

D-Penicillamine metabolism in an *in-vivo* model of inflamed synovium

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Abstract

Oxidation to disulphides is the chief metabolic transformation of D-penicillamine (D-pen) in patients with rheumatoid arthritis. Oxidation also occurs in many biological fluids *in-vitro*. Reduction of oxygen species may accompany the oxidation of D-pen under appropriate conditions and may mediate the anti-rheumatic action of D-pen. The transformation of D-pen therefore was examined in an *in-vivo* model of inflamed synovium.

Subcutaneous air-pouches of groups of rats were treated with saline, 10% serum or 10% zymosan activated serum (ZAS). The transformation of D-pen to low molecular weight (LMW) metabolites and protein conjugates within the pouch was then assessed. The concentrations of total protein were significantly higher in the serum and ZAS-treated groups than in the saline-treated group and the inflammatory cell counts were significantly higher in the ZAS-treated group than in either of the other groups, as expected. D-pen oxidised rapidly to LMW metabolites and smaller amounts of D-pen-protein conjugate (D-pen-protein) in the air pouches of all animals. The rates of oxidation to LMW metabolites were greater in the ZAS-treated animals than the saline-treated group ($p < 0.005$). The concentrations of D-pen-protein conjugate were also greater for the serum-treated and ZAS-treated animals than for the saline controls ($p < 0.005$ in each case) at all times.

Oxidation of D-pen therefore occurs at this site of inflammation and is influenced by local conditions. This may be important to understanding the forms in which D-pen exists in inflamed synovial joints and the way it may exert its antirheumatic activity.

Introduction

The mode of action of D-penicillamine (D-pen) in rheumatoid arthritis is unknown. It may act, however, through reduction of oxygen or oxygen-derived free radicals [1-5]. Interactions may occur both directly and through reduction of oxidised transition metals [6], which may then reduce oxygen. D-pen is oxidised in the process. Oxidised products of D-pen are also its major metabolites *in-vivo* and comprise D-pen-protein (apparently

through disulphide linkage to the single free cysteine group of albumin) together with low molecular weight (LMW) metabolites, such as D-pen-L-cysteine mixed disulphide and symmetrical D-pen disulphide [7-10]. The metabolism of D-pen *in-vivo* therefore may be linked to its mode of action.

D-pen also oxidises rapidly in plasma, blood [11] and urine [12] *in-vitro* by a process which is considered analogous to oxidation *in-vivo*. Total protein concentration is the major determinant of the rate

of oxidation in albumin solutions [11]. Protein-containing exudates, such as synovial fluid are therefore also likely sites of D-pen oxidation *in-vivo*. The biotransformation of D-pen within synovial fluid will then substantially determine the concentrations of parent drug and metabolites in the adjacent synovium. The formation of metabolites from D-pen therefore was examined in an *in-vivo* model of a synovial cavity, in order to gain an understanding of its biotransformation in synovial fluid. The rat air-pouch model, which was studied, has considerable similarity to a synovial joint in its histology and its biochemical, vascular and cellular responses to inflammatory stimuli [13]. Various aspects of inflammatory fluids, such as cell count and protein concentration may be investigated through appropriate choice of stimuli. Direct introduction of D-pen into the pouch permitted examination of local biotransformation without the confounding influence of systemic metabolism.

Methods

Preparation of inflammatory stimuli

Whole blood was collected from male Wistar rats (approximately 400 g) by cardiac puncture for preparation of zymosan-activated serum (ZAS). The blood was allowed to clot at room temperature for 30 minutes and then centrifuged at 2000 g for 15 minutes at 4°C. The complement system was activated by incubation of the serum with zymosan (10 mg/ml; Sigma Chemical Co.) at 37°C for 30 minutes. Zymosan was removed by centrifugation (15 minutes at 2000 g) and activated serum was stored in 500 µl aliquots at -20°C. Non-activated serum was similarly treated, but incubated in the absence of zymosan. ZAS and non-activated serum were diluted to 10% in endotoxin-free saline immediately prior to injection.

