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Influence of Conversion of Penicillin G into a Basic Derivative on Its Accumulation and Subcellular Localization in Cultured Macrophages

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β-Lactam antibiotics do not accumulate in phagocytes, probably because of their acidic character. We therefore synthesized a basic derivative of penicillin G, namely, ^{14}C-labeled *N*-(3-dimethylaminopropyl)benzylpenicillinamide (ABP), and studied its uptake and subcellular localization in J774 macrophages compared with that of ^{14}C-labeled penicillin G. Whereas the intracellular concentration (C_i) of penicillin G remained lower than its extracellular concentration (C_e), ABP reached a C_i/C_e ratio of 4 to 5. Moreover, approximately 50% of intracellular ABP was found associated with lysosomes after isopycnic centrifugation of cell homogenates in isoosmotic Percoll or hyperosmotic sucrose gradients. The behavior of ABP was thus partly consistent with the model of de Duve et al. (C. de Duve, T. de Barsy, B. Poole, A. Trovet, P. Tulkens, and A. Van Hoof, Biochem. Pharmacol. 23:2495–2531, 1974), in which they described the intralysosomal accumulation of weak organic bases in lysosomes. Although ABP is microbiologically inactive, our results show that β-lactam antibiotics can be driven into cells by appropriate modification. Further efforts therefore may be warranted in the design of active compounds or prodrugs that may prove useful in the chemotherapy of intracellular infections.

Penicillins, and β-lactam antibiotics in general, are not accumulated by phagocytic or nonphagocytic cells (6, 12, 13, 15, 18, 19, 22, 32, 43). This behavior may explain their poor efficacy against intracellular bacteria (1, 16, 24, 40). Thus, the design of β-lactam derivatives or prodrugs with enhanced cellular accumulation appears to be a promising approach in the chemotherapy of intracellular infections. This property is indeed an essential one, albeit not the only one, that must be displayed by an antimicrobial agent to show activity in this respect.

In contrast to penicillins, which are acidic compounds, several basic antibiotics are accumulated by phagocytes (15, 19, 20, 25, 26, 32). Other weak organic bases, such as chloroquine and propranolol, are also accumulated by cells and have been shown to largely localize in the lysosomes (11, 30, 31, 47). This behavior probably results from the well-known ability of weak organic bases to become concentrated in acidic, membrane-bound compartments (for reviews, see references 9 and 27). Thus, we reasoned that conversion of a penicillin into a basic molecule could promote its intracellular accumulation. As a test for this hypothesis, we examined and report here on the synthesis, cellular accumulation, and subcellular distribution of *N*-(3-dimethylaminopropyl)-[^{14}C]benzylpenicillinamide ([^{14}C]ABP; Fig. 1), i.e., a derivative of penicillin G in which the carboxylic acid group of the molecule has been substituted by a basic, aminated moiety. Although it was anticipated that this particular compound would be microbiologically inactive (14), we considered that positive results could provide the incentive for further development of β-lactam prodrugs in this direction.

(Part of these data were presented at the 14th International Congress of Chemotherapy, Kyoto, Japan [C. Renard, A. Zenebergh, H. J. Vanderhaeghe, P. J. Claes, and P. M. Tulkens, Proceedings of the 14th International Congress of Chemotherapy, p. 331–332, 1986].)

