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BENZYLPENICILLIN TRANSPORT AND SUBCELLULAR DISTRIBUTION IN MOUSE PERITONEAL MACROPHAGE MONOLAYERS

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Abstract—[^{14}C]Benzylpenicillin was rapidly taken up into and eluted from mouse peritoneal macrophage monolayers and entered cytosol, lysosomes and phagolysosomes containing *Staphylococcus aureus*. Equilibrium concentrations in cytosol and lysosomes were consistent with transport by diffusion but partial membrane carrier dependence was evidenced by saturability (K_m and V_{max} 110 mM and 117 nmoles $(10^6$ cells$)^{-1}$ $(3$ min$)^{-1}$ respectively) and inhibition by probenecid. Partial dependence of transport on metabolic energy was shown by inhibition with cyanide plus 2-deoxyglucose and the temperature coefficient (Q_{10}) was about 2. Some of the intralysosomal radiolabel accumulated as benzylpenicilloic acid.

Benzylpenicillin is relatively ineffective against intracellular bacteria and it has been thought that this might be due either to defective penetration into mammalian cells [1] or to a phenotypic resistance of the intracellular bacteria imposed on them by the intracellular growth conditions [2]. Previous attempts to directly assess the penetration of benzylpenicillin into mammalian cells by using the radiolabelled antibiotic have yielded conflicting results. Early studies suggested that the drug might penetrate HeLa- and L-strain tissue culture cells, reaching a diffusion equilibrium within a few hours [3]. In contrast, in more recent studies leukocytes in suspensions freshly prepared from human peripheral blood appeared to be completely impermeable to the antibiotic over a 2-hr incubation period [4] and rabbit alveolar macrophages in suspension took up minimal amounts in 2 hr [5]. Nevertheless, autoradiography has supported bacteriological evidence that the antibiotic could rapidly penetrate human peripheral blood leukocytes when the cells were maintained as monolayers [6].

In the present study the characteristics of radiolabelled benzylpenicillin penetration of mouse peritoneal macrophage monolayers were assessed and analytical subcellular fractionation was used to define the distribution of radiolabel among the subcellular organelles of normal macrophages and macrophages infected with *Staphylococcus aureus*.

MATERIALS AND METHODS

Macrophages. Resident (non-elicited) peritoneal macrophages were obtained from mice of the CFLP outbred strain and maintained as monolayers in serum-rich, antibiotic-free, medium in 5-cm diameter plastic petri dishes for about 2 wk [2]. Monolayers contained 3–5 × 10^6 macrophages per dish as estimated by DNA content [2]. On the day that they were used for penicillin transport studies the apparent intracellular water space of the cells was determined [7]. For subcellular fractionation studies about 2 × 10^7 cells were plated per 11-cm diameter dish.

Bacteria. Staphyloccus aureus Oxford was grown to mid-exponential phase at 37° in nutrient broth (Oxoid No. 2) containing [methyl-^{3}H]thymidine (1.0 μCi ml^{-1}; 24 Ci mmole^{-1}) and stored in liquid N_2. Before use the bacteria were washed 5 times by centrifugation for 5 min at 18,000 *g* in Hanks' balanced salt solution (HSS). The final suspension in HSS was subjected to brief ultrasonication [2] to disrupt clumps and the viability of the bacteria was estimated as about 17 per cent by comparison of the total bacterial concentration (determined by light microscopy using a Thoma counting chamber) with the concentration of viable bacteria (determined as colony-forming units (cfu) on blood agar).

Measurement of transport of benzylpenicillin, sucrose and 3-O-methyl-D-glucose. In preliminary experiments transport was measured using macrophages in maintenance medium. Medium was aspirated from the monolayers and replaced with medium (2 ml/dish) either containing ^{14}C-labelled penicillin (about 1 μCi ml^{-1}, potassium 6 phenyl-[^{14}C]-acetamidopenicillinate, 59 mCi mmole^{-1}; Radiochemical Centre, Amersham, Bucks, U.K.) and non-labelled sodium benzylpenicillin (Glaxo Ltd., Greenford, Middx, U.K.) or [^{14}C]sucrose (1 μCi ml^{-1}, 10.1 mCi mmole^{-1}; Radiochemical Centre) and non-labelled sucrose. Total, labelled plus non-labelled, substrate concentrations were 1 mM. All penicillin solutions were used on the day of preparation. At intervals during subsequent incubation of the monolayers with the substrates at either 0 or 37° uptake was measured by rapidly rinsing duplicate monolayers with 5 × 2 ml of ice-cold Hanks' balanced

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salt solution buffered with 20 mM-HEPES at pH 7.3 (HH), digesting the rinsed monolayers in 0.2 M NaOH (1 ml) and determining the radioactivity in digest samples by liquid scintillation counting. Cell-associated radioactivity was calculated from counts of experimental dishes minus counts of control, cell-free, dishes that had been treated identically to the monolayer dishes. Control counts were equivalent to $<0.2\ \mu l$ of the original solution. To assess elution of penicillin from pre-loaded cells the medium containing the extracellular radiolabel was removed by aspiration and replaced with fresh medium (2 ml). Incubation was continued and at intervals cell-associated radioactivity was estimated as described above.

In the majority of experiments penicillin transport was measured using macrophages in HH instead of maintenance medium. Monolayers were first rinsed once with HH (2 ml) and HH was substituted for maintenance medium in the above procedures. Other details were as described in the figure legends. In each figure the data shown were obtained from one batch of monolayers unless otherwise stated.

[^{14}C]*Benzylpenicilloic acid.* [^{14}C]Benzylpenicillin (425 nmoles) was incubated in HH (250 μl) with penicillinase (10 μl, Wellcome Reagents Ltd., Beckenham, Kent, U.K.) for 1 hr at 37°. HH (740 μl) was then added and the enzyme removed by centrifugal ultrafiltration (Centriflo CF25 membrane, Amicon Ltd., High Wycombe, Bucks, U.K.). The percentage of radioactivity present as benzylpenicilloic acid was assessed after separation from benzylpenicillin by thin-layer chromatography on silica in acetone : chloroform : acetic acid (10 : 9 : 1 by volume) [8]. Radioactivity distribution on the developed chromatograms was detected (Packard radiochromatogram scanner, Model 7201) then quantified by liquid scintillation counting after elution of the peaks with methanol : water. Over 90 per cent of [^{14}C]benzylpenicillin was converted to [^{14}C]benzylpenicilloic acid by the penicillinase treatment.

