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DRUG–PROTEIN CONJUGATES—XVII

THE EFFECT OF STORAGE ON THE ANTIGENICITY AND IMMUNOGENICITY OF BENZYLPENICILLIN IN THE RAT

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Abstract—The disposition and immunogenicity of freshly prepared and stored solutions of benzylpenicillin (BP) and benzylpenicillenic acid (BPE), a degradation product of BP, were studied. No IgG anti-benzylpenicilloyl (BPO) antibodies were detected by enzyme-linked immunosorbent assay (ELISA) following daily i.p. or i.m. administration to male Wistar rats of BP (2.7 mmol/kg) freshly dissolved in 0.5% glucose, for 4 consecutive days at 4-week intervals. In contrast, IgG anti-BPO antibodies were detected following both chronic i.p. and i.m. administration of BP (2.7 mmol/kg) stored for 24 hr at room temperature in 0.5% glucose. An IgG anti-BPO response was obtained only after the high dose, following daily i.m. administration of BPE (27 μmol/kg, 2.7 μmol/kg, 0.24 μmol/kg). The specificity of the IgG antibody for the BPO-determinant was confirmed by ELISA inhibition with BPO-aminocaproate. Circulating BPO plasma–protein antigens were detected by a modified ELISA following i.p. and i.m. administration of both stored and fresh BP. Significantly lower BPO-antigen levels were detected in serum following BPE administration. Irreversible binding of BP to 75% rat plasma proteins was of the same magnitude when freshly dissolved in phosphate buffer or in 0.5% glucose (2.63 ± 0.32% and 2.55 ± 0.25% bound, respectively after 3 hr incubation at 37°). Irreversible binding was significantly greater ($P < 0.05$) when the BP was stored prior to incubation with the protein (3.81 ± 0.27%). The major degradation product of stored BP was benzylpenicilloic acid; a small amount of BPE (0.2% of incubated BP) was detected in stored but not fresh BP. Thus, the increased immunogenicity of BP stored for 24 hr at room temperature may be due to the formation of reactive degradation products such as BPE *in vitro*, which can then form immunogenic drug–protein conjugates *in vivo*. These experiments also show that although BP and BPE form drug–protein conjugates *in vivo*, circulating levels of antigen do not relate to the immunogenicity of either of the compounds.

Hypersensitivity reactions are the most serious adverse effects associated with penicillin therapy [1], and may be manifested in a variety of clinical forms, including anaphylaxis [2], haemolytic anaemia [3] and various skin rashes [4]. Our present understanding of drug-induced hypersensitivity is based on the assumption that the drug must first become covalently bound to a macromolecular carrier, in order to be recognised as foreign by the immune system and act as an immunogen for induction of antibody synthesis [5]. Consistent with this hypothesis, antibodies directed against the protein–conjugated benzylpenicilloyl group (BPO; the major antigenic determinant formed from penicillins [6]) have been detected in both patients [7] and experimental animals [8].

However, there is still debate as to the nature and origin of the immunogen responsible for stimulating the immune response [1]. It has been suggested that penicillin immunogens may be formed by conjugation of the drug to proteins during the manufacturing process [9]. Accordingly, in the rat, we observed an immune response to 0.84 nmol BPO/kg bound to keyhole limpet haemocyanin, whereas pure free BP was non-immunogenic at 10^6-fold higher doses [10]. It has also been suggested that reactive intermediates are formed by rearrangement of penicillin in solution, which then react with autologous proteins, and thus form immunogens *in vivo* [11]. To investigate this second possibility, we have investigated the immunogenicity, and protein reactivity, of freshly prepared and stored (24 hr) solutions of benzylpenicillin.

MATERIALS AND METHODS

Chemicals

[Phenyl-4-(*n*)-^{3}H]benzylpenicillin ([^{3}H]-BP; 8.8 Ci/mmol) was obtained from Amersham International (Bucks, U.K.). Benzylpenicillin (BP, sodium salt), human serum albumin (HSA), benzylpenicillenic acid (BPE), *o*-phenylenediamine dihydrochloride, keyhole limpet haemocyanin (KLH), Tween-20 and Freund's Complete Adjuvant were all obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Glucose solution (5% Viaflex, lot 86E 30DB, 500 ml for infusion) was obtained from Travenol Laboratories (Norfolk, U.K.). Dimethyl sulphoxide and Tween-80 were obtained from British Drug Houses (Poole, Dorset, U.K.). Scintillation fluid (Aqua Luma plus) was

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† Abbreviations used: BP, benzylpenicillin; BPO, benzylpenicilloyl; HSA, human serum albumin; BPE, benzypenicillenic acid; KLH, keyhole limpet haemocyanin; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography.

