Biochemical Pharmacology, Vol. 37, No. 4, pp. 737–742, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

DRUG–PROTEIN CONJUGATES—XV

A STUDY OF THE DISPOSITION OF D-PENICILLAMINE IN THE RAT AND ITS RELATIONSHIP TO IMMUNOGENICITY

JOHN W. COLEMAN, AMANDA L. FOSTER, JOHN H. K. YEUNG* and B. KEVIN PARK†
Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool, L69 3BX, U.K.

(*Received* 1 *June* 1987; *accepted* 21 *August* 1987)

Abstract—The disposition of [^{14}C]D-penicillamine (PA) was investigated *in vitro* and *in vivo* with male Wistar rats. Irreversible binding of [^{14}C]PA to isolated rat plasma proteins *in vitro* reached a maximum of 20.6% of total radioactivity at 6 hr. Irreversibly bound [^{14}C]PA could be dissociated with dithiothreitol, demonstrating that conjugation was via disulphide linkage. Three hours after i.v. administration of [^{14}C]PA (27 μmol/kg) to rats 100% of plasma radioactivity was irreversibly bound, representing approximately 3.5% of the dose. Further studies on the disposition of PA–plasma protein conjugates showed that dissociation occurred readily *in vivo*: the plasma half-life of the conjugate was approximately 3 hr. Free [^{14}C]PA was the major urinary metabolite after administration of both free and conjugated drug. These studies show that the disposition of PA is similar to that reported for the structurally related sulphydryl drug captopril (CP). Free PA (340 μmol/kg and 3.4 mmol/kg) administered i.p. and i.m. daily for 4 days at one monthly intervals, and also PA–KLH conjugate (100 μg/rat) administered by single i.p. injection at monthly intervals with and without Freund's complete adjuvant, failed to induce PA-specific IgG or IgM antibody responses detectable by ELISA. In contrast, CP (270 μmol/kg), administered by the same protocol as free PA, induced a CP-specific IgG antibody response after the third series of monthly injections. These data suggest that the difference in immunogenicity between PA and CP arises from a difference in the intrinsic immunogenicity of the haptens, rather than from their disposition.

D-Penicillamine (PA, β,β-dimethylcysteine)‡ is used widely in the treatment of Wilson's disease, cystinuria, rheumatoid arthritis, lead poisoning, primary biliary cirrhosis and active chronic hepatitis [1–3]. The drug is active orally, and doses as high as 3 g daily are recommended in certain disorders [2]. The therapeutic effects of the drug are related to its metal chelating activity (in Wilson's disease and lead poisoning) and its capacity to dissociate cysteine (in the form of PA-cysteine) from cystine in cystinuria. In the case of rheumatoid arthritis the mechanism of action of the drug is still debated. The clinical use of PA is associated with a high prevalence of adverse reactions, which include fever, rash, loss of taste, thrombocytopenia, neutropenia, proteinuria, and less commonly, autoimmune-type disorders such as myasthenia gravis, pemphigus and Goodpasture's syndrome [1–3]. The nature of many of these side effects is indicative of an interaction of the drug with the immune system, and it has been suggested that such immunotoxicity may result from conjugation of PA to macromolecules to generate potentially antigenic drug–carrier complexes [4, 5]. Drug–carrier conjugation is thought to provide the basis for many drug hypersensitivity reactions, but the chemical nature of the postulated antigenic determinant, and the relationship between conjugate formation and immunogenicity are generally poorly understood [6].

Previous studies with the anti-hypertensive agent captopril (CP), which like PA is an amino acid derivative incorporating a free sulphydryl group, have shown that this compound becomes extensively conjugated to plasma proteins *in vivo*: 34% of circulating CP, representing 3% of the dose, is bound 3 hr after i.v. administration in the rat [7]. Disulphide-linked CP-foreign protein conjugates are immunogenic in rabbits [8], and CP-specific IgG antibodies have been detected in patients receiving this drug [9], supporting the conclusion that CP is potentially immunogenic.

In the case of PA, there appears to be little quantitative information on the covalent binding of the drug to plasma proteins either *in vitro* or *in vivo*. Moreover, although PA has been reported to be immunogenic in experimental animals [5, 10], those studies have employed free or conjugated PA in conjunction with Freund's complete adjuvant. The aims of the present study were thus to quantify conjugation of PA to plasma proteins *in vitro* and *in vivo*, and to investigate the immunogenicity of free PA administered i.v. and i.m. without adjuvant to rats.