Other reagents. Probenecid (*p*-dipropylsulphamoyl benzoic acid; Sigma Chemicals Ltd., London, U.K.) was dissolved as 0.5 M solution in 0.1 N NaOH and the solution adjusted to pH 7.3. Phloretin (3-(4-hydroxyphenyl)-1-(2,4,6-trihydroxyphenyl)-1-propanone; K and K Laboratories, Kodak Ltd., Liverpool, U.K.) was dissolved in 1% (v/v) ethanol in Dulbecco A salt solution (Oxoid Ltd., Basingstoke, Hampshire, U.K.).

Partition coefficients. Partition of radiolabelled sucrose, 3-*O*-methyl-D-glucose, benzylpenicillin and benzylpenicilloic acid between n-octanol and HH at 37° was measured as described by Graff *et al.* [9].

Analysis of subcellular distribution of benzylpenicillin in control and infected macrophages. Control monolayers, containing a total of about 5×10^7 macrophages, were incubated for 2 hr at 37° with [^{14}C]benzylpenicillin in the maintenance medium at a concentration of 0.5 μCi ml^{-1} and a total benzylpenicillin concentration of 1 mM. The medium was then removed and the monolayers were rinsed with 5×10 ml of ice-cold HSS then with with 10 ml of ice-cold 0.25 M-sucrose containing 20 mM-ethanol, 5 units heparin ml^{-1} and 1 mM-Na_2EDTA, pH 7.4 (SVEH). The cells were then scraped free into 7 ml SVEH using a rubber policeman and disrupted with 15 strokes of a tight-fitting (B) pestle in a Dounce homogeniser (Kontes Glass Co., Vineland, N.J., U.S.A.). Subcellular organelles were then fractionated essentially as previously described [10]. Homogenate (5 ml) was layered on a 28-ml sucrose gradient extending, linearly with respect to volume, from a density of 1.05 to 1.32 g(cm^3)$^{-1}$ and resting on a 6 ml cushion, density 1.34 g(cm^3)$^{-1}$, in a Beaufay small-volume automatic zonal [11] or a vertical tube rotor (VTi50; Beckman RIIC Ltd.). All solutions contained 1 mM-Na_2EDTA, pH 7.4, 20 mM-ethanol and 5 units heparin ml^{-1}. After centrifugation at 35,000 rpm at 4° for 35 min (Beaufay rotor; integrated angular velocity 3.3×10^{10} rad^2(S)$^{-1}$) or 45,000 rpm for 45 min (VTi50 rotor; integrated angular velocity 4.55×10^{10}) some 15 fractions were collected. Fractions were weighed and their densities determined. The fractions were assayed for [^{14}C]benzylpenicillin by liquid scintillation counting [2] and for protein and the marker enzymes for subcellular organelles, *N*-acetyl-β-glucosaminidase, neutral α-glucosidase, cytochrome *c* oxidase, 5′-nucleotidase [12] and catalase [13]. Results from isopycnic-centrifugation experiments were expressed as frequency–density histograms [10]. Data from the Beaufay and VTi50 rotors were indistinguishable and were combined.

The distribution of [^{14}C]benzylpenicillin in infected macrophages was examined in the same way. Monolayers were infected with ^{3}H-labelled *Straphylococcus aureus* by incubation for 1.5 hr at 37° with a suspension of 3×10^7 cfu ml^{-1} in HSS containing 2.5% (v/v) newborn-calf serum. bacteria that had not been ingested by the macrophages at the end of this phagocytosis period were selectively removed by rinsing the monolayers with 2×10 ml HSS and incubation at 37° for 15 min with lysostaphin (Sigma; 10 μg ml^{-1} in HSS). The lysostaphin solution was then removed and the monolayers were incubated for 30 min in fresh maintenance medium to allow internalisation of any surface-adherent bacterial remnants and then for 1 hr in maintenance medium containing [^{14}C]benzylpenicillin (0.5 μCi ml^{-1}, 1.0 mM). Preliminary experiments had shown that neither lysostaphin nor penicillin affected the viability of the intracellular bacteria under these conditions. The infected macrophages were then rinsed, disrupted and their subcellular organelles fractionated as described above. The distribution of bacterial cfu and tritium in the fractions was assessed in addition to the distribution of organelle marker enzymes and [^{14}C]benzylpenicillin. Marker enzyme distributions were corrected when necessary for any contribution by bacterial enzymes.

Binding of benzylpenicillin by subcellular organelles from macrophages. Monolayers that had not been exposed to either bacteria or penicillin were rinsed and disrupted in SVEH containing [^{14}C]benzylpenicillin (2.25 nCi ml^{-1}). The subcellular organelles were then fractionated as described above except that density gradients were made from solutions containing an identical concentration of [^{14}C]benzylpenicillin (2.25 nCi ml^{-1}). Thus before organelle fractionation the radiolabel was uniformly distributed throughout the sucrose gradient. Any difference between radioactivity distribution in a

gradient in which macrophage homogenate had been fractionated and a control gradient without homogenate was considered to indicate binding to macrophage organelles and was plotted in the same way as the marker enzyme activities.

Binding of benzylpenicillin by Staphylococcus aureus. A suspension of ^{3}H-labelled mid-logarithmic phase *Staphylococcus aureus* Oxford (6.6×10^{8} total bacteria) in HSS was thawed from liquid N_2, pelleted by centrifugation (18,000 *g*, 5 min, 4°) and resuspended in Medium 109 (1.0 ml) that had been adjusted to pH 5.4 and contained [^{14}C]benzylpenicillin (1.22 μCi nmole^{-1}). After incubation for 18 min at 37° the bacteria were pelleted by centrifugation and washed 5 times by resuspension in ice-cold Medium 109, pH 5.4, and once using SVEH. The bacteria were finally suspended in SVEH and subjected to 15 strokes in a Dounce homogeniser before centrifugation into a sucrose gradient under conditions identical to those used for macrophage subcellular fractionation. Gradient fractions were collected and assayed for ^{3}H, ^{14}C and viable bacteria.