Formation of air-pouch

The rat subcutaneous air pouches were formed as previously described [14]. Male Wistar rats, weighing between 150 and 180 g were anaesthetised with methohexitone sodium (Breital, 25 mg/kg intraperitoneally). The hair on the dorsal and nuchal regions was shaved and the area swabbed with isopropyl alcohol. Approximately 20 ml of air was passed through a 0.2 µm Millipore filter and in-

jected subcutaneously via a 25 gauge needle. The injection site was just behind the scapulae and the injection formed a regular ellipse on the dorsum. The air-pouch was re-injected with 5-10 ml of air 2 and 4 days later to maintain inflation. Air-pouches so formed were used for experiments between 3 and 7 days after the final injection of air.

D-Pen metabolism in-vivo

Rats were anaesthetised for study with intraperitoneal methohexitone sodium and a 1.3 mm × 5.1 cm teflon catheter (Angiocath) was introduced into the air pouch and retained with tape. Fifteen ml of saline (6 animals), 10% rat serum (6 animals) or 10% ZAS (6 animals) was introduced into the air-pouch via the catheter. Two hours later, animals were re-anaesthetised and 500 µl of 3.5 mmol/l D-pen (Fluka) in pyrogen-free saline, containing 440 nCi of ¹⁴C-labelled D-pen (Amersham) was injected into the pouch via a 25 gauge needle at a site away from the catheter. The pouch was massaged to distribute the drug. Samples (500 µl) were collected immediately prior to injection and at 5, 15, 25, 40, 60, 120, 180 and 240 minutes via the catheter under methohexitone anaesthesia. Specimens were added to 20 µl of 0.25 mol/l EDTA in 1.5 ml microcentrifuge tubes, agitated and immediately centrifuged for 30 seconds (Eppendorf microcentrifuge). Four hundred µl of supernatant was transferred to a fresh tube containing an equal volume of 18% trichloroacetic acid (TCA), stood for 5 minutes at 0°C and then centrifuged for 30 seconds. Supernatant was removed and immediately frozen. Supernatant is stable for at least a week under these conditions [11]. Protein precipitate was washed twice by resuspending in 1 ml of 5% TCA and centrifuging. The amount of protein lost through dissolution in 5% TCA is negligible [15]. After removal of the final wash, the precipitate was frozen until assay. An additional 200 µl of fluid was collected with the initial specimen and with the 2 hour and 4 hour specimens for determination of total cell count and total protein concentration (Biuret). Blood samples were collected from each animal by cardiac puncture at the time of the final pouch fluid specimen. Plasma (500 µl) was treated as for pouch fluid. Additionally, complete plasma profiles of D-pen, LMW metabolite and D-pen-protein concentrations were obtained from one saline-

treated and one ZAS-treated animal by tail vein blood sampling. Plasma (100 μ l) was added to 200 μ l 18% TCA and supernatants and precipitates were then treated as for pouch fluid.

D-Pen and metabolite assays

D-pen was measured utilising a Varian 5020 HPLC and a BAS LC-4B/19 electrochemical detector equipped with a BAS TC-6A mercury-coated gold working electrode (Bioanalytic Systems Inc.), glassy carbon auxiliary electrode and Ag/AgCl reference electrode [16, 17]. The detector output was integrated and plotted by a Hewlett-Packard 3390 A integrator. Twenty μ l samples of supernatant were injected, using a Kortek 65A autosampler (ETP-Oxford Pty Ltd, Ermington, Australia) and separated on a C-18 Rainin "Short One" column. The mobile phase consisted of 100 mmol/l monochloroacetic acid/NaOH (B.D.H.), pH 3.0, with 6% acetonitrile (Mallincrodt) and 1 gm/l heptane sulphonic acid (Regis). Flow rate was 0.6 ml/min. The elution time of D-pen was identified using authentic standard and samples were quantitated by comparison with standards. Standards were made up in saline and treated as for pouch fluid and plasma samples. Four point standard curves were run before and after each group of twenty samples to compensate for variation in detector sensitivity. The mercury surface of the working electrode was replaced when assay sensitivity exceeded 1 μ mol/l.