MATERIALS AND METHODS

Reagents. D-Penicillamine, D-penicillamine disulphide, human serum albumin (fraction V, HSA),

* Present address: Department of Pharmacology, Chinese University of Hong Kong.
† To whom correspondence should be addressed.
‡ Abbreviations used: PA, D-penicillamine; CP, captopril; HSA, human serum albumin; OVA, ovalbumin; KLH, keyhole limpet haemocyanin; FCA, Freund's complete adjuvant; ELISA, enzyme-linked immunosorbent assay.

chicken ovalbumin (grade V, OVA), keyhole limpet haemocyanin (KLH), *o*-phenylenediamine dihydrochloride, Tween 20 and Freund's complete adjuvant were obtained from Sigma Chemical Co., U.K. D-Penicillamine-cysteine and D-penicillamine-glutathione mixed disulphides, and D-[carboxyl-^{14}C]-penicillamine (specific activity 46 μCi/mg) were kindly provided by Lilly Research Centre Ltd. (Surrey, U.K.). [^{14}C]Captopril (specific activity 4.66 μCi/mg), labelled at the amide-carbonyl carbon atom, captopril and captopril disulphide were supplied by the Squibb Institute (NJ, U.S.A.). PA–KLH [8], PA–HSA [11] and CP–OVA [8] conjugates were synthesised as described previously. All other general reagents and chemicals were obtained from British Drug Houses (Poole, Dorset, U.K.). Horseradish peroxidase-labelled rabbit anti-rat IgG was from Nordic Immunological Reagents Ltd. (Berkshire, U.K.). Horseradish peroxidase-labelled sheep anti-rat IgM was from Serotec Ltd. (Oxfordshire, U.K.) ELISA microtitre plates (Immunolon B) were from Dynatech (Surrey, U.K.).

In vitro *binding of* [^{14}C]-*penicillamine*. [^{14}C]PA (0.15 mg; 0.5 μCi) in phosphate buffer (0.75 ml; 0.05 M; pH 7.4) was incubated with rat plasma (0.25 ml) in a shaking water bath at 37°. Immediately after mixing (control) and after 0.25, 0.5, 1, 2, 3, 6, 24 and 48 hr incubation, *N*-ethylmaleimide (1 mg) in acetone (1 ml) was added to the incubation mixture. The protein precipitate was washed again in 3 ml of chilled acetone, and then dissolved in 0.1 M phosphate buffer pH 7.4 containing 2% sodium dodecyl sulphate. The amount of [^{14}C]PA covalently bound to plasma proteins was determined using the method of Sun and Dent [12] as previously described for captopril [7]. The chemical nature of the linkage between [^{14}C]PA and plasma proteins was determined by incubation of conjugate (0.4 ml; 0.8 μCi) with various concentrations of dithiothreitol (0–10 mM) in 0.1 M phosphate buffer (pH 7.4; 0.4 ml) at 37° for 16 hr, after which the degree of binding was determined as described above.

Disposition of [^{14}C]-*penicillamine* in vivo. Male Wistar rats (250–300 g) were anaesthetized with urethane (14% in saline; 10 ml/kg, i.p.) and the trachea, carotid artery and the jugular vein cannulated with polypropylene tubing of the appropriate size. After heparinisation of the animals (400 units/kg) [^{14}C]PA (10 μCi, 27 μmole/kg) in saline (1 ml/kg) was infused over 2 min via the jugular vein and blood samples taken at 5, 30, 60, 120 and 180 min. Blood (1 ml) was collected into a solution of *N*-ethylmaleimide (1.0 mg) in acetone (125 μl) and mixed thoroughly and plasma obtained by centrifugation. The irreversible binding of [^{14}C]PA to plasma proteins was determined by equilibrium dialysis after precipitation in acetone, as described above. At 3 hr the animals were killed, a terminal blood sample was obtained and the liver, lungs, kidneys, brain, heart and spleen removed for the determination of radioactive content. Aliquots of tissue (50 mg) were dissolved in NCS tissue solubiliser (1 ml) at 50° overnight, decolourised with hydrogen peroxide, neutralised with acetic acid, and then dissolved in scintillant (12 ml) and the tissue radioactivity determined by liquid scintillation counting.