obtained from May and Baker Chemical Division (Manchester, U.K.). All other reagents were of analytical grade. Microtitre plates (Immulon B) were purchased from Dynatech Laboratories Ltd. (Surrey, U.K.). Rabbit anti-rat IgG (Fc-specific) and horseradish peroxidase-labelled goat anti-rabbit IgG (H and L) were obtained from Nordic Immunological Laboratories (Maidenhead, U.K.). *N*-(α-D-Benzylpenicilloyl) aminocaproic acid (BPO-aminocaproate) was synthesized according to the method of Levine [12]. Benzylpenicilloic acid and benzylpenilloic acid were synthesised as described [13, 14]. Benzylpenicilloylated human serum albumin (BPO–HSA) and BPO–keyhole limpet haemocyanin (BPO–KLH) were synthesised and characterised as described elsewhere [10]. The epitope densities (molar ratio of hapten to protein) of the conjugates were 5:1 for BPO–HSA and 40:1 for BPO–KLH (assuming a molecular weight for KLH of 10^6).

Treatment of rats

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

Group 1. BP (2.7 mmol was added to 1 ml 0.5% glucose) freshly prepared or stored for 24 hr at room temperature was injected i.p. into two groups of 8 rats. The injections were performed daily for four consecutive days, and further series of injections were repeated 4 weeks and 8 weeks after the first. Blood samples (~0.5 ml) were removed from the tail vein prior to immunization, daily for the first three days during the first series of injections and 1 week and 2 weeks after completion of each series of injections.

Group 2. BP (2.7 mmol was added to 1 ml 0.5% glucose) freshly prepared or stored as above was injected intramuscularly into alternate hind limbs into two groups of 8 rats. The injection schedule being as described in experiment 1. Blood (~1 ml) was obtained 1 week and 2 weeks after completion of each series of injections.

Group 3. BPE dissolved immediately prior to injection in 10% dimethyl sulphoxide, 90% 0.15 M NaCl, was injected i.m. into three groups of eight rats. Separate groups received either 27, 2.7 or 0.27 μmol/kg of BPE intramuscularly into alternate hind limbs according to the injection schedule described in experiment 1. Blood (~0.5 ml) was obtained 1 week and 2 weeks after completion of each series of injections. Blood (~0.5 ml) was also obtained for the first three days during the first series of injections of 27 μmol/kg BPE.

Group 4. BPE (27 μmol/kg in 1.0 ml/kg 5% Tween-80, 95% 0.15 M NaCl) was injected i.m. into eight rats for three consecutive days. Blood (~0.5 ml) was obtained before dosing and daily after each injection.

Immunization of rabbit with BPO–KLH

A male New Zealand white rabbit (5 kg) was immunized by injection of BPO–KLH emulsified in Freund's Complete Adjuvant, into intramuscular and subcutaneous sites on days 0 (5 mg of conjugate), 14, 28 and 170 (2.5 mg of conjugate). The rabbit was bled out on day 197 and serum obtained. The IgG anti-BPO–HSA activity was determined by enzyme-linked immunosorbent (ELISA) as described elsewhere [15], except that BPO–HSA (10 μg/ml) was used as the coating antigen. The antibody titre expressed as the dilution of antiserum giving half maximal end point absorbance was 37910. The antisera was shown to be specific for the BPO–hapten by inhibition with BPO-aminocaproic acid.

ELISA for detection of IgG anti-BPO antibodies

IgG anti-BPO antibody activity in the serum samples obtained 1 and 2 weeks after completion of each series of injections from experiments 1 to 3 was determined by ELISA. The method employed was similar to that used previously [10]. Briefly microtitre plates were coated with BPO–HSA or HSA (10 μg/ml) in 0.05 M phosphate buffer, pH 7.2, overnight at 4° (125 μl/well). The plates were then washed three times over a 2-min cycle with 0.15 M phosphate buffered saline containing 0.05% Tween 20 (PBS–Tween, pH 7.2). 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 1/10); 100 μl of rabbit anti-rat IgG (diluted 2000:1 in PBS–Tween); 100 μl of peroxide labelled-goat anti-rabbit IgG (diluted 5000:1 in PBS–Tween); substrate as previously described [10]. The absorbances were read as previously following termination of the enzyme–substrate reaction after 10 min. IgG anti-BPO activities were calculated as the difference in the optical density (Δ OD) between end-point OD following coating with BPO–HSA conjugate and unconjugated HSA at a serum dilution of 1/30 minus the difference in OD in the absence of serum ($\Delta OD_{1/30} - \Delta OD_0$). Hapten inhibition experiments were performed on pooled serum samples obtained from the final bleed of each group of rats. ELISAs for IgG anti-BPO activity were performed as above, except that at the second step of the assay a fixed amount of BPO–caproate (final concentration of 1 mg/ml) was added to each dilution of the serum samples.

