of 2.7 mmol/kg (~1 g/kg) of BP i.v., neither IgG nor IgM anti-BPO activity was detected 2 weeks after the first series of injections. No further i.v. injections of this dose were administered because tail necrosis developed in the recipients. After i.m. injections of the same dose (2.7 mmol/kg) of the drug, neither IgG nor IgM anti-BPO activities were detected after each of the three series of injections.

DISCUSSION

Penicillin allergy has become accepted as the classical example of drug-induced hypersensitivity and is one of the few instances of an immunotoxic reaction in which anti-drug antibodies have been definitively characterized [1]. Although the major antigenic determinant has been identified as the penicilloyl group [4], the exact nature and origin of the immunogen responsible for stimulation of the immune system is still unknown. It is thought that for a drug to generate an immunogen *in vivo* it must become covalently bound to an autologous carrier macromolecule, for example a plasma protein, thereby forming a hapten and converting the carrier from an immunologically tolerated macromolecule to one recognized as foreign by the immune system. The difficulty in establishing this mechanism in the case of penicillin allergy has been the detection of penicillin–protein conjugates formed *in vivo*. Although penicillin will react readily with proteins *in vitro* at alkaline pH [15] the reaction is much slower at physiological pH.

Determination of covalent binding of penicillin to plasma proteins *in vivo* has been hampered by the lack of a specific and sensitive assay for penicilloylated proteins. The existence of low circulating

levels of penicilloylated albumin has been demonstrated in patients by radioimmunoassay [13]. However, it was found that proteolysis of the conjugated albumin resulted in an increase in apparent penicilloylation of up to 200 times the original values. Thus radioimmunoassay is unsuitable for accurate quantification of the absolute amount of penicilloylation and, moreover, has been shown to be sensitive to variations in hapten density on individual carrier molecules [12]. In this study we have used high specific activity radiolabelled benzylpenicillin (BP) combined with rapid sample processing (to avoid *ex vivo* binding of BP to plasma proteins) and carefully controlled dialysis (ensuring removal of BP metabolites as well as parent compound) in order to quantify the degree of irreversible binding of BP in the rat. Such irreversible binding is largely due to covalent binding of penicilloyl groups to lysine residues in proteins [20].

The major finding was that BP does not conjugate extensively with rat plasma proteins either *in vivo* or *in vitro*; the irreversible binding values obtained represented less than 0.004% of the dose after both BP doses. In absolute mass terms, these figures mean that after a BP dose of 27 μmol/kg the amount irreversibly bound to plasma proteins was only 3.3 pmol/mg. This represents a mean epitope density of 1.5×10^{-4}, assuming an average molecular weight of proteins of 68,000 and an equal distribution of hapten amongst the proteins. In a similar study with dinitrofluorobenzene (DNFB) we have shown that the same (equimolar) i.v. dose of DNFB leads to an epitope density of 2.80×10^{-2} [21]. DNFB provides a useful comparison with penicillin since it reacts selectively with lysine residues in proteins, as does penicillin, and because it functions as an immunogen, even when administered in its free (non-conjugated) form. It is apparent from these studies that there is a 100-fold lower degree of haptenation of autologous proteins with penicillin compared with DNFB. In order to attain the same level of haptenation as seen with DNFB, doses of 2.7 mmol/kg of BP were required; however, in the present study no anti-BPO response was obtained following administration of BP. Such doses are one or two orders of magnitude higher than therapeutic doses typically used in man to treat minor infections, but are equivalent to the mega-unit doses used in the treatment of conditions such as meningitis and endocarditis [22].

When large (2.7 mmol/kg) doses of BP were administered in these experiments, there was a marked change in its disposition (Fig. 1). The availability of BP, as demonstrated by the plasma concentration–time curve and AUC_{0-3h}, was greatly increased. This change may represent saturation of active renal tubular secretion, a conclusion which is supported by the fact that tissue levels of radioactivity were raised in line with the increase found in plasma and that there appeared to be a reduction in urinary radioactivity. Moreover, a similar increase in BP availability was observed when probenecid was administered prior to a low dose (27 μmol/kg) of BP (unpublished observation); probenecid is known to compete with BP for active tubular secretion. Whatever the mechanism, the increased availability of BP found after the high dose did not appear to result in a proportional increase in covalent binding. Thus 'exposure' of proteins to BP apparently is not the determining factor in protein binding. This suggests that either the binding affinity of albumin (or other proteins) for BP decreases as penicilloylation proceeds, or that binding of BP is not direct but proceeds through a minor metabolite formed enzymically by a saturable enzyme.