Urine was aspirated from bladders at 3 hr into *N*-ethylmaleimide (1 mg/ml). [^{14}C]PA metabolites were chromatographed, along with authentic standards of PA, PA-disulphide, PA-cysteine mixed disulphide, PA-glutathione mixed disulphide and the *N*-ethylmaleimide derivative of PA, on silica gel TLC plates with methanol–water–acetic acid (80:10:10 v/v) as eluant. With this solvent system it was possible to separate the *N*-ethylmaleimide derivative of PA from the other standards; it was not possible to achieve satisfactory separation of the various disulphide standards in urine samples.

Preparation of [^{14}C]*penicillamine–protein conjugates*. The method used to prepare [^{14}C]PA–protein conjugates for disposition studies was essentially that used previously to prepare disulphide-linked protein conjugates of captopril [7]. Rat serum (1 ml) was incubated with [^{14}C]PA (12.5 μCi; 0.27 mg) at 37°, with shaking, overnight. The plasma was then diluted with 0.9% saline (4 ml) and concentrated to 1 ml in an Amicon B15 protein concentrator. The Amicon B15 concentrator has a molecular weight cut-off at 15,000. This process was repeated 4 times. The amount of [^{14}C]PA covalently bound to protein was estimated to be $\geq$ 99%, by equilibrium dialysis after acetone precipitation of an aliquot.

Disposition of [^{14}C]*penicillamine–protein conjugates* in vivo. Male Wistar rats (250–300 g) were anaesthetised and cannulated as described for the [^{14}C]PA disposition experiment. Autologous rat serum (0.5 ml) containing covalently bound [^{14}C]PA (*ca.* 1.16 μCi) was infused into the jugular vein over 4 min. Blood samples were obtained from the carotid artery at 5, 30, 60, 120 and at 180 min when the animals were killed for the determination of tissue radioactivity. Tissues, plasma and urine were analysed as described above.

Immunization of rats. D-Penicillamine (PA, 340 μmol/kg, 3.4 mmol/kg i.p. and i.m.), captopril (CP, 270 μmol/kg i.p. and i.m.) and PA–KLH conjugate (100 μg/rat i.p. with and without Freund's complete adjuvant) were injected in 0.15 M NaCl into male Wistar rats (250 g, 7–8 animals per group). Injections of PA and CP were administered on four consecutive days at four-weekly (28 day) intervals over a period of four months; PA–KLH conjugate was administered as a single injection at four-weekly (28 day) intervals over the same period. Blood samples were obtained from the tail vein two weeks after the first series of injections and one and two weeks after all subsequent series of injections. Serum was separated from clotted blood by centrifugation.

ELISA for IgG and IgM anti-PA antibodies. Microtitre plates were coated overnight at 4° with PA–HSA (hapten density 4.5:1) or HSA (100 μg/ml in 0.05 M phosphate buffer, pH 7.2, 125 μl/well). The plates were then washed three times in phosphate-buffered saline containing 0.05% Tween 20 (PBS-Tween) and emptied. Rat serum was then serially diluted threefold down columns in PBS-Tween (dilutions 1/10–1/7290, 100 μl/well) and plates incubated for 1 hr at room temperature. The plates were washed as before and 100 μl of horseradish peroxidase-labelled rabbit anti-rat IgG

(diluted 1/5000 in PBS-Tween) or horseradish-peroxidase labelled sheep anti-rat IgM (1/2000) added per well. Incubation was for a further 1 hr at room temperature. The plates were washed again and 100 μl of substrate solution, containing 0.4 mg/ml of *o*-phenylenediamine dihydrochloride and 0.1% hydrogen peroxide (30% w/v) in 0.15 M citrate–phosphate buffer, pH 5.0, was added to each well. The enzyme–substrate reaction was terminated after 5 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, interfaced with an Apple II microcomputer.

ELISA for IgG anti-CP antibodies. Rat IgG anti-CP antibodies were assayed by the method used for IgG anti-PA antibodies except that wells were coated with CP-OVA and OVA in place of PA-HSA and HSA respectively.