ELISA for detection of circulating BPO antigens

Microtitre plates were coated overnight at 4° in a moist box with duplicate aliquots (125 μl/well) of rat serum (diluted 1000:1 in 0.05 M phosphate buffer, pH 7.2), obtained from experiments 1 to 4. Serum samples were immediately frozen to prevent *in vitro* reactivity between any residual penicillin and protein. The plates were then washed three times over a 2-minute cycle in 0.15 M phosphate buffered saline containing 0.05% Tween 20 (PBS–Tween, pH 7.2). 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 rabbit anti-BPO–KLH (diluted 5000:1 in PBS–Tween); 100 μl of peroxidase labelled goat anti-rabbit IgG (diluted 5000:1 in PBS–Tween); substrate as previously described [10]. Absorbances were read as before following termination of the enzyme–substrate reaction after 4 min.

The specificity of the ELISA for the BPO determinant was tested by hapten inhibition experiments on pooled serum samples obtained 1 week after completion of the third series of injections. Aliquots of the pooled sera were coated onto microtitre plates as before, however, at the second step of the assay a fixed amount of BPO–caproate (final concentration 10 μg/ml) was added.

Irreversible binding of radiolabelled material to rat plasma proteins in vitro

Rat plasma (750 μl) was added to [^{3}H]BP (0.27 mol, 5 μCi) in 0.5% glucose (100 μl) or phosphate buffer (100 μl, pH 7.4, 0.05 M) in 10 ml glass tubes, diluted to 1 ml with phosphate buffer and incubated for 3 hr in a water bath at 37°. [^{3}H]-BP (0.27 mol, 5 μCi) in 0.5% glucose (100 μl) was also stored for 24 hr at room temperature prior to addition of the rat plasma. At the end of the incubation period the protein was precipitated by addition of 200 μl of trichloracetic acid (6 M). The extent of irreversible binding of radiolabelled material to the precipitated rat plasma proteins was determined by equilibrium dialysis according to the method of Sun and Dent [16] with modifications as previously described [17].

Identification of the degradation products of BP in vitro

The degradation products of [^{3}H]-BP were analysed by co-chromatography with authentic standards using reversed phase HPLC as described previously [10]. The retention times of the standards were 14 min for benzylpenilloic acid, 19 min for benzylpenicilloic acid and 23 min for BP. Aliquots (100 μl) of [^{3}H]-BP (930 mg/ml; 15 μCi) stored for 24 hr at room temperature were analysed without prior treatment, the eluate being collected in 30 sec intervals and dissolved in scintillant (4 ml) for measurement of radioactivity (Packard Tricarb 4640).

A modification of the spectrophotometric method described by Levine [18] was used to detect BPE. Briefly, the ultraviolet absorption spectrum of BPE (30 nmol/ml) in 95% ethanol and diluted samples (1 in 10) of BP (2.7 mmol/ml) freshly dissolved or stored for 24 hours at room temperature in 0.5% glucose were obtained using a Cecil double beam ultraviolet spectrophotometer (CE 505). The amount of BPE present in the stored BP solution was determined using the molar extention coefficient obtained for BPE at 322 nm.

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, tables and figures are mean ± SEM.

RESULTS

IgG anti-BPO antibodies

The IgG anti-BPO antibody activity expressed as $\Delta OD_{1/30} - \Delta OD_0$ following chronic (three series of four daily injections at 4-weekly intervals) i.p. and