Protein precipitates were initially digested with 250 μ l of NCS tissue solubiliser (Amersham) and neutralised with 7.5 μ l glacial acetic acid. 14 C-D-pen activities in digests and supernatants (200 μ l) were measured by liquid scintillation spectroscopy after addition of 12 ml of PCS scintillant (Amersham). Total D-pen concentration in supernatant and D-pen-protein concentration were then calculated using the specific activity of the injectate. This was determined from measured D-pen concentrations (HPLC) and 14 C-D-pen activities in diluted aliquots of the injectate. The radiochemical purity of the 14 C-D-pen was checked prior to use using HPLC and scintillation counting of collected fractions. A mean of 93.5% of total activity co-eluted with the D-pen peak. LMW metabolite concentrations were calculated by subtraction of free D-pen concentration (HPLC) from total supernatant D-pen concentration (liquid scintil-

lation spectroscopy). Concentrations of D-pen and metabolites are expressed as molar equivalents of D-pen.

Mathematical modelling and statistics

The initial D-pen concentration and elimination rate constant were estimated by fitting D-pen concentration data to a mono-exponential decay model using a non-linear, least squares fitting procedure (Marquart's). The volume of distribution (volume of the air-pouch), and clearance of free D-pen were then estimated for each animal. Formation and elimination of LMW metabolites were also modelled (Fig. 1) using an iterative non-linear least squares fitting programme (Funfit). Reduction of D-pen containing disulphides by thiol-disulphide interchange occurs *in-vitro*, but is slow [18], so the transformation of LMW metabolites back to D-pen was not modelled. The model describes the period between 5 and 240 minutes, as there were no points between 0 and 5 minutes to allow accurate modelling of the rapid formation of LMW metabolites during this period. Areas under the concentration-time curves for LMW metabolites were estimated using the trapezoidal rule. Statistical comparisons were made using the Mann-Whitney "U" test. A probability of <0.05 was taken as significant. Results are expressed as mean \pm SEM.

Results

The inflammatory response to ZAS

Cell counts in pouch fluids were highest at all times in ZAS treated animals ($P < 0.005$ vs saline at 4

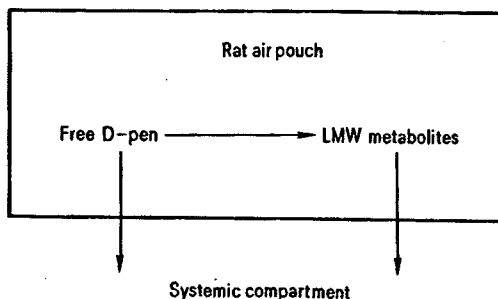


Figure 1
Rat air-pouch: model for D-pen egress, oxidation of D-pen to LMW metabolites and egress of LMW metabolites.

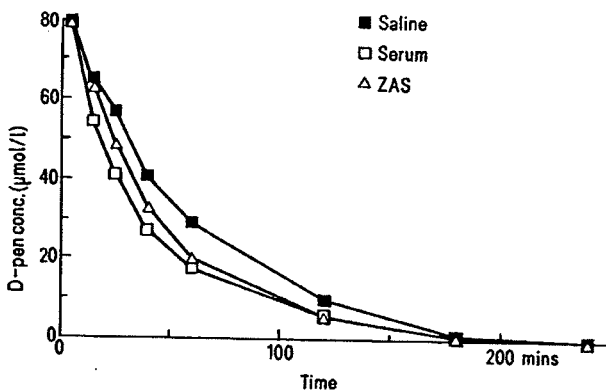


Figure 2
Mean concentrations of D-pen in pouch fluid of saline-treated, serum-treated and ZAS-treated animals over 4 hours ($n=6$ for each group).

hours; $P < 0.05$ vs serum at 4 hours) and were higher in serum treated animals ($P < 0.05$ at 4 hours) than saline treated animals. Cell counts rose with time, peaking at $0.3 \pm 0.2 \times 10^6$ per ml, $1.5 \pm 0.6 \times 10^6$ per ml and $4.7 \pm 1.3 \times 10^6$ per ml in the saline, serum and ZAS groups respectively. Total protein concentration in the fluid also rose with time in all animals. Mean peak (final) protein concentrations were 6.1 ± 0.9 mg/ml, 11.8 ± 1.3 mg/ml and 13.2 ± 1.5 mg/ml in the saline, serum and ZAS groups respectively. Mean concentrations were higher at all times in the serum ($P < 0.02$) and ZAS ($P < 0.005$) treated animals than the saline controls.