RESULTS

Disposition studies

The irreversible binding of [^{14}C]PA to rat plasma proteins, *in vitro*, increased with time and reached a maximum of 20.6% of incubated radioactivity at 6 hr (Fig. 1). Extraction with acetone, followed by equilibrium dialysis in SDS removed 98% of radioactivity from control incubations, to which *N*-ethylmaleimide was added directly after addition of [^{14}C]PA to derivatise the free sulphydryl group. The irreversibly bound [^{14}C]PA was found to dissociate from protein in the presence of dithiothreitol (Fig. 2), which indicates that [^{14}C]PA becomes conjugated to protein via a covalent disulphide bond. From Fig. 3 it can be seen that [^{14}C]PA also becomes extensively and irreversibly bound to plasma protein *in vivo*. After 3 hr essentially all the radioactivity in plasma was irreversibly bound to plasma protein and this represented, in total, approximately 3.5% of the dose given, assuming a blood volume of 20 ml [13].

Drug–protein conjugates were synthesised *in vitro* from rat plasma proteins, and then infused into the rat from which the plasma had been obtained. They thus represent autologous protein conjugates. The plasma concentration–time profile for the [^{14}C]PA-plasma protein conjugates is shown in Fig. 4. The plasma concentration of the [^{14}C]PA-protein conjugate declined over the time period of the experiment, with an apparent plasma half-life of 166 ± 48 min. The high concentrations at 5 min indicate that, initially, radioactivity is distributed mainly in the blood.

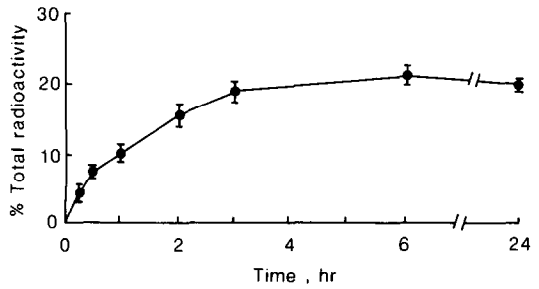


Fig. 1. Concentrations of [^{14}C]PA irreversibly bound to rat plasma proteins *in vitro*. Results are means ± SE for triplicate determinations.

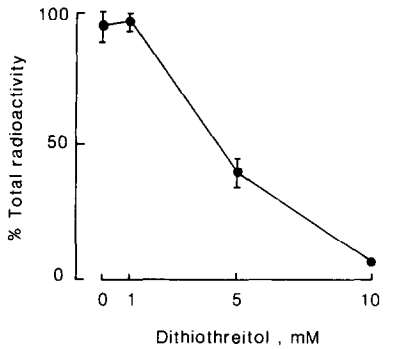


Fig. 2. Dissociation of [^{14}C]PA from rat plasma proteins by dithiothreitol *in vitro*. Results are means of triplicate determinations. SEs are shown where larger than symbols.

The tissue distribution at 3 hr after administration of either free [^{14}C]PA or [^{14}C]PA conjugated to plasma proteins is shown in Fig. 5. There was a significantly greater retention of radioactivity in plasma and in most of the tissues investigated after administration of drug–protein conjugates, compared with administration of free drug. Accordingly, urinary excretion of radioactivity was greater ($P < 0.001$) 3 hr after administration of free drug (70.6 ± 16.2% dose) than after administration of the conjugate (10.6 ± 2.3% dose). Free [^{14}C]PA, identified as the *N*-ethylmaleimide derivative by thin layer chromatography was found to be the major constituent in urine after administration of either conjugated (46.2 ± 2.5%) and non-conjugated [^{14}C]PA (63.6 ± 4.8%; $P < 0.05$). The former result indicates that PA–protein conjugates dissociate *in vivo* to generate free drug.

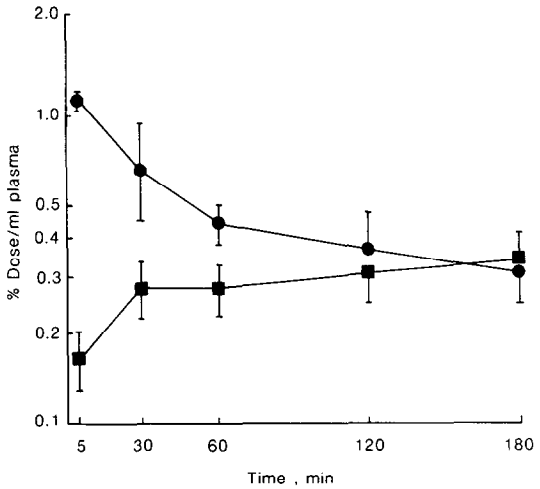


Fig. 3. Concentrations in plasma of total radioactivity (●) and irreversibly protein-bound radioactivity (■) *in vivo* after administration of [^{14}C]PA to rats. Results are means ± SE for four experiments.