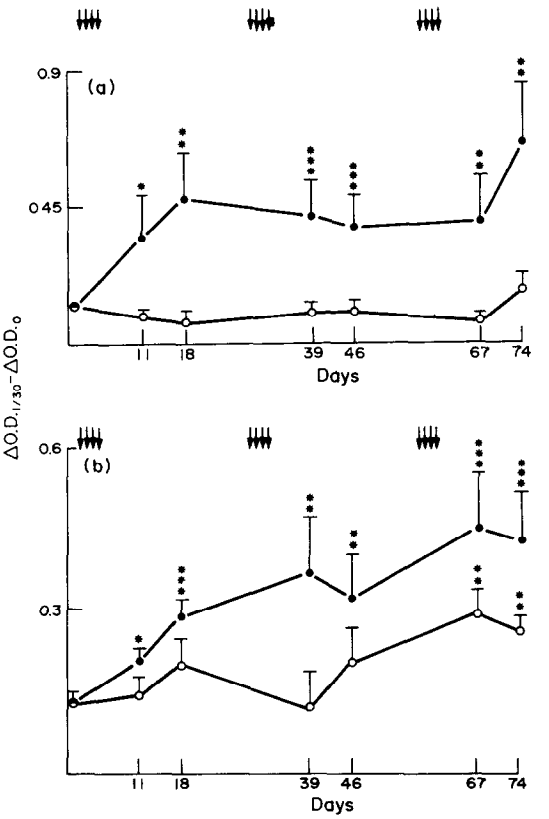

Fig. 1. IgG anti-BPO antibody activities ($\Delta OD_{1/30} - \Delta OD_0$) in serum from rats administered BP (2.7 mmol/kg) freshly dissolved (○,N = 8–7), or stored for 24 hr at room temperature (●, N = 4–8) in 0.5% glucose (a) i.p. and (b) i.m. Arrows indicate injections. Error bars represent SEM. *P < 0.05, **P < 0.005, ***P < 0.001 compared to pre-injection (day 0) levels.

i.m. injection of both stored and fresh BP is shown in Fig. 1. The results are given as the mean of 4–8 animals as it was not possible to obtain repeated bleeds from the tail vein of all animals during the course of the study. There was a significant increase in the antibody activity following chronic injection of stored BP i.p. (Fig. 1a) compared to pre-immune levels. Hapten inhibition with BPO–aminocaproate (1 mg/ml) produced a 54% reduction in the antibody activity exhibited by a pooled serum sample from the terminal bleed. There was no significant increase in the antibody activity following i.p. administration of fresh BP (Fig. 1a).

Following the chronic i.m. injection of stored BP, there was a significant increase in antibody activity above pre-immune levels, the activity ($\Delta OD_{1/30} - \Delta OD_0$) being greater than 0.3 following completion of the second and third series of injections (Fig. 1b). BPO–aminocaproate (1 mg/ml) produced a 53% reduction in the antibody activity obtained with a pooled serum sample from the terminal bleed. There was a significant increase in antibody activity above pre-immune levels following completion of the third series of i.m. injections of fresh BP, the mean activity was less than 0.3 (Fig. 1b) and BPO–aminocaproate produced a 42% reduction in the

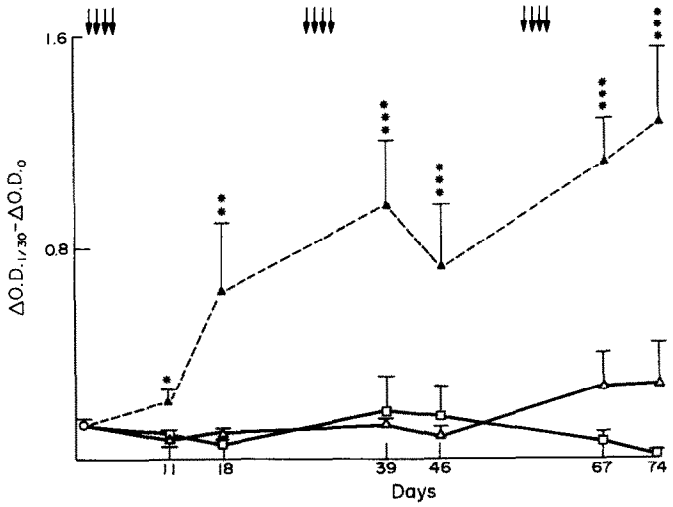

Fig. 2. IgG anti-BPO antibody activities ($\Delta OD_{1/30} - \Delta OD_0$) in serum from rats administered i.m. BPE at doses of 27 μmol/kg (▲, broken line, N = 8–6), 2.7 μmol/kg (△, solid line, N = 8) or 0.27 μmol/kg (□, solid line, N = 8–6). Arrows indicate injections. Error bars represent SEM. *P < 0.05, **P < 0.005, ***P < 0.001 compared to pre-injection levels (day 0).

activity exhibited by a pooled serum sample from the terminal bleed.

Figure 2 shows the IgG anti-BPO antibody activity ($\Delta OD_{1/30} - \Delta OD_0$) following chronic i.m. injection of BPE. There was a significant increase in activity above pre-immune levels, following 27 μmol/kg BPE. Hapten inhibition with BPO–aminocaproate (1 mg/ml) produced a reduction of 60% in the activity obtained with a pooled serum sample from the terminal bleed after injection of 27 μmol/kg BPE.