Presence of benzylpenicillin within lysosomes. Monolayers containing a total of about 8×10^{7} macrophages were incubated for 30 min at 37° with [^{14}C]benzylpenicillin in HSS (1.0 μCi ml^{-1}; 51 mCi mmole^{-1}), then rinsed, harvested and homogenised in SVEH as described above. Homogenate (5.5 ml) was layered on a 13-ml discontinuous sucrose gradient comprising 6 ml of density 1.25 g(cm^{3})$^{-1}$ and 7 ml of density 1.18 g(cm^{3})$^{-1}$ in a 3×25 ml swing-out rotor (MSE Ltd.). All solutions contained 1 mM-Na_2EDTA, pH 7.4, 20 mM-ethanol and 5 units heparin ml^{-1}. After centrifugation at 30,000 rpm (17,000 *g*) at 4° for 1 hr some 2.5 ml was collected from the 1.18 to 1.25 g(cm^{3})$^{-1}$ interface and the volume of this *N*-acetyl-β-glucosaminidase-rich fraction was adjusted to 10 ml by adding ice-cold SVEH. This organelle suspension (density approx 1.09 g(cm^{3})$^{-1}$) was layered on 10 ml of sucrose solution, density 1.11 g(cm^{3})$^{-1}$ and centrifuged as above for 2.5 hr. The resultant pellet was extracted with 50% methanol in water, and the [^{14}C]benzylpenicillin and [^{14}C]penicilloic acid content were then quantified after thin-layer chromatography. To assess whether or not the pelleting radioactivity was trapped within the organelles rather than surface-bound, an *N*-acetyl-β-glucosaminidase-rich fraction was divided into three equal (1 ml) parts so that the effect of membrane disruption on sedimentation could be examined. To one part (control) 2.5 ml SVEH was added, to another part 2.5 ml SVEH was added and the mixture was then ultrasonicated for 1 min, to the third part 2.5 ml SVEH containing 0.7 mg digitonin ml^{-1} was added. Each part was then layered on 17 ml of 1.11 g(cm^{3})$^{-1}$ sucrose and centrifuged as above for 2.5 hr to separate the particulate material (pellet) from the soluble material (top 6 ml). After centrifugation 10×2 ml fractions were collected from the top, lightest fraction first, the pellet being resuspended in the last fraction, and assayed for radioactivity, *N*-acetyl-β-glucosaminidase and cholesterol. Cholesterol (total) was used as a marker for organelle membrane and was assayed as described by Gamble *et al.* [14].

RESULTS

Uptake of [^{14}C]benzylpenicillin from maintenance medium by macrophage monolayers was rapid at 37° (Fig. 1a) and the amount of cell-associated antibiotic reached equilibrium within about 30 min. The

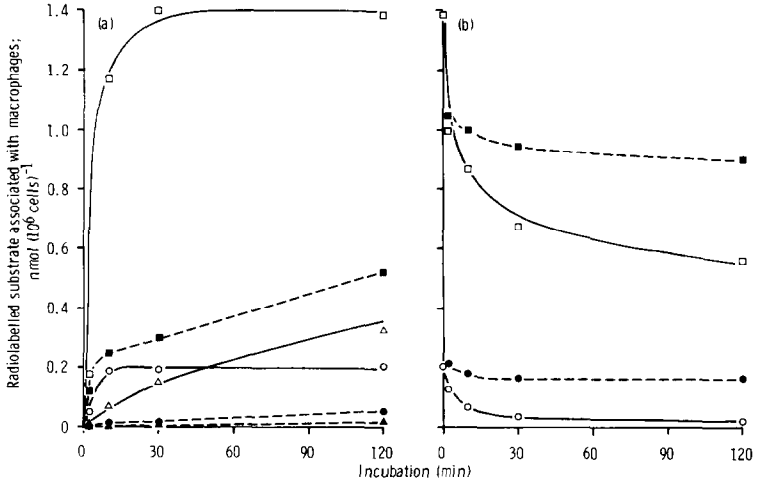

Fig. 1. Time course of benzylpenicillin uptake into and elution from macrophage monolayers in comparison with 3-*O*-methyl-D-glucose and sucrose. (a) Radiolabel uptake was assessed in monolayers incubated with 1 mM-[^{14}C]benzylpenicillin (○,●) or [^{14}C]sucrose (△,▲) in maintenance medium or with 1 mM-3-*O*-methyl-[^{3}H]-D-glucose (□,■) in glucose-free Hanks' balanced salt solution buffered at pH 7.3 with 20 mM-HEPES. Open and closed symbols represent results from incubation at 37 and 0° respectively. (b) Radiolabel elution was assessed in monolayers that had been pre-loaded by incubation for 2 hr at 37° with 1 mM radiolabelled substrate. The pre-loading medium was removed and incubation was continued in substrate-free maintenance medium (benzylpenicillin-loaded cells; ○,●) or glucose-free Hanks' HEPES (3-*O*-methyl-D-glucose-loaded cells; □,■) at 37 or 0° (open and closed symbols respectively).

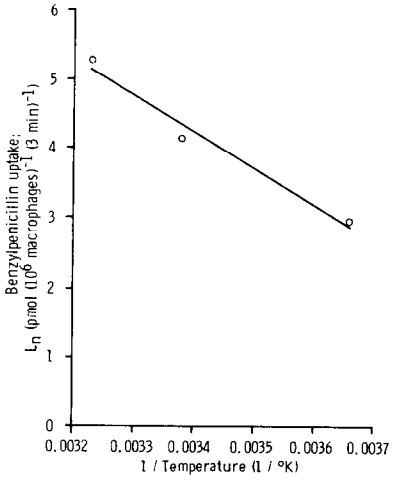

Fig. 2. Arrhenius plot of initial rate of uptake of [^{14}C]benzylpenicillin from a 1 mM-solution in Hanks' HEPES at 0, 27 and 37°. The slope of this line corresponds to a Q_{10} of 1.98.