MATERIALS AND METHODS

Synthesis of ABP. ABP was prepared by the procedure described by Barnden et al. (2) for various carboxy derivatives of benzylpenicillin. Ethylchloroformate (108 mg [1 mmol] in 2 ml of tetrahydrofuran [THF]) was added to a cooled (0°C) suspension of benzylpenicillin sodium salt (356 mg [1 mmol] in 10 ml of THF) containing 0.2 mmol of pyridine. The suspension was stirred for 30 min (at 0°C), and 3-dimethylaminopropylamine (102 mg [1 mmol]) in 2 ml of THF was added. The reaction mixture was stirred for 2 h at 0°C, 1 ml of HI (1 N aqueous solution) was added, and the solution was adjusted to an apparent pH of 6.5 with HI. THF was evaporated under reduced pressure at room temperature, and the remaining powder was dissolved in 20 ml of water and freeze-dried. The resulting product was triturated with anhydrous ether, filtered, and dried over P_2O_5, yielding approximately 300 mg of the crude ABP (HI salt) as a powder. Thin-layer chromatography (TLC) of this product was performed on silanized silica gel plates (Gel F-254; E. Merck AG, Darmstadt, Federal Republic of Germany) by using a 3:7 mixture of acetone and a 2 M ammonium acetate buffer (pH 5) as a mobile phase. On exposure to I_2 vapors, one major spot at $R_f = 0.4$ and two minor spots at R_fs = 0.6 and 0.7 were detected. The starting penicillin G in this system shows a single spot with an R_f of 0.5. The crude ABP

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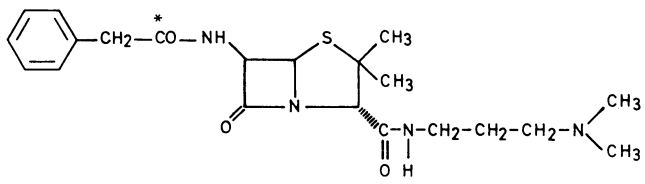

FIG. 1. Molecular structure of [^{14}C]ABP. The asterisk shows the ^{14}C-labeled atom.

(150 mg) was further chromatographed on a 40-ml resin column (100-200 mesh; XAD-2; Servachrome). Elution was carried out with an acetone-water gradient (10 to 14% acetone), yielding approximately 100 mg of ABP. The loss of material observed was due to peak tailing caused by the absence of salts and buffers in the mobile phase.

The purified HI salt of ABP was analyzed by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy. Spectral data were in agreement with the proposed structure: IR (KBr): $\bar{\nu}$ 3280 (NH), 1780 (β-lactam), 1660 and 1520 (amide) cm^{-1}; ^{1}H NMR (90 MHz, in D_2O containing the sodium salt of 3-(trimethylsilyl)propanesulfonic acid as internal standard): δ 1.46 and 1.63 (s, CMe_2), 2.15 (m, C-CH_2-C), 2.88 (s, NMe), 3.04 and 3.42 (m, N-CH_2), 3.68 (s, $CH_2C_6H_5$), 4.33 (s, 3-H), 5.55 (s, 6-H and 5-H), 7.37 (s, C_6H_5) ppm. TLC of the recovered compound showed a major spot with an R_f value of 0.4.

^{14}C-labeled ABP (HI salt) was synthesized by the procedure described above, starting with 50 mg of benzylpenicillin containing approximately 100 μCi of [^{14}C]benzylpenicillin (6-phenyl-[1-^{14}C]acetamidopenicillinate; 40 to 60 Ci/mol; Amersham International plc, Amersham, England; 95% of the radioactivity was associated with benzylpenicillin, as shown by TLC). After neutralization by HI, unreacted benzylpenicillin was further removed as follows. The pH was brought to 3 with diluted HI. The cooled solution was rapidly extracted twice with 2 ml of ethyl acetate, and the aqueous layer was adjusted to pH 6 with diluted NaOH and freeze-dried, yielding approximately 50 mg of powder. TLC showed that 82% of the radioactivity was associated with ABP (R_f = 0.4), and 4 and 14% of the radioactivity was found in side products, decomposition products, or both, with R_f values of 0.6 and 0.7. The specific radioactivity of ABP was approximately 0.5 mCi/g. Concentrations given for [^{14}C]ABP (HI salt) were corrected for purity, which was estimated at 80%. Lactose was added to the dried [^{14}C]ABP (3:1; wt/wt); and the mixture was dissolved in water, freeze-dried, and used as such.

Cells and culture conditions. All experiments were performed with J774 macrophages. These cells were derived from a mouse reticulosarcoma (33). Cells were cultured at 37°C in 95% air–5% CO_2 in RPMI 1640 medium supplemented with 10% calf serum (GIBCO Ltd., Paisley, Scotland). Cells were seeded in petri dishes (diameter, 6 cm) at a density of approximately 10^5 cells per cm^2 and grown until confluency (2 to 3 days).