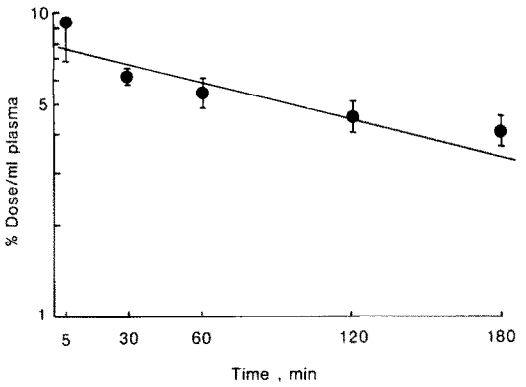
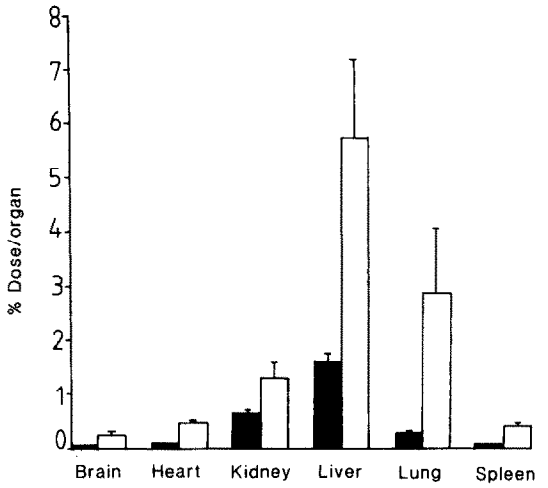


Fig. 4. Concentrations in plasma of irreversibly bound [^{14}C]PA *in vivo* after administration of conjugated [^{14}C]PA-plasma proteins to rats. Results are means ± SE for four experiments.



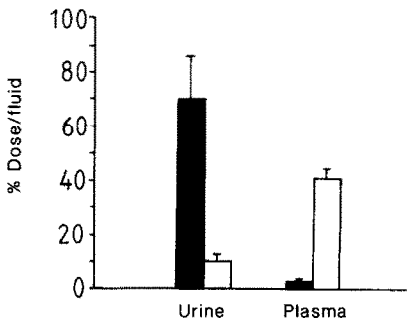


Fig. 5. Distribution of radioactivity into tissues and fluids after administration of [^{14}C]PA (filled columns) and conjugated [^{14}C]PA–plasma proteins (open columns) to rats. Results are means ± SE for four experiments.

Immunogenicity studies

Following chronic administration of PA by i.p. and i.m. routes (340 μmol/kg and 3.4 mmol/kg), no serum IgG nor IgM antibodies directed against PA–HSA or HSA were detected in either individual or pooled serum samples (see Table 1 for IgG results). Following the second and subsequent i.p. injections of PA–KLH, emulsified in FCA, high titre serum IgG antibody activity directed against PA–HSA and HSA was detected (Table 1). Weak IgM anti-PA–HSA and anti-HSA activities were detected after the third injection of PA–KLH in adjuvant (data not shown). The antibody responses were not PA-specific, since anti-PA–HSA activity was accompanied by anti-HSA activity. In the absence of FCA, PA–KLH failed to induce IgG or IgM anti-HSA or anti-conjugate responses (see Table 1 for IgG results). In contrast to PA, chronic i.p. injection of free CP (270 μmol/kg) led to a clear IgG anti-CP-OVA response, which was most marked after the third series of CP injections (Table 1). The IgG anti-CP–OVA activity was not accompanied by anti-OVA activity, thus demonstrating specificity for a CP-induced determinant.

DISCUSSION

The present studies show that D-penicillamine (PA) becomes extensively and irreversibly bound by disulphide linkage to rat plasma proteins both *in vitro* and *in vivo* after i.v. injection. *In vitro*, a maximum of 20% of radiolabelled drug was plasma protein-bound; in comparison, the structurally related sulphydryl drug captopril attained a level of binding to rat plasma proteins of 60% [7, 14] whereas benzylpenicillin attained a level of binding of only 1.6% [15]. *In vivo*, 3 hr after i.p. administration of PA (27 μmol/kg), 3.5% of the dose was bound to plasma proteins, representing 100% of total plasma drug. By comparison, 3 hr after i.p. administration of captopril (CP) (27 μmol/kg), approximately 3% of the dose was conjugated to plasma proteins, representing 34% of the total plasma drug [7]. In the case of benzylpenicillin, 3 hr after i.p. administration (27 μmol/kg), less than 0.05% of the dose, representing 5.6% of plasma drug, was protein-conjugated [15].