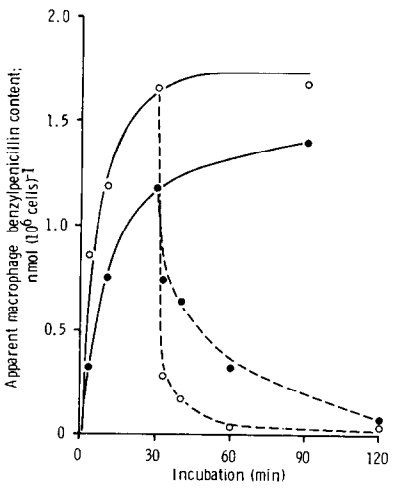

Fig. 3. Effect of metabolic energy depletion on transport of benzylpenicillin. Macrophage monolayers were pre-incubated twice for 30 min at 37° in inhibitor solution, comprising glucose-free Hanks' HEPES with 1 mM-NaCN and 50 mM-2-deoxyglucose, then incubated with 1 mM-[^{14}C]benzylpenicillin in inhibitor solution and radiolabel uptake was assessed at intervals (●——●). After 30 min this solution was removed from some of the monolayers, replaced with penicillin-free inhibitor solution and radiolabel elution from these monolayers was assessed during continued incubation (●- - -●). Inhibitor-free monolayers were assessed for radiolabel uptake (○——○) and elution (○- - -○) in an identical manner except that incubations were in conventional (glucose-containing) Hanks' HEPES.

shape of the uptake curve was different from that for [^{14}C]sucrose, a marker for fluid-phase endocytosis [15, 16], and resembled that for 3-*O*-methyl[^{3}H]-D-glucose which is taken up by facilitated diffusion [17, 18]. After uptake from medium containing the same molar concentrations of penicillin or 3-*O*-methyl-D-glucose the amount of cell-associated penicillin found at equilibrium was consistently much less than the amount of 3-*O*-methyl-D-glucose. At 0° uptake rates were much slower (Fig. 1a).

Penicillin resembled 3-*O*-methyl-D-glucose in readily eluting from pre-loaded cells (fig. 1b), suggesting that the antibiotic was not irreversibly bound or sequestered in the cells. Elution rates of penicillin and glucose analogue, were similarly inhibited at 0° (Fig. 1b).

Similar curves of penicillin uptake were obtained using HH instead of maintenance medium but at equilibrium uptake was 3-fold greater in HH.

An Arrhenius plot of the effect of temperature on the initial velocity (0–3 min) of penicillin uptake in HH was linear in the range from 0 to 37° (Fig. 2). The Q_{10} was calculated to be 1.98.

When intracellular sources of metabolic energy had been depleted by incubation of the cells with cyanide (1 mM) plus 2-deoxyglucose (50 mM) the rates of both uptake and elution of penicillin were substantially inhibited (Fig. 3); in three separate experiments the initial velocities of uptake from 1 mM-penicillin were suppressed by 63, 83 and 88 per cent.

When initial velocities of uptake of penicillin were determined using penicillin concentrations ranging from 0.3 to 100 mM saturability of the uptake process was evident. From a Lineweaver–Burk plot (Fig. 4a) the apparent K_m and V_{max} were calculated, using a non-parametric method [19, 20], to be 110 mM and 117 nmoles (10^6 cells)$^{-1}$ (3 min)$^{-1}$ respectively.

The energy poisons cyanide and 2-deoxyglucose together depressed uptake rates by eliminating the saturable component of uptake (Fig. 4b).

The effect of probenecid at 10 mM was similar to that of energy poisoning in eliminating the saturable transport mechanism (Fig. 4b). Phloretin (1 mM) had no effect on penicillin uptake in phosphate-buffered saline (not shown).

When the uptake of [^{14}C]penicillin was allowed to reach equilibrium (80 min) with a standard concentration of radiolabel and different concentrations of non-labelled penicillin the equilibrium amount of cell-associated radiolabel increased with increase in non-labelled extracellular penicillin (Fig. 5). No effect of penicillin on uptake of [^{14}C]sucrose was seen (Fig. 5).

Cells that had been incubated for 80 min at 37° with non-labelled 30 mM-penicillin subsequently showed the same initial velocity of ^{14}C uptake from labelled 30 mM-penicillin as did cells that had not been pre-incubated with penicillin (not shown). In contrast, the initial velocity of elution of radiolabel from cells in equilibrium with 30 mM-penicillin was about one-sixth of the rate of elution from cells in equilibrium with 0.3 mM-penicillin (Fig. 6).

[^{14}C]Benzylpenicilloic acid was present as a contaminant in the [^{14}C]benzylpenicillin solutions used and the ratio of the acid to the parent molecule was increased in macrophages (Table 1).

Substantial variation between batches of macrophages was noted in both initial rate of uptake and content of benzylpenicillin at equilibrium. The basis of this variability was not systematically explored but appeared to be related to the degree to which the individual macrophages had matured and spread

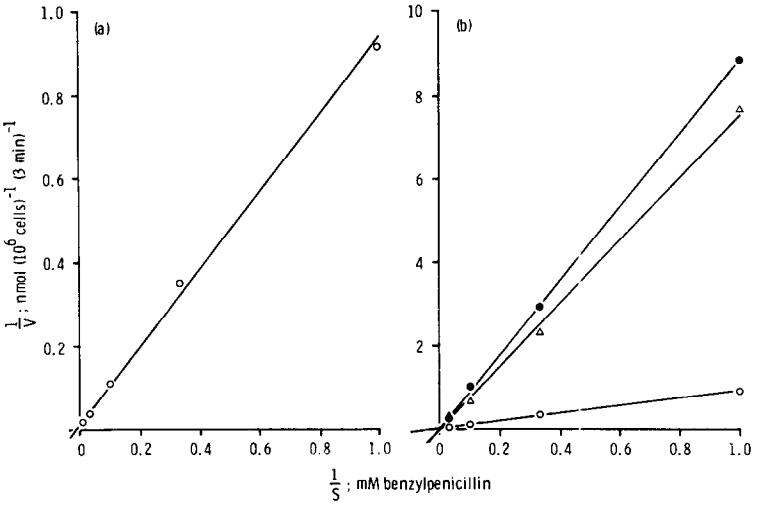

Fig. 4. Lineweaver–Burk plots of the uptake of [^{14}C]benzylpenicillin by macrophages in the presence and absence of inhibitors. Plotted are the reciprocal initial concentrations of the antibiotic in Hanks' HEPES against the corresponding reciprocal cell contents after 3 min at 37°. The straight lines were fitted using a non-parametric method to data obtained with initial antibiotic concentrations from 0.3 to 100 mM but for clarity the values obtained with 0.3 mM-antibiotic are not shown. (a) Uptake by inhibitor-free monolayers. (b) Uptake after pre-treatment with and in the continued presence of 1 mM-NaCN plus 50 mM-2-deoxyglucose as described in Fig. 4 (●) and uptake in the presence of 10 mM-probenecid (△). Control data are replotted on the same scale to facilitate comparisons (○).

out in the dish. Accumulated data revealed a positive correlation between the size of the apparent intracellular water space and either rate or extent of penicillin uptake (Fig. 8).