Pharmacokinetics of D-Pen and metabolites

Free D-pen concentrations at the time of first sampling (Fig. 2) were 80.0 ± 9.8 $\mu\text{mol/l}$ (saline), 79.3 ± 5.6 $\mu\text{mol/l}$ (serum) and 79.1 ± 6.9 $\mu\text{mol/l}$ (ZAS). The volumes of distribution (pouch volumes) were 14.3 ± 0.4 ml (saline), 15.1 ± 1.3 ml (serum) and 15.8 ± 0.8 ml (ZAS). They were not significantly different. Elimination rate constants were higher ($P < 0.005$) in the ZAS-treated group ($3.41 \pm 0.48 \times 10^{-2}$ per min) than in the saline-treated group ($2.09 \pm 0.09 \times 10^{-2}$ per min). The mean elimination rate constant in the serum treated group was $3.40 \pm 0.57 \times 10^{-2}$ per min. Free D-pen clearance was also significantly greater ($P < 0.005$) in the ZAS-treated group (0.55 ± 0.10 ml/min) than in the saline treated group (0.30 ± 0.01 ml/min). Clearance in the serum treated

group was 0.52 ± 0.09 ml/min. LMW metabolites formed rapidly in all groups and median times to peak concentration were 40 mins, 25 mins and 40 mins in the saline, serum and ZAS groups respectively. Concentrations peaked at 55.0 ± 5.9 $\mu\text{mol/l}$ (saline), 58.9 ± 7.3 $\mu\text{mol/l}$ (serum) and 51.7 ± 1.4 $\mu\text{mol/l}$ (ZAS). There were no significant differences in the peak concentrations, times to peaks or areas under concentration-time curves (data not shown) between the groups (Fig. 3). The results of modelling the data indicated that virtually all elimination of D-pen was through formation of LMW metabolites in all animals. The rate constants for LMW metabolite formation were 0.028 ± 0.006 per min (saline), 0.050 ± 0.016 per min (serum) and 0.029 ± 0.002 per min (ZAS). Mean rate constants for free D-pen passing out of the pouch were less than 0.0005 per min in all three groups. The rate constants for LMW metabolite loss from the pouch were 0.027 ± 0.003 per min (saline), 0.027 ± 0.003 per min (serum) and 0.029 ± 0.005 per min (ZAS). They were not significantly different.

D-pen-protein concentrations rose throughout the experiment in all animals (Fig. 4). Concentrations were significantly higher at 5 minutes and at all subsequent times ($P < 0.005$ for each time) in the serum and ZAS treated groups than in the saline treated group. Peak (final) concentrations were 5.5 ± 0.3 $\mu\text{mol/l}$ (saline), 9.9 ± 0.8 $\mu\text{mol/l}$ (serum) and 8.9 ± 0.4 $\mu\text{mol/l}$ (ZAS). There was no significant difference between the serum and ZAS treated groups. The rate of formation was initially

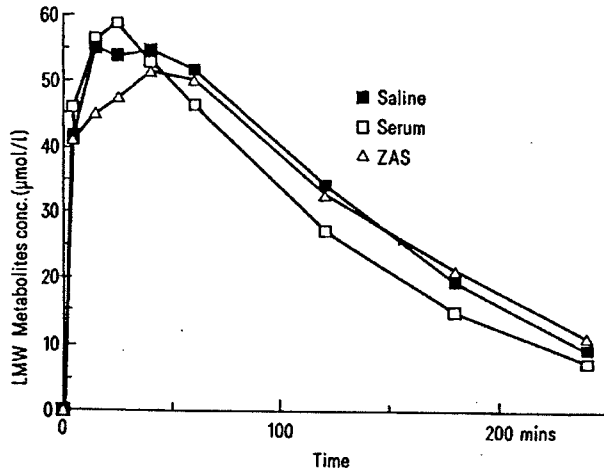


Figure 3
Mean concentrations of LMW metabolites in pouch fluid of saline-treated, serum-treated and ZAS-treated animals over 4 hours ($n=6$ for each group).