Circulating BPO–antigens

Figure 3 shows the levels of circulating BPO–plasma–protein antigens detected in the serum from rats during the first series of i.p. injections of stored and fresh BP and BPE (27 μmol/kg) i.m. Antigen levels increased after each injection of BP. Significantly greater levels of antigen ($P < 0.001$) were detected on days 2 and 3 after administration of stored BP compared to fresh BP. There was a slight but significant increase in BPO–antigen levels, compared to pre-immune levels, during the first series of i.m. injections of BPE (27 μmol/kg) in DMSO/saline. However, BPO–antigen levels were significantly lower ($P < 0.001$) after BPE i.m. compared to those obtained after either stored or fresh BP i.p. There was no significant increase ($P > 0.05$) in antigen levels following i.m. administration of BPE in Tween/saline, compared to pre-immune levels (0.356 ± 0.011 (N = 5) on day 0; 0.317 ± 0.008 (N = 3) on day 4 during 1st series of injections).

Figure 4 shows the levels of cirulating BPO–antigens 7 and 14 days after completion of each series of injections of stored and fresh BP. Circulating BPO–antigens were clearly detectable 7 days after completion of each series of i.p. injections of stored and fresh BP (Fig. 4a), 14 days after the second and third series of stored BP and 14 days after the third series of fresh BP. Antigen levels were significantly greater ($P < 0.001$) after stored compared to fresh BP i.p. 7 and 14 days after completion of the second series of injections. The specificity of the assay for the BPO–determinant was confirmed by the inclusion of BPO–aminocaproate (10 μg/ml) which produced 60 and 62% reductions in the optical density produced

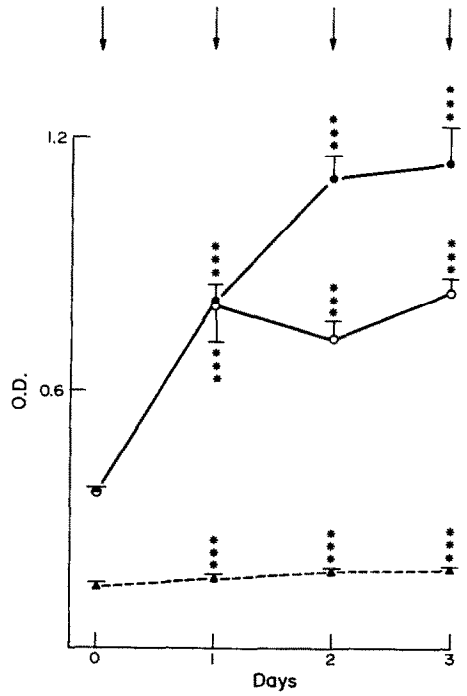

Fig. 3. Circulating BPO–antigen levels on days, 0, 1, 2 and 3 in the serum from rats administered BP (2.7 mmol/kg) freshly dissolved (○, N = 8) or stored (●, N = 5) in 0.5% glucose i.p., or BPE 27 μmol/kg i.m. (▲, N = 8). Arrows indicate injections. Error bars represents the mean ± SEM. ***P < 0.001 compared to pre-injection (day 0) levels.