Analytical subcellular fractionation of [^{14}C]-penicillin-loaded macrophages in sucrose density gradients showed that the great majority of the radiolabel was not associated with the sedimenting organelles (Fig. 9). However, a small peak of radioactivity was consistently found at a modal density of about 1.23 g(cm^3)$^{-1}$, coincident with the modal density of

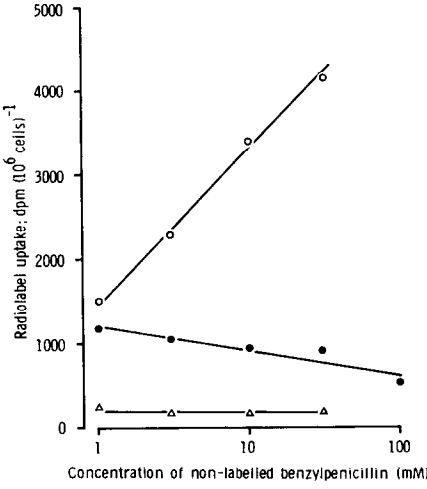

Fig. 5. The contrasting effects of increasing the initial extracellular concentration of non-labelled benzylpenicillin on the net uptake of radiolabel at equilibrium with [^{14}C]benzylpenicillin and on the rates of uptake of [^{14}C]benzylpenicillin and [^{14}C]sucrose. Macrophages were incubated at 37° with Hanks' HEPES containing different concentrations of non-labelled benzylpenicillin and either [^{14}C]benzylpenicillin at a concentration of 1.1×10^6 dpm (ml)$^{-1}$ or [^{14}C]sucrose at 8.2×10^5 dpm (ml)$^{-1}$. Cell associated radiolabel was measured after incubation with [^{14}C]penicillin for either 80 min (○; equilibrium values) or 3 min (●; data from Fig. 4) and after incubation with [^{14}C]sucrose for 80 min (△).

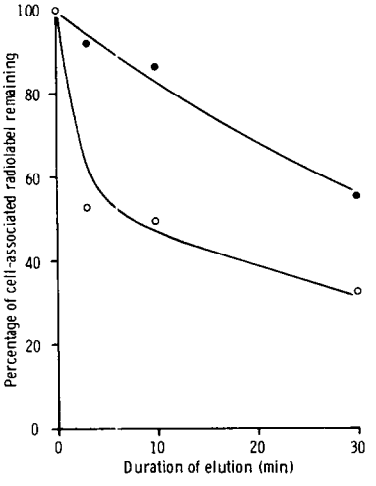

Fig. 6. Suppression by non-labelled benzylpenicillin of radiolabel elution from macrophages that had been preloaded by incubation with [^{14}C]benzylpenicillin. Monolayers were first incubated with Hanks' HEPES containing [^{14}C]benzylpenicillin at a concentration of 1.1×10^6 dpm (ml)$^{-1}$ and non-labelled penicillin at either 0.3 or 30 mM for 30 min at 37°. The solutions were then replaced with Hanks' HEPES containing non-labelled penicillin at the same concentration but without radiolabel and the decrease in cell-associated radiolabel was determined during continued incubation at 37°. Monolayers incubated with 0.3 mM-penicillin (○) contained 503 dpm (10^6 cells)$^{-1}$ at the start of elution, those incubated with 30 mM-penicillin (●) contained 1660 dpm (10^6 cells)$^{-1}$.

Table 1. Increase in the ratio of [^{14}C]benzylpenicilloic acid to [^{14}C]benzylpenicillin within macrophages

Source of radioactivity	Percentage of total radioactivity present as: Penicillin (1)	(2)	Mean	Penicilloic acid (1)	(2)	Mean
Fresh solution	82.6	85.4	84.0	17.4	14.6	16.0
Solution after incubation with macrophages	84.5	85.3	84.9	15.5	14.7	15.1
Cell extract	78.0	81.8	79.9	22.0	18.2	20.1

Duplicate macrophage monolayers (3×10^6 cells) were incubated with a freshly prepared solution of [^{14}C]-benzylpenicillin (25 μCi ml^{-1}) at 37° for 80 min then rinsed with 5×2 ml of ice-cold buffer solution.

Cell-associated radioactivity was extracted into 50% (v/v) methanol in water, fractionated by thin-layer chromatography and quantified by liquid scintillation counting.

N-acetyl-β-glucosaminidase and distinct from the modal densitities of the other subcellular organelle marker enzymes.

In *Staphylococcus aureus*-infected cells the distribution of radiolabel was similar to that seen with the uninfected cells. The modal density of *N*-acetyl-β-glucosaminidase was slightly less at 1.22 g(cm^3)$^{-1}$ and coincident with a small peak of protein. Bimodality was now evident in the profiles of 5′-nucleotidase and neutral α-glucosidase, their respective peaks at 1.14 and 1.15 g(cm^3)$^{-1}$ being diminished and new peaks being present at 1.22 g(cm^3)$^{-1}$ coincident with *N*-acetyl-β-glucosaminidase. Cytochrome *c* oxidase and catalase were unchanged in modal density, at 1.17 and 1.19 g(cm^3)$^{-1}$ respectively. The bacteria showed a major peak at 1.22 g(cm^3)$^{-1}$ in contrast with the restriction of bacteria to the denser regions in control gradients in which bacteria alone had been centrifuged. The addition of bacteria to homogenates of uninfected cells did not affect the distribution profiles of bacteria or marker enzymes.

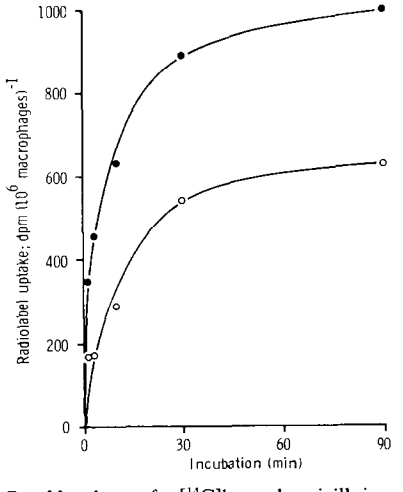

Fig. 7. Uptake of [^{14}C]benzylpenicilloic acid and [^{14}C]benzylpenicillin compared. Macrophages were incubated at 37° with Hanks' HEPES containing 30 mM-non-labelled benzylpenicillin and either [^{14}C]benzylpenicilloic acid at 5.6×10^5 dpm (ml)$^{-1}$ (○) or [^{14}C]benzylpenicillin at 5.5×10^5 dpm (ml)$^{-1}$ (●).