In the present study chronic administration of BP (three series of four daily injections at 4-week intervals) at doses up to 270 μmol (0.1 g)/kg i.v. and up to 2.7 mmol (1.0 g)/kg i.m. failed to induce either IgG or IgM anti-BPO responses detectable by ELISA. In those rats administered a single series of four daily injections of 2.7 mmol/kg i.v., tail necrosis developed and injections were discontinued, but again no circulating anti-BPO antibodies were detected. In contrast, single injections of 20 μg/kg and 200 μg/kg of BPO–KLH without adjuvant at 4-week intervals induced readily detectable IgG and IgM anti-BPO responses. The lower dose of BPO–KLH used (20 μg/kg) represented a dose of conjugated BP of 0.8 nmol/kg, which is 3.4×10^5-fold less than the intermediate dose of free BP (270 μmol/kg), and 3.4×10^6-fold less than the high dose of free BP (2.7 mmol/kg) employed in the chronic administration experiments. These results show that BP is at least to 10^6 times less immunogenic in free form than when conjugated to a foreign protein. In addition, hapten–protein conjugates derived from autologous proteins are less immunogenic than those derived from heterologous proteins, since only the latter possess inately the requisite carrier (T cell) determinants for immune recognition. In order for an autologous protein to be rendered immunogenic by substitution, not only must the haptenic (B cell) determinant be generated, but so also must novel carrier (T cell) determinants. These considerations make it highly unlikely that conjugation of BP to autologous proteins *in vivo* leads to immunogen formation. Furthermore, if a preparation of BP is shown to be immunogenic either in animals or humans, the possibility that one BP molecule per 10^6 is covalently bound to a foreign contaminant in the drug preparation must be excluded before *in vivo* generation of the immunogen is implicated.

Perhaps the best evidence that penicillin generates an immunogen *in vivo* is provided by Schneider and de Weck [6] who induced anti-BPO antibody responses in rabbits immunized with benzylpenicillin and phenoxymethyl-penicillin emulsified with complete Freund's adjuvant. Selective hydrolysis of the penicillin preparations to yield the non-reactive penicilloic acid led to loss of immunogenic activity, whereas in control experiments the structural and functional integrity of penicilloylated proteins was unaltered by the same treatment. Nevertheless, complete Freund's adjuvant appears necessary for induction of anti-BPO antibody response by penicillin. Ahlstedt *et al.* [7] failed to detect anti-ampicillin antibodies by radioimmunoassay in mice treated by daily injection of 50 mg/kg of ampicillin without adjuvant. Similarly, Kristofferson *et al.* [8] failed to induce IgE anti-BPO responses in mice injected with 60 mg/kg/day of BP without adjuvant, whereas contamination of BP preparations with as little as 3 μg

of BPO–bovine γ-globulin per g of penicillin led to IgE responses readily detectable by passive cutaneous anaphylaxis. Our own experiments in rats support the conclusion that penicillin is non-immunogenic in free form when administered without adjuvant. The lack of immunogenicity of free BP (27 μmol–2.7 mmol/kg) contrasts with the graded immune response observed when DNFB (0.027–27 μmol/kg) was given using an identical injection schedule [21]. This difference may be partly accounted for by the relative degrees of conjugation observed with these two compounds *in vivo*, as discussed above. In addition, it is known that BP's reactivity towards proteins is equivalent to dinitro*chloro*benzene [23], making BP several times less reactive than DNFB [24]. The faster relative rate of reactivity of DNFB may result in the formation of individual protein molecules with epitope densities much higher than the mean value. The rapid distribution and slower reactivity of BP is likely to result in a more uniform distribution of epitopes on plasma proteins.

In conclusion, we have found that BP does not readily form conjugates that are immunogenic in the rat. This lack of immunogenicity of BP may be partly accounted for by: (i) its rapid excretion; (ii) its rapid hydrolysis to breakdown products such as penicilloic acid; and (iii) its low degree of conjugation to plasma proteins. The marked immune response to small (10^6-fold less) doses of BP conjugated to a foreign protein is consistent with the hypothesis that BP–protein conjugate impurities may provide a source of penicilloylated immunogens.

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