[^{14}C]ABP or [^{14}C]benzylpenicillin (4 μCi/g) was added to the culture medium at a concentration of 0.3 mM (i.e., 100 and 160 mg/liter for benzylpenicillin and ABP, respectively). After incubation with the cells the medium was aspirated; and the cell sheet washed once with ice-cold, drug-free medium and twice with ice-cold phosphate-buffered saline, scraped off with a rubber policeman, and suspended in ice-cold distilled water. The protein concentration was determined by the method described by Lowry et al. (23). The radioactivity of the cell lysate was measured by liquid scintillation counting. The molecular nature of the radioactivity found in the cell lysate and the culture medium was ascertained by TLC, as described above. The ratio of intracellular to extracellular drug concentration was calculated, with the consideration that 1 mg of cell protein is equivalent to a cellular volume of 5 μl, as in peritoneal macrophages (41) or cultured fibroblasts (45).

Cell fractionation experiments. Cells incubated with [^{14}C]ABP or [^{14}C]benzylpenicillin, as indicated above, were collected, washed in 0.25 M sucrose–3 mM EDTA–3 mM imidazole (pH 7.4), and homogenized in the same medium in an all-glass Dounce tissue grinder with 7 strokes of the tightly fitting pestle. Results of preliminary studies showed that these conditions cause a satisfactory liberation of soluble, cytosolic enzymes, whereas enzymes associated with mitochondria and lysosomes remain largely latent and sedimentable. The homogenate was separated into a nuclear fraction and a cytoplasmic extract, as described previously (44). In some experiments (differential centrifugation) this cytoplasmic extract was further fractionated into a granule fraction and a soluble fraction (supernatant) by centrifugation at 40,000 rpm (100,000 × *g*) for 30 min in a rotor (Ti 50; Beckman Instruments, Inc., Fullerton, Calif.). In other experiments, the cytoplasmic extract was fractionated by isopycnic centrifugation in Percoll or sucrose gradients. In Percoll gradients 10 ml of cytoplasmic extract was mixed with 10 ml of 54% Percoll (Pharmacia AB, Uppsala, Sweden) in 0.25 M sucrose–3 mM EDTA–3 mM imidazole (pH 7.4). The centrifugation was performed at 20,000 rpm (35,000 × *g*) during 90 min in a fixed-angle rotor (no. 30; Beckman) containing 2.5 ml of 2.5 M sucrose as a cushion. For sucrose gradients a 5-ml sample of cytoplasmic extract was deposited on top of a 30-ml, preformed linear sucrose gradient (density limits, 1.10 to 1.30) resting on a cushion of 1.5 ml of sucrose with a density of 1.34 and centrifuged at 49,000 rpm (200,000 × *g*) for 90 min in a vertical type rotor (VTi 50; Beckman). After centrifugation, 12 to 15 fractions were collected and weighed, and their densities were measured by refractometry. In each fraction the amount of radioactivity was measured and the activity of marker enzymes of the main subcellular components was determined as follows: for plasma membrane, alkaline phosphodiesterase; for lysosomes, *N*-acetyl-β-hexosaminidase and α-galactosidase; for mitochondria, cytochrome oxidase; for cytosol, lacticodehydrogenase, as established for mouse peritoneal marcophages by previous work from our laboratory (7, 8). The methods were those described previously (7, 8), except for *N*-acetyl-β-hexosaminidase, for which the method described by Sellinger et al. (38) was used. Protein was assayed by the method described by Lowry et al. (23), with serum albumin used as a standard. For linear sucrose gradients, results are presented as density distribution histograms, because this method provides the most meaningful representation of the distribution patterns obtained by this technique (for a general review and discussion, see reference 3). For Percoll the gradient was not linear and its shape was therefore difficult to determine accurately from a finite number of points. We therefore adopted a more factual mode of representation, i.e., volume distribution histograms (adapted from those described previously [4]). To facilitate the comparison of the distribution profiles obtained from different experiments, the histograms were systematically standardized by dividing the abscissa (density or volume scale) in 15 sections of equal increments. Moreover, in Fig. 3 and 4 the ordinate

is the relative concentration or frequency, so that the area of each histogram is consistently equal to 1.