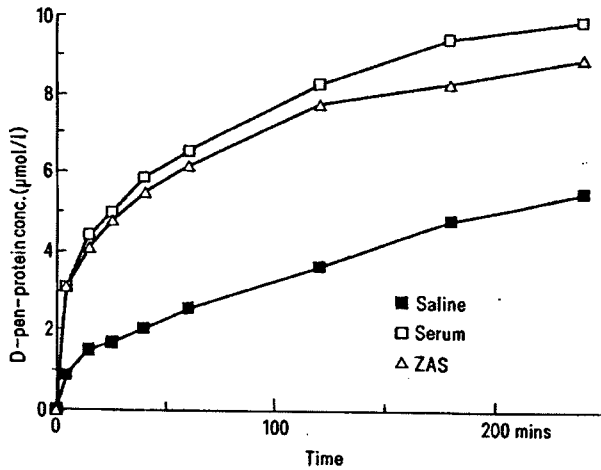


Figure 4
Mean concentrations for D-pen-protein in pouch fluid of saline-treated, serum-treated and ZAS-treated animals over 4 hours ($n=6$ for each group).

rapid in all animals and subsequently slower. Concentrations continued to rise after all free D-pen had been eliminated, but LMW metabolites were still present. The mean pouch protein concentration correlated ($R^2=0.64$; linear regression) with peak (final) D-pen-protein concentration in the 18 animals.

D-pen was not detected in the final blood sample (4 hr) from any animal. Final plasma LMW metabo-

lite concentrations were $0.9 \pm 0.08 \mu\text{mol/l}$ (saline), $0.5 \pm 0.08 \mu\text{mol/l}$ (serum) and $0.8 \pm 0.15 \mu\text{mol/l}$ (ZAS). Final plasma D-pen-protein concentrations were $6.1 \pm 0.3 \mu\text{mol/l}$, $5.8 \pm 0.7 \mu\text{mol/l}$ and $5.5 \pm 0.3 \mu\text{mol/l}$ respectively in the three groups. There were no significant differences in concentrations of either metabolite between the groups. No free D-pen was detectable in plasma at any time after administration into the pouch in either of the two

animals from which complete plasma profiles were obtained. Peak LMW metabolites concentration (4.1 $\mu\text{mol/l}$, saline and 3.6 $\mu\text{mol/l}$, ZAS) occurred at 60 mins and 40 mins respectively. Plasma concentrations of D-pen-protein rose throughout the experiment in both animals.

Discussion

The rat air pouch lining resembles normal synovium and its biochemical, cellular and vascular responses to inflammatory stimuli model the responses of a synovial joint [13]. Appreciable differences exist, however, between an inflamed joint and an inflamed air pouch, including the absence of cartilage and a true synovial fluid. For these experiments, inflammation was induced in air pouches of one group of rats with ZAS (a source of C5a), while another group, treated with non-activated serum, served as controls. Pyrogen-free saline provided a non-protein, non-inflammatory stimulus for the third group. D-pen was introduced directly into the pouch so that metabolism occurring locally could be distinguished from that occurring systemically.

The number of inflammatory cells in the fluid was increased in the ZAS treated group compared with the saline treated group, as expected. An intermediate number of cells were seen in fluid from the serum-treated animals due to the irritant effect of serum. Protein concentrations were higher in the serum- and ZAS- treated animals than in the saline-treated group. D-pen itself is not known to alter cell numbers or protein concentrations in ZAS or serum treated animals but is mildly pro-inflammatory in saline treated animals [19]. This may contribute to the rise in cell numbers and protein concentration seen in this group during the period of sampling.