The rapid disappearance of free PA from plasma *in vivo* observed in the present study is consistent with previous estimates of a plasma half-life for the drug of 0.83 hr [16].

The extensive conjugation of PA and CP to plasma proteins *in vivo* raises the possibility that these drugs may generate immunogenic hapten–protein conjugates provided that functional T and B cell determinants are generated. Antibody responses resulting from drug–protein conjugation are thought to form the basis for drug hypersensitivity reactions [6]. The disulphide bond responsible for PA–plasma protein conjugation is a relatively labile covalent linkage, and *in vivo* may not be sufficiently stable to carry the conjugate through the sequence of antigen processing and presentation which leads ultimately (via macrophages and T cells) to antibody synthesis by B cells. In this study the *in vivo* plasma half-life of [^{14}C]PA-autologous protein conjugate generated *in vitro* in plasma was approximately 3 hr. This compares with an even shorter plasma half-life for CP–plasma protein conjugates of 1–1.5 hr [7]; in spite of which, CP–protein conjugates are immunogenic in the rabbit [8]. Three hours following administration

Table 1. IgG antibody titres directed against PA–HSA and HSA in pooled serum samples

Treatment	Day							
	14	35	42	63	70	91	98
	IgG antibody titres* (anti-PA-HSA/anti-HSA)						
PA, 340 μmol/kg i.p.	12/-	10/-	-/-	20/11	-/-	-/-	27/23
PA, 3.4 mmol/kg i.p.	16/-	-/-	-/-	20/17	-/-	-/-	-/-
PA, 340 μmol/kg i.m.	13/-	-/-	-/-	23/15	-/-	20/21	-/-
PA, 3.4 mmol/kg i.m.	18/-	-/-	-/-	22/10	-/-	-/-	-/-
PA-KLH, 100 μg/rat + FCA i.p.	16/-	390/269	>7290/5950	4481/1891	>7290/>7290	>7290/>7290	>7290/>7290
PA-KLH, 100 μg/rat − FCA i.p.	23/-	10/-	-/-	17/11	10/-	63/10	-/-
CP, 270 μmol/kg i.p.	-/-	17/-	38/-	193/-	211/-	115/-	94/44

Single (PA–KLH) and chronic (PA, CP) injections were completed on days 0, 28, 56 and 84.
* Titres calculated as reciprocal of the serum dilution giving an optical density of 0.5. Only titres > 10 are shown.

of [^{14}C]PA-plasma protein conjugate, a greater proportion of radioactivity was retained in the tissues, including plasma, than following administration of free [^{14}C]PA. Protein-conjugation thus prolongs retention of the drug. The tissue and body fluid distribution of PA after administration of free and conjugate drug is similar to that reported for CP [7]. Because of limitations in sensitivity of disposition studies utilising radiolabelled drug, we were unable to evaluate the long-term fate of free or conjugated PA, but it seems reasonable to suggest that during chronic administration, as for example in therapy, small amounts of potentially immunogenic conjugated PA may persist in the tissues and plasma.

Having demonstrated that PA–plasma protein conjugates generated *in vitro* and *in vivo* are disulphide-linked, we utilised a disulphide-linked PA–HSA conjugate as antigen in an ELISA for anti-PA antibodies; this conjugate had been characterised and used successfully to detect anti-PA antibodies in rabbits in a previous study (preparation A in [11]). Chronic administration of PA at high doses i.p. and i.m. failed to induce an IgG or IgM response in the rat. Injection of PA conjugated to a highly immunogenic carrier (KLH) likewise failed to induce drug-specific antibodies, although IgG and IgM antibodies cross-reactive with HSA were generated when Freund's adjuvant was co-administered, presumably because of the complex protein composition of the adjuvant. In contrast to the lack of immunogenicity of PA, chronic administration of CP did lead to an IgG CP-specific antibody response. This difference in immunogenicity between PA and CP in the rat is consistent with our findings showing that CP–KLH is highly immunogenic, whereas PA–KLH is weakly immunogenic, in rabbits [11]. This difference in immunogenicity does not reflect differences in the degree of *in vivo* conjugation of PA and CP to plasma, nor the stability of plasma conjugates, since these are similar for both drugs, but may be accounted for by differences in intrinsic immunogenicity of the two drugs. In the same rat model, dinitrofluorobenzene [17] and the rearrangement product of benzylpenicillin, benzylpenicillenic acid (G. Christie, unpublished observations), both of which form stable amide bonds with lysine groups in proteins, are immunogenic, whereas benzylpenicillin is non-immunogenic [15].