RESULTS

Chemistry and properties of ABP. IR and ^1H NMR spectra of the purified, unlabeled product were in agreement with the proposed structure of a N-substituted penicillinamide. After synthesis in the presence of ^{14}C-labeled benzylpenicillin, crude ^{14}C-labeled ABP was obtained with a radiopurity of 82%. Further purification by chromatography on Servachrome XAD-2 resin (in the absence of buffer, salts, or both) resulted in a considerable loss of material due to extensive peak tailing. Chromatography on silanized silica gel, with acetone–0.1 M aqueous KCl (1:9) as a mobile phase, followed by removal of KCl was attempted. However, ABP was found to be unstable in concentrated solutions. Thus, partial decomposition (formation of more polar components with R_fs = 0.6 and 0.7) occurred on concentration of effluents before and after removal of the KCl. The purity of the product thus obtained was lower than that of the crude reaction product. Therefore, purification was limited to extraction of the benzylpenicillin remaining in the reaction mixture. The radiopurity of the resulting ABP was about 80%. In view of the partial accumulation of ABP in lysosomes (see below), we checked its stability in the presence of a soluble fraction of purified lysosomes (42) at pH 5 and 37°C for 24 h in the presence of 2 mM cystein (to fully activate thiol-dependent hydrolases). No difference was observed by TLC between incubated and control [^{14}C]ABP. ABP was inactive against penicillin G-susceptible *Staphylococcus aureus* ATCC 25923 and *Streptococcus agalactiae* Lancefield group B.