Bacteria that had been incubated with 21 μM [^{14}C]benzylpenicillin (13 units ml^{-1}) at pH 5.4 for 18 min, washed and then centrifuged into a sucrose density gradient were efficiently separated from unbound penicillin and the distribution profile of bound penicillin closely followed that of the bacteria (not shown). Calculations showed they bound 0.85 pmole (10^8 bacteria)$^{-1}$ or 5000 molecules (bacterium)$^{-1}$.

When penicillin-free macrophages were fractionated on a gradient that contained uniformly distributed [^{14}C]benzylpenicillin at low concentration and high specific activity the passage of the sedimenting organelles into the gradient resulted in a depletion of penicillin from the upper regions that was balanced by accumulation in lower regions (Fig. 10). This effect was small, being a redistribution of 1.0 pmole

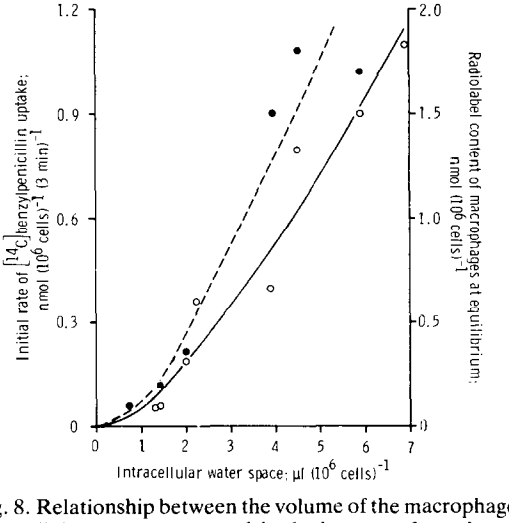

Fig. 8. Relationship between the volume of the macrophage intracellular water space and both the rate of uptake of benzylpenicillin and the amount associated with the cells at equilibrium. Monolayers were incubated with 1 mM-[^{14}C]benzylpenicillin in Hanks' HEPES at 37°. Radiolabel uptake was measured after 3 min (○; initial uptake rate) or 80 min (●; content at equilibrium) for 9 separate batches of monolayers. The intracellular water space was estimated for each batch using 3-*O*-methyl-D-glucose uptake.

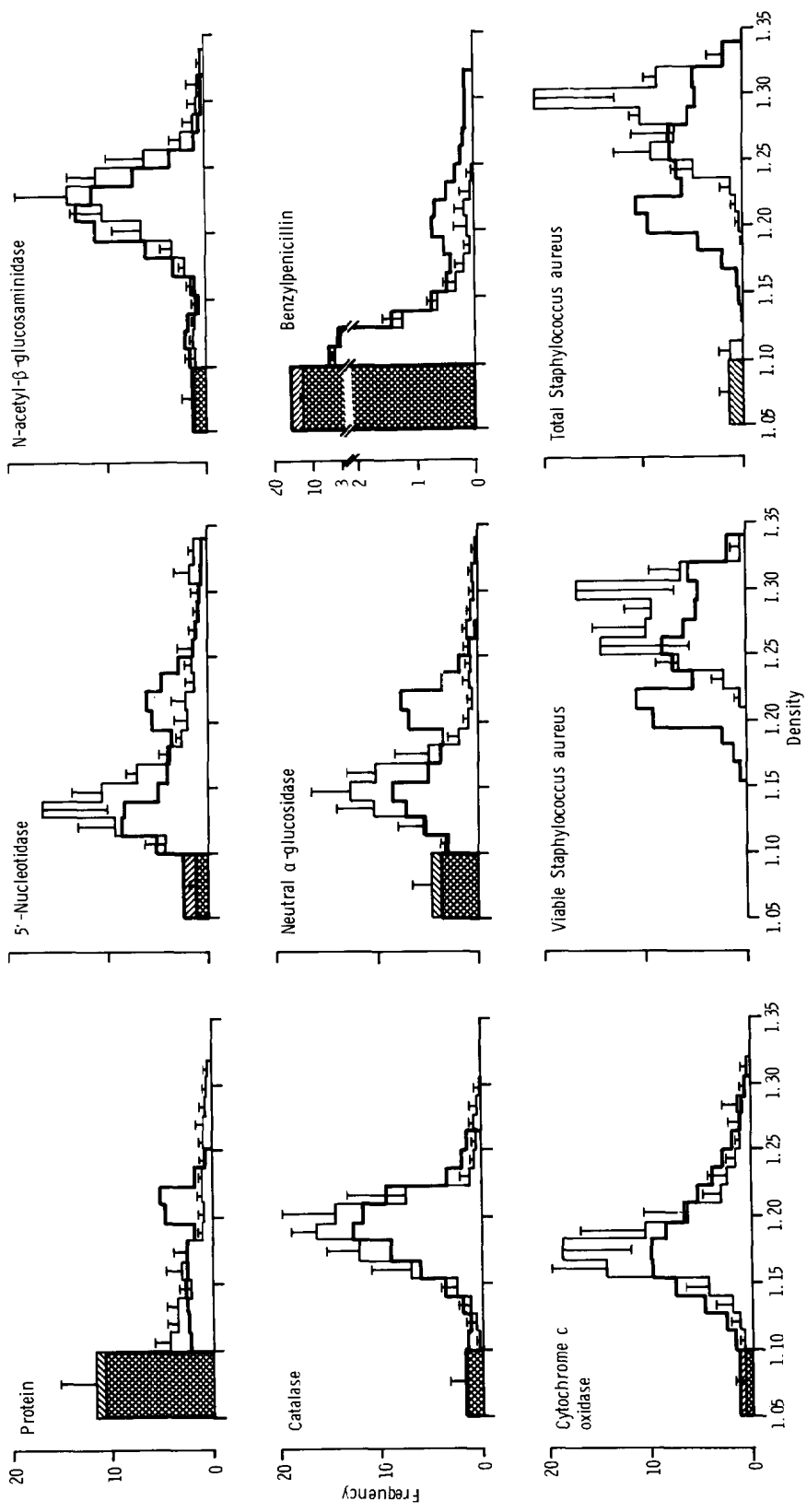

Fig. 9. Isopycnic fractionation of subcellular organelles from normal and *Staphylococcus aureus*-infected mouse peritoneal macrophage monolayers. Monolayers were equilibrated with [^{14}C]benzylpenicillin and thoroughly rinsed before homogenisation. Graphs show frequency–density distributions for benzylpenicillin, protein and organelle marker enzymes from normal monolayers (——; average ± S.D. from 4 to 5 experiments) and from infected monolayers ——; one experiment). The distributions of total and viable bacteria from the infected macrophages (——) are shown against the corresponding distribution observed with bacteria alone (——; averaged from 4 experiments). Frequency is defined as the fraction of the total recovered activity that was present in the subcellular fraction divided by the density span covered. The cross-hatched area represents, over an arbitrary abscissa interval, the activity remaining in the sample layer.