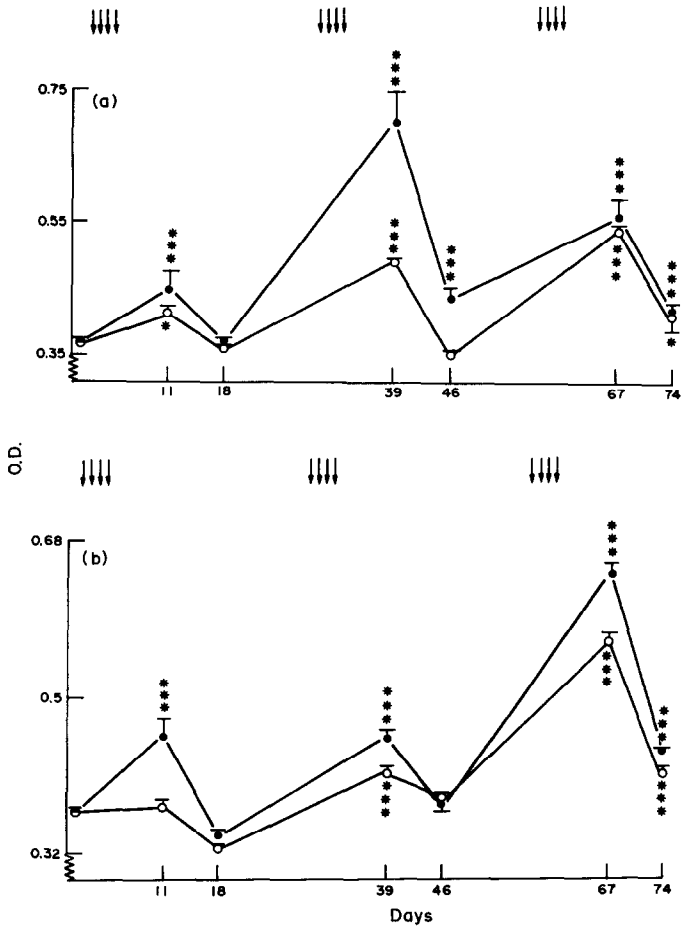

Fig. 4. Circulating BPO antigens 1 week and 2 weeks after completion of each series of injections from rats administered BP (2.7 mmol/kg) freshly dissolved (○, N = 8–6) or stored for 24 hr at room temperature (●, N = 8–4) in 0.5% glucose (a) i.p. and (b) i.m. Arrows indicate injections. Error bars represent SEM. *P < 0.05, ***P < 0.001 compared to pre-injection (day 0) levels.

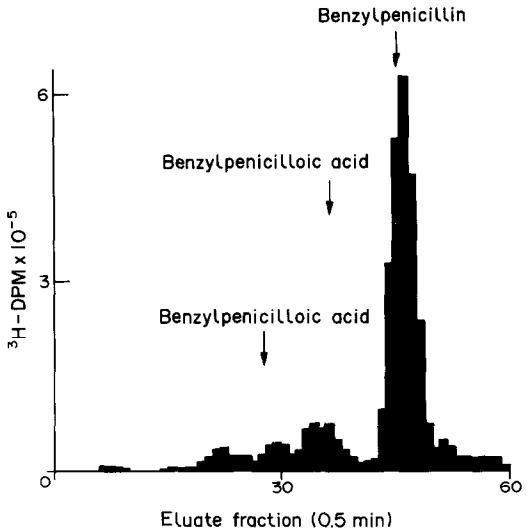

Fig. 5. Reverse-phased high performance chromatograph of the [^{3}H]-labelled degradation products of [^{3}H]-BP (2.7 mmol/ml; 15 μCi) stored at room temperature for 24 hr (each fraction 30 sec). Degradation products were identified by co-chromatography with authentic standards.

by pooled serum samples from day 67 following i.p. stored and fresh BP respectively. Following i.m. administration of stored BP, circulating BPO–antigens were detectable 7 days after each series of injections and 14 days after completion of the third series (Fig. 4b) significantly lower ($P < 0.05$) BPO–antigens were detected after fresh compared to stored BP i.m. on days 39, 67 and 74 (Fig. 4b). BPO–aminocaproate (10 μg/ml) produced 49% and 54% reductions in the optical density produced by pooled serum samples from day 67 following i.m. stored and fresh BP respectively. Circulating BPO–antigens following stored BP were significantly greater ($P < 0.01$) following i.m. compared to i.p. injection. However, following the administration of fresh BP there was no clear difference in antigen levels between the two injection routes. There were no detectable circulating BPO–antigens 7 or 14 days after completion of each series of i.m. injections of BPE at doses of 27, 2.7 or 0.27 μmoles/kg.

Irreversible binding of tritiated material in vitro

Following incubation of [^{3}H]BP freshly dissolved in phosphate buffer with rat plasma proteins *in vitro*, 2.63 ± 0.32% (N = 3) of the incubated radioactivity

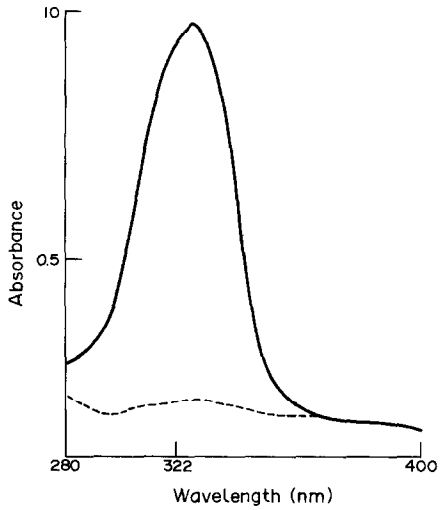

Fig. 6. Ultraviolet absorption spectrum of BP (2.7 mmol/ml) immediately dissolved (broken line) and stored for 24 hr at room temperature (solid line) in 0.5% glucose. Both solutions were diluted 1/100 and the spectra were ran against 0.5% glucose.

became irreversibly bound. There was no significant difference when the [^{3}H]BP was freshly dissolved in 0.5% glucose (2.55 ± 0.25%, N = 4). However, there was a significant increase ($P < 0.05$) in irreversible binding when the [^{3}H]BP was stored for 24 hr at room temperature in 0.5% glucose prior to incubation with the protein (3.81 ± 0.27%, N = 4).