Studies of drug immunogenicity require that antigens formed *in vivo* must be structurally defined as a prerequisite for antibody tests [6]. In the case of PA, we have established that disulphide linkage is responsible for conjugation to plasma proteins, but have failed to demonstrate antibodies against this form of the drug in rats injected chronically with free drug. This does not exclude the possibility that PA may conjugate via a different chemical linkage (thiazolidine ring linkage has been suggested [18]) to form an alternative immunogen in non-plasma tissues.

It has been suggested recently that PA may induce drug-specific cell-mediated responses. Injection of PA-conjugated isologous spleen cells into recipient mice leads to induction of sensitized T cells in draining lymph nodes which proliferate *in vitro* in response to stimulation with haptenated cells [19]. In view

of our demonstration that the major route of PA conjugation to plasma proteins does not lead to immunogen formation, the hypothesis that direct haptenation of lymphoid cells may lead to drug-specific immune responses certainly merits further investigation.

Acknowledgements—This work was supported by the Mersey Regional Health Authority and by E.R. Squibb and Sons. BKP is a Wellcome Senior Lecturer. We thank Miss Susan Oliphant for typing the manuscript.

REFERENCES

1. Multicentre Trial Group (F. M. Andrews, D. N. Golding, A. M. Freeman, J. R. Golding, A. T. Day, A. G. S. Hill, A. V. Camp, E. Lewis-Fanning and W. H. Lyle), *Lancet* **i**, 275 (1973).
2. *Distamine Data Sheet*, Dista Products Limited (1984).
3. T. E. W. Feltkamp (Ed.), *Scand. J. Rheumatol.* Suppl. 28 (1979).
4. H. E. Amos, *Int. Archs Allergy appl. Immunol.* **45**, 218 (1973).
5. J. M. Dewdney, in *Drugs and Immune Responsiveness* (Eds. J. L. Turk and D. Parker), p. 161. Macmillan, London (1979).
6. B. K. Park, J. W. Coleman and N. R. Kitteringham, *Biochem. Pharmac.* **36**, 581 (1987).
7. B. K. Park, P. S. Grabowski, J. H. K. Yeung and A. M. Breckenridge, *Biochem. Pharmac.* **31**, 1755 (1982).
8. J. H. K. Yeung, J. W. Coleman and B. K. Park, *Biochem. Pharmac.* **34**, 4005 (1985).
9. J. W. Coleman, J. H. K. Yeung, D. H. Roberts, A. M. Breckenridge and B. K. Park, *Br. J. clin. Pharmac.* **21**, 161 (1986).
10. E. S. K. Assem and M. R. Vickers, *Postgrad. Med. J.* **50** (Aug. Suppl.), 10 (1974).
11. A. L. Foster, B. K. Park and J. W. Coleman, *Int. Archs Allergy appl. Immunol.* **84**, 271 (1987).
12. J. D. Sun and J. G. Dent, *Chem. Biol. Interact.* **32**, 41 (1980).
13. M. K. Bijsterbosch, A. M. Duursma, J. M. W. Bouma and M. Gruber, *Experientia* **37**, 381 (1981).
14. J. H. K. Yeung, A. M. Breckenridge and B. K. Park, *Biochem. Pharmac.* **32**, 2467 (1983).
15. N. R. Kitteringham, G. Christie, J. W. Coleman, J. H. K. Yeung and B. K. Park, *Biochem. Pharmac.* **36**, 601 (1987).
16. K. Kyogoku, K. Inoue, T. Otake, K. Noda and M. Ozeki, *Yakugaku Zasshi* **102**, 322 (1982).
17. B. K. Park, M. D. Tingle, P. S. Grabowski, J. W. Coleman and N. R. Kitteringham, *Biochem. Pharmac.* **36**, 591 (1987).
18. H. E. Amos, in *Allergic Drug Reactions*. Edward Arnold, London (1976).
19. N. Nagata, U. Hurtenbach and E. Gleichmann, *J. Immunol.* **136**, 136 (1986).