Table 2. Partition of radiolabelled solutes between octanol and Hanks' HEPES buffer at pH 7.3

Solute	Partition coefficient
Benzylpenicillin	0.0462
Benzylpenicilloic acid	0.0295
3-O-methyl-D-glucose	0.0081
Sucrose	0.0004

Table 3. Percentage of radiolabel found associated with *N*-acetyl-β-glucosaminidase-rich organelles as benzylpenicillin and as benzylpenicilloic acid on fractionation of [^{14}C]benzylpenicillin-loaded macrophages.

	Benzylpenicillin	Benzylpenicilloic acid
Experiment 1	24.5	75.5
Experiment 2	34.4	65.6

Table 4. Parallel release of enzyme and radioactivity by disruption of a *N*-acetyl-β-glucosaminidase-rich fraction of subcellular organelles from [^{14}C]benzylpenicillin-loaded macrophages*

Disruptive treatment	Subfraction	N-acetyl-β-glucosaminidase (mU)	^{14}C (dpm)	Cholesterol (μg)
None	Supernatant	1.6	241	2.8
	Pellet	40.5 (100)†	294 (100)	21.2 (100)
Ultrasonics	Supernatant	30.5	465	5.2
	Pellet	11.6 (29)	72 (25)	22.9 (108)
Digitonin	Supernatant	40.1	517	6.0
	Pellet	1.9 (4.7)	13 (4.5)	22.3 (105)

* Monolayer macrophages were rinsed and disrupted after equilibration with [^{14}C]benzylpenicillin at 1.0 μCi ml^{-1}. A *N*-acetyl-β-glucosaminidase-rich fraction was prepared by centrifugation of the organelles on a discontinuous sucrose gradient and collection of the material at a 1.18 to 1.25 g(cm^3)$^{-1}$ interface. The fraction was divided into three equal parts and adjusted to density <1.09 g(cm^3)$^{-1}$. One part was not given disruptive treatment, one part was ultrasonicated for 1 min and to one part digitonin (0.5 mg ml^{-1}) was added. The three preparations were then layered on 1.11 g(cm^3)$^{-1}$ sucrose, centrifuged to pellet membranes and then the distribution of *N*-acetyl-β-glucosaminidase, radioactivity and cholesterol (membrane marker) between supernatants (soluble fraction) and pellet (particulate fraction) was assessed.

† In parentheses the pelleted activity is expressed as a percentage of the pelleted activity with the control (undisrupted) preparation.

(110 dpm) when organelles from 6×10^7 cells were centrifuged into a gradient containing 44 nM [^{14}C]benzylpenicillin (500 dpm ml^{-1}). The peak of penicillin accumulation was broad, with a modal density at about 1.24, coincident with *N*-acetyl-β-glucosaminidase in these experiments, and penicillin depletion appeared to be impaired in the density region 1.14 to 1.15, the region where 5′-nucleotidase and neutral α-glucosidase peaked.

In a 1.18 to 1.25 g(cm^3)$^{-1}$ density fraction of [^{14}C]penicillin-loaded cells about 30 per cent of the radioactivity was present as [^{14}C]benzylpenicillin and the remainder as [^{14}C]benzylpenicilloic acid (Table 3). This radioactivity appeared to be trapped together with *N*-acetyl-β-glucosaminidase within membrane-limited structures. Exposure to either ultrasonication or the detergent effect of digitonin caused parallel conversion of radioactivity and enzyme to forms that no longer pelleted with cholesterol on high-speed centrifugation (Table 4).