Identification of BP degradation products

The degradation products of [^{3}H]BP stored in 0.5% glucose for 24 hr at room temperature, were resolved into one major and a number of minor components by HPLC (Fig. 5). The recovery of tritiated material from the column was 96%, of which 74% was identified, on the basis of co-chromatography, as BP (retention time 23 min), 12% as benzylpenicilloic acid (retention time 19 min), the remaining radiolabelled material (14%) was present as polar products which were not identified further by HPLC. BP (2.7 mmol/ml) stored, but not freshly dissolved in 0.5% glucose exhibited a strong and distinctive absorbance peak at 322 nm (Fig. 6). This characteristic absorbance peak has been attributed to the presence of BPE or BPE disulphide [19]. The amount of BPE equivalents in the stored BP solution was calculated, using the molar extinction coefficient of BPE to be 46.7 nmoles.

DISCUSSION

In this study we have shown that storage of benzylpenicillin, at room temperature for 24 hr in aqueous glucose solution has a pronounced effect on the immunogenicity of the drug in the rat. Intraperitoneal administration of stored solutions of a high dose (930 mg/kg) of penicillin for four days produced a detectable IgG anti-benzylpenicilloyl antibody response, whereas administration of the same dose, but freshly prepared, did not produce a detectable response, even when given over a period of three months. The effect of storage on penicillin immunogenicity was confirmed in a second series of experiments in which the drug was given chronically, by intramuscular administration. Thus, freshly prepared penicillin solutions did not produce a detectable antibody response in the rat as shown previously [10], whereas stored solutions of penicillin gave rise to a specific IgG anti-BPO response after a single series of four injections, which appeared to increase after the second and third series of injections. The specificity of IgG anti-BPO response was confirmed by hapten inhibition experiments, which are essential for the detection of anti-drug antibodies by ELISA (enzyme-linked immunosorbent assay) in experimental animals [10] and man [7].

These observations are of clinical significance since it has been reported that storage of penicillin solution may contribute to the immunogenicity of benzylpenicillin in patients [20]. Neftel *et al.* [20] reported a significant difference in anti-benzylpenicillin antibody levels and sensitization of lymphocytes, between patients given intravenous infusions of penicillin solutions stored for 4–24 hr at 4°, and untreated controls. There was no antibody response when the penicillin solution was freshly prepared and given as a bolus, rather than a slow infusion. It was therefore suggested that the causitive antigen (immunogen) was a degradation and/or transformation product of penicillin, rather than high molecular weight (protein conjugate) impurities in the penicillin preparations. It is thought that for a low molecular weight compound, such as a drug, to function as an immunogen it must become irreversibly bound to a carrier molecule [5]. Studies with model haptens indicate that the immunogenicity of such drug–protein conjugates is dependent on both the epitope density (drug–protein molar ratio) and on the nature of the carrier molecule [5]. We therefore investigated the chemical reactivity towards proteins *in vitro*, of freshly prepared and stored solutions of radiolabelled penicillin. It was found that the degree of irreversible binding to rat plasma proteins was significantly greater with the stored solution compared to freshly prepared solutions. It should be noted the reaction of penicillin with proteins was measured after 3 hr, and that the binding observed for freshly prepared penicillin solutions may be due to the formation of protein-reactive degradation products during this incubation period.

Chromatographic analysis of the stored solution revealed that benzylpenicillin was the major component, but that substantial hydrolysis had occurred, the principal product, as identified by chromatography being benzylpenicilloic acid. Benzylpenicilloic acid may be formed either by direct hydrolysis of the B-lactam ring [21], or via hydrolysis of the highly unstable rearrangement product benzylpenicillenic acid [22]. Spectroscopic analysis of the stored benzylpenicillin solution showed a strong absorbance at 322 nm, which indicated that the solution contained 0.2% benzylpenicillenic acid [18]. Benzylpenicillenic acid is unstable in aqueous solutions and therefore could not be quantified by chromatographic analysis.