D-pen metabolism was seen in all groups. The predominant products were LMW metabolites. These were likely to be predominantly oxidised D-pen products [11], namely the symmetrical disulphide and disulphides with other thiols. Similar patterns of metabolism are seen in other biological fluids [10, 12]. S-methyl penicillamine [20] is unlikely to have been formed locally as the required S-methyl transferase [21] and cofactor would have been virtually absent from the fluid. D-pen-metal complexes [22] may have comprised a small proportion of the LMW metabolites. D-pen-protein, probably

a disulphide with albumin [9, 23, 24] was the other main metabolite. D-pen-containing disulphides also may be reduced to free D-pen after systemic administration [25]. Thiols which may reduce D-pen disulphides through thiol-disulphide interchange, such as glutathione, have not been quantitated in pouch fluid. However the rapid disappearance of D-pen, despite the presence of appreciable amounts of disulphides, indicates that the conditions in the pouch do not favour disulphide reduction. The pattern of metabolism is therefore similar to that observed after systemic administration of D-pen to animals [8] and humans [8, 10].

Kinetic analysis ("Funfit") indicated that free D-pen was largely eliminated by conversion to LMW metabolites, rather than by diffusion out of the pouch. Total protein concentration and cell count were both increased in the pouch fluid of these two groups, compared with saline controls. A relationship between protein concentration and D-pen oxidation rate has been observed in cell-free albumin solutions [11]. Activated neutrophils, however, also release superoxide radicals [26] which may increase D-pen oxidation in the presence of a transitional metal [2, 3]. An independent effect of neutrophil-derived oxygen radicals on the oxidation of D-pen was not apparent in this data. The relatively small rise in total protein concentration in pouch fluid over 4 hours indicated a limited permeability of the pouch lining to the influx of protein. This is consistent with prior observations in the air-pouch [13] and is similar to the response of synovial lining [27]. It indicates that the D-pen-protein which was found in the pouch fluid was formed locally and had not simply diffused in from the systemic circulation. D-pen-protein formed more slowly than LMW metabolites. Formation was biphasic with an initial rapid phase and a subsequent slower phase, which continued after disappearance of free D-pen in all animals. D-pen-protein therefore appeared to be derived from both direct reaction of free D-pen with protein and from thiol-disulphide interchange between LMW metabolites (presumably disulphides) and protein thiol groups. Differences in D-pen-protein formation between groups related to the protein concentration in the pouch fluid.

The absence of free D-pen in plasma is consistent with limited loss from the pouch. Low concentrations of LMW metabolites were detectable in plasma of all animals four hours after D-pen adminis-

tration and appeared early in the two rats from which full profiles were obtained. Kinetic analysis indicated that D-pen predominantly left the pouch as LMW metabolites. LMW metabolites were presumably the source of D-pen found conjugated to protein systemically, since the pouch is relatively impermeable to protein. Disulphide exchange between D-pen containing disulphides and endogenous thiols occurs *in-vitro* [18] and has been implicated *in-vivo* [25]. The formation of appreciable quantities of D-pen-protein confirms that the drug may readily form a hapten with protein *in-vivo*. Hapten formation with tissue protein, and the subsequent recognition of the conjugate by an overactive immune system may be a basis of the immunological toxicity of D-pen. Antibody formation to D-pen-protein disulphide [24] has been achieved experimentally in animals, confirming its immunogenicity.

These results indicate that D-pen metabolism occurs in inflammatory fluids and that the concentrations of D-pen and its metabolites are influenced by local protein concentration. Similar metabolism is expected in joint effusions of patients with rheumatoid arthritis. Oxygen and oxygen radicals are reduced during the oxidation of D-pen, under some conditions. The production [2, 3] and quenching [4, 5] of reduced oxygen species have been proposed as mechanisms of D-pen's action in rheumatoid arthritis, so local articular drug metabolism may influence efficacy. D-pen's interaction with reduced oxygen species, which is determined by the drug concentration [4, 6, 28, 29], therefore may be influenced by local drug metabolism in inflamed joints.

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