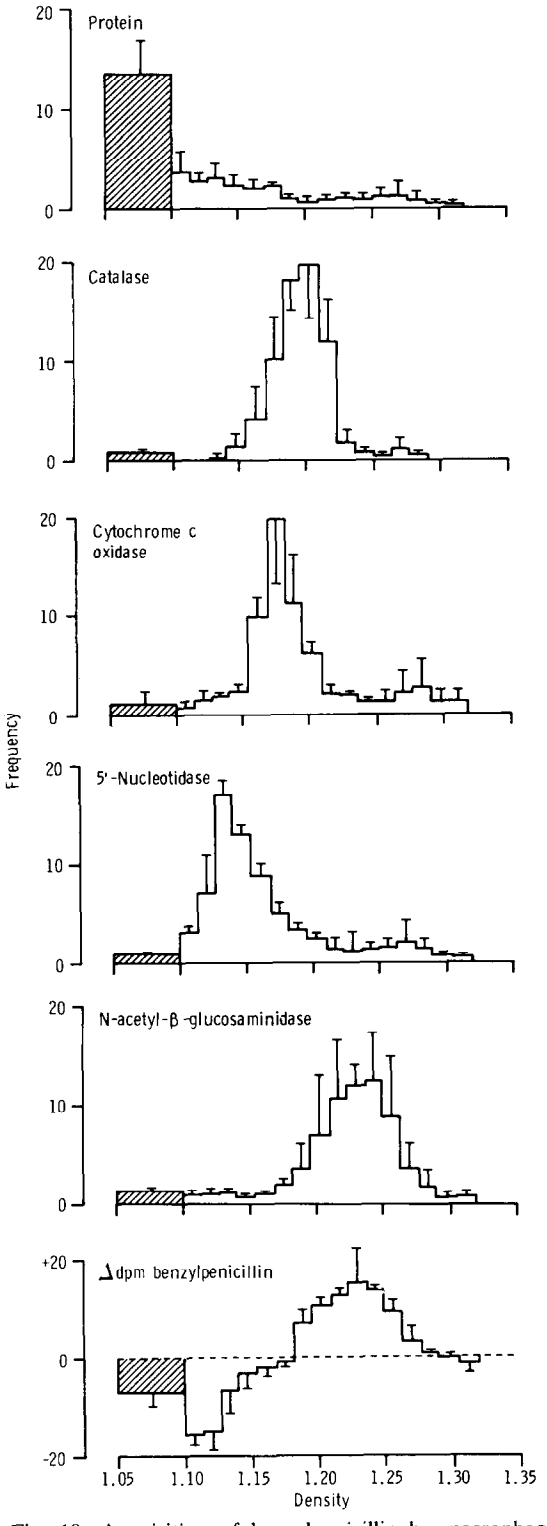

Fig. 10. Acquisition of benzylpenicillin by macrophage subcellular organelles during isopycnic fractionation. Penicillin-free macrophages were homogenised then fractionated as in Fig. 1 except that [^{14}C]benzylpenicillin was present at a uniform concentration in homogenate and density gradient before centrifugation. Organelle marker enzymes and the depletion and accumulation of penicillin in the gradient fractions relative to a homogenate-free gradient were determined and expressed as in Fig. 1. Results are averages (±S.D.) from two experiments.

DISCUSSION

We have here confirmed earlier observations that under physiological conditions benzylpenicillin rapidly associates with macrophages in monolayer culture and within minutes reaches a steady-state equilibrium at which the rate of uptake is balanced by the rate of loss and there is no net concentration [2]. Direct evidence that the antibiotic enters lysosomes, and phagolysosomes in infected cells, has been obtained.

The kinetics of transport were strikingly different from those reported for aminoglycoside antibiotics in fibroblast monolayers where accumulation occurs slowly over several days to give concentrations that, on a cell volume basis, are several-fold higher than the extracellular concentrations and much higher within lysosomes [21].

The saturability and substantial inhibition of uptake rate by metabolic inhibitors and probenecid (Fig. 4) strongly suggest that benzylpenicillin uptake can involve a carrier-mediated transport process that is energy-dependent, in addition to a simple diffusion process. Susceptibility of elution to metabolic inhibitors (Fig. 3) and saturation (Fig. 6) indicates active transport operating in both directions. Carrier and metabolic energy-dependent transport is also responsible for active secretion of penicillin in renal tubules and salivary glands [22, 23].

Possibly the active transport seen here was a property of a subpopulation of the macrophages and the variation between cell batches reflected variation in the proportion of cells with both active and passive uptake relative to those (less mature?) with only passive uptake. An analogous situation has been evidenced for glucose analogue uptake by other cell types [24, 25].

If intracellular penicillin was uniformly distributed in the cell water space then at equilibrium with 1 mM-penicillin in buffer at pH 7.3 the intracellular concentration was about 0.3 mM (Fig. 8) or about one-third of the extracellular concentration. Since benzylpenicillin is a weak acid with pK_a 2.7 [26] this would be consistent with a diffusion equilibrium across a membrane that is permeable only to the uncharged (protonated) molecule if the intracellular pH is 6.8 [27]. The partition coefficient of benzylpenicillin between octanol and buffer might be compatible with equilibration within 30 min by simple transmembrane diffusion [9]. However, transport solely by diffusion would not account for the observed energy dependence.

On subcellular fractionation the patterns of distribution of the various macrophage enzyme activities resembled those reported by Canonico *et al.* [28] for similar macrophages and sustained the use of the enzymes as markers for subcellular organelles in accord with established principles [28, 29].

The presence of the great majority of benzylpenicillin at the top of the density gradients on fractionation of penicillin-loaded macrophages (Fig. 1) showed that it was predominantly present in the soluble (cytosolic) fraction, with a trace associated with lysosomes (*N*-acetyl-β-glucosaminidase) in uninfected cells and with phagolysosomes in staphylococcus-infected cells.

Phagolysosome formation was evidence by the

coincident peaks of bacteria, lysosomal and plasma membrane (5′-nucleotidase) marker enzymes at a new position in the gradient. Some neutral α-glucosidase also moved to the phagolysosome peak but it is unclear whether this activity was present in endoplasmic reticulum [12], plasma membrane or secretory granules [28]. Because of possible confusion with an acid α-glucosidase [28] assays were also done at pH 9 but activity distribution profiles were identical to those at pH 7.5. Despite reports that macrophage catalase can transfer into phagolysosomes [30, 31] only minimal change in the distribution of catalase was seen in contrast to 5′-nucleotidase and neutral α-glucosidase. Calculations based upon the number of bacteria present (about 10 per macrophage) and their penicillin binding capacity indicated that this would have no perceptible effect on the amount of penicillin associated with phagolysosomes. The estimate of penicillin binding capacity agreed with reported values [32].

Although lysosomes and perhaps other organelles showed a slight capacity to acquire radiolabelled penicillin during subcellular fractionation (Fig. 10) the lysosome-associated radiolabel found with [^{14}C]penicillin pre-loaded cells was within rather than bound to the surface of the lysosomes (Table 4) and about 70 per cent was present as penicilloic acid. This suggests that the antibiotic entered the lysosomes and was partially hydrolysed there during incubation of the intact cell at 37°. Since the acid has a lower affinity for the transport system (Fig. 7) this would account for the intralysosomal increase in the ratio of the acid form to the parent antibiotic and the enhancement of net radiolabel uptake at equilibrium for an increase in extracellular concentration of non-labelled benzylpenicillin (Fig. 5).

Assuming that the volume of the lysosomes is 10 per cent of the cell volume [33, 34], that the fraction of the lysosome volume available for solvation [35] is half the value for the cell as a whole [7, 18], and that the intralysosomal penicillin is free in solution, the intralysosomal penicillin concentration would be about a thirtieth of the cytosol concentration. This is compatible with calculations for a diffusion equilibrium when the intralysosomal pH is 5.0 [36, 37] and the cytosol pH is 6.8.

The nature of the energy-dependent, carrier-mediated transport system is unknown. Since it enhances both influx and efflux rates and the lysosomal and cytosolic penicillin concentrations are compatible with equilibration by transmembrane diffusion an energy-dependent mechanism for facilitating diffusion seems indicated. One possibility is that this reflects the operation of membrane recylcing or shuttle [38–40] whereby plasma membrane is internalised together with associated solutes, transported through the intracellular membrane compartments and returned to the cell surface [39].

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