It has been shown that benzylpenicillenic acid readily reacts with proteins, and is forty times more

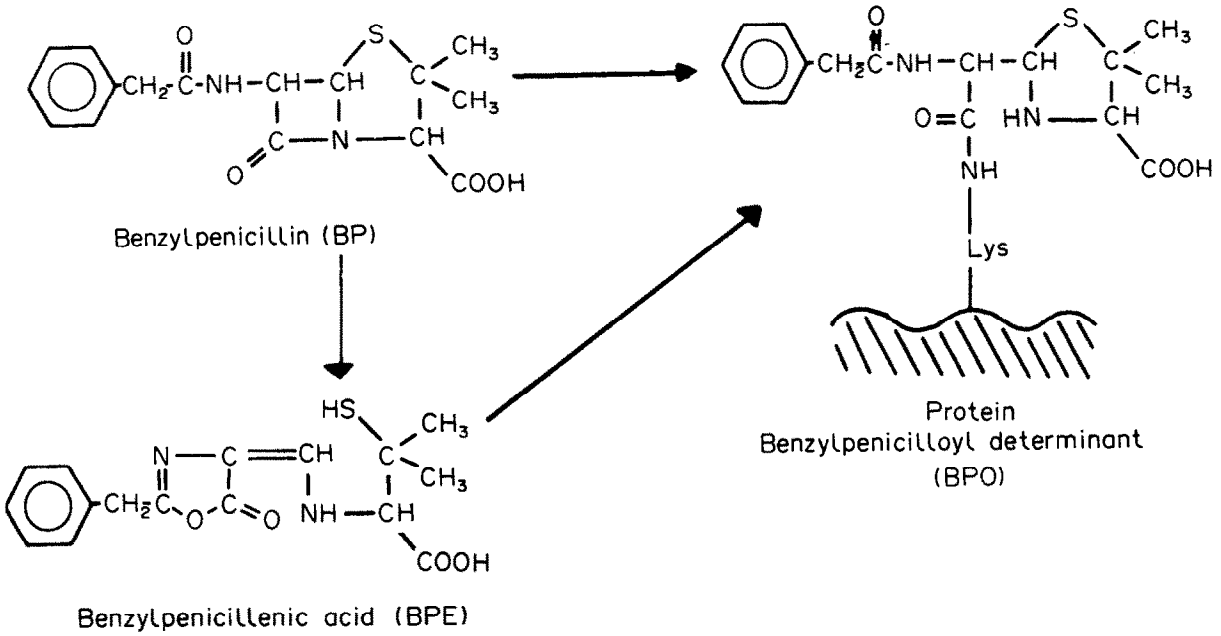

Fig. 7. Scheme summarizing the formation of benzylpenicilloyl (BPO)-protein-conjugates. BP reacts directly with lysine groups on albumin, or via the reactive rearrangement product, benzylpenicillenic acid (BPE) to form covalently bound conjugates.

reactive than BP towards cellular proteins [23]. Accordingly benzylpenicillenic acid was found to be highly immunogenic in the rat, the dose required to produce a specific IgG anti-BPO response was 100-fold less than the maximum dose of penicillin investigated (Fig. 2).

Finally, we considered analysis of protein–conjugation *in vivo*. In previous studies we have used radiometric analysis for the determination of protein–conjugates in blood [10]. This method was not sufficiently sensitive or specific for experiments which involve chronic administration of the drug due to the low level of protein-conjugates observed *in vivo* [10]. We therefore turned to highly sensitive immunochemical analysis of penicilloylated proteins [24, 25]. By modification of the ELISA method it was possible to determine relative quantities of circulating BPO–antigens after intraperitoneal and intramuscular administration of BP by using a rabbit anti-BPO antiserum. However, it is known that such methods are critically dependent upon epitope density [26] and therefore cannot be used to measure antigen concentration in absolute terms. There was a small but statistically significant increase in circulating BPO–plasma–protein antigen after administration of stored solutions, compared with freshly prepared solutions, but little or no detectable circulating BPO–antigens after administration of BPE. Therefore, it is clear that circulating levels of BPO–antigens do not relate to the immunogenicity of the preparation. We have shown in previous studies that the clearance of drug–protein conjugates from blood, is critically dependent upon epitope density [27]. We suggest that the BPE present in penicillin preparations will react rapidly with autologous proteins at the injection site after administration to form drug–protein conjugates with a high epitope density, which will be rapidly cleared by immune and non-immune mechanisms [27]. Studies with model haptens have shown that such highly conjugated proteins will also be effective immunogens, at low concentrations.

In conclusion, we have shown that the simple process of storate is sufficient to produce a marked enhancement of the immunogenicity of penicillin solutions. Chemical anlaysis, indicates that penicillin rearranges to a protein-reactive intermediate, thought to be benzylpenicillenic acid *in vitro*, which reacts more readily with proteins compared to benzylpenicillin *in vivo* (Fig. 7); however, BPE is only one possible reactive impurity in stored BP, and other unidentified polar products (14% of incubated BP) may be the causitive immunogens.

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