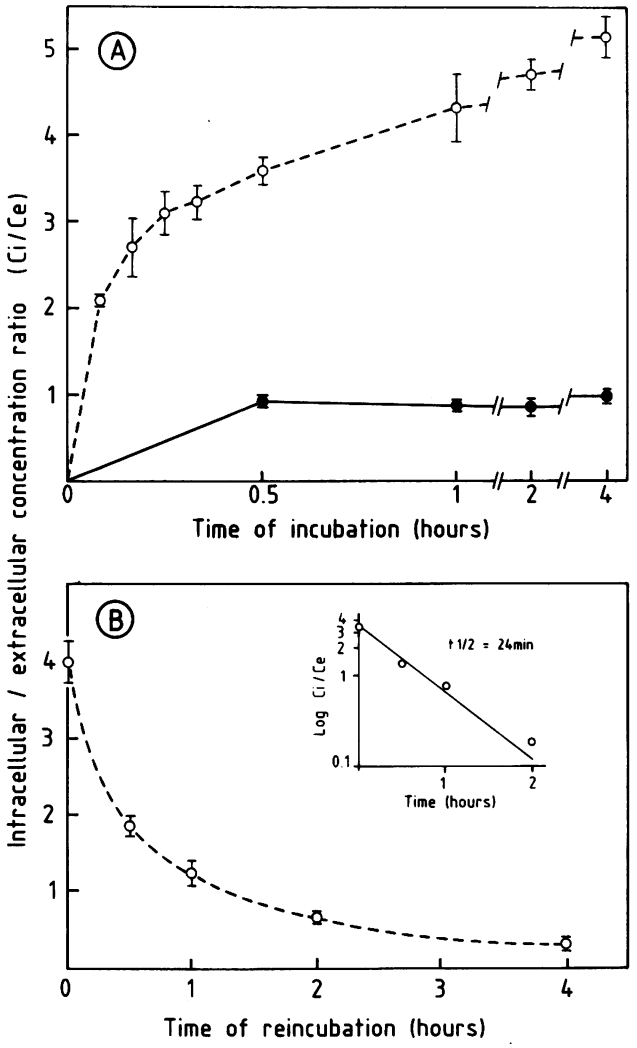

FIG. 2. (A) Uptake of [^{14}C]ABP and [^{14}C]benzylpenicillin by J774 macrophages. Cells were incubated in the presence of 3 mM (160 mg/liter) [^{14}C]ABP (○) or 3 mM (100 mg/liter) [^{14}C]benzylpenicilline (●) (means ± SD; n = 3). (B) Release of [^{14}C]ABP from J774 macrophages. Cells were preincubated for 1 h in the presence of 3 mM (160 mg/liter) [^{14}C]ABP. The cells were washed and reincubated in drug-free medium (means ± SD; n = 3). The inset shows the data on a semilogarithmic scale ($t_{1/2}$, half-life). The equation was calculated from the direct plot by the least-squares method, after the value recorded at 4 h (plateau) was subtracted.

TABLE 1. Distribution of protein, marker enzymes, and [^{14}C]ABP between subcellular fractions obtained by differential centrifugation

Enzyme	Percentage of enzyme or constituent in the following fractions:			Recovery (%)[a]
	Nuclear	Granule	Supernatant	
Protein	24.8	46.3	28.9	77.3
Lacticodehydrogenase	14.2	20.8	65.0	57.0
N-Acetyl-β-hexosaminidase	9.6	79.0	11.4	90.6
α-Galactosidase	8.6	76.0	15.4	96.4
Alkaline phosphodiesterase	5.9	83.4	10.7	70.2
[^{14}C]ABP	5.4	62.8	31.8	113.4

[a] Recovery is (sum of the activities [or amount] collected in each fraction/activity [or amount] in the unfractionated homogenate) × 100.

Intracellular accumulation and release. The accumulation of [^{14}C]ABP in cultured J774 macrophages is shown in Fig. 2A. After a rapid initial uptake the intracellular concentration rose more slowly. About 60% of the maximal intracellular accumulation was reached within 15 min. After 60 min of incubation the ratio of intracellular to extracellular concentration was 4.2 ± 0.4 (standard deviation [SD]; n = 6) and 5.4 ± 0.4 (SD; n = 3) after 4 h. The molecular nature of the radioactivity that was accumulated by the cells was checked by TLC. The pattern observed was similar to that of the radioactive compound in the culture medium and in the stock solutions of [^{14}C]ABP; i.e., approximately 80% of the radioactivity was associated with the main spot at an R_f = 0.4, the remaining being at R_fs = 0.6 and 0.7 (decomposition or side products; see above). In contrast to [^{14}C]ABP, ^{14}C-labeled penicillin accumulated markedly less in cells, and its intracellular concentration remained below the extracellular concentration throughout the duration of the incubation period (intracellular/extracellular concentration ratio = 0.9 ± 0.1 [SD; n = 3] after 4 h).

The rate of release of cell-associated [^{14}C]ABP was determined in cells that were incubated with the drug for 1 h and thereafter transferred to drug-free medium. This release obeyed first-order kinetics with a half-time of elimination of 24 min (Fig. 2B). A small proportion of the drug (approx. 12%) was not released after 4 h, however.

Intracellular distribution. The distribution of [^{14}C]ABP and marker enzymes between the nuclear fraction, the granule fraction, and the cell supernatant after differential centrifugation of cell homogenate is shown in Table 1.

Enzymes of the lysosomes and the pericellular membrane were mostly collected in the granule fraction, whereas lacticodehydrogenase was largely soluble. A significant proportion of the latter enzyme was found, however, in the nuclear and the granule fraction, suggesting insufficient homogenization, reabsorption, or both. About two-thirds of [^{14}C]ABP was collected in the granule fraction, and one-third was collected in the final supernatant.

The distribution patterns of [^{14}C]ABP and marker enzymes in cytoplasmic extracts after isopycnic centrifugation in sucrose or Percoll gradients are shown in Fig. 3A and 4A. In both types of gradients part of the [^{14}C]ABP pattern overlapped those of the lysosomal marker enzymes *N*-acetyl-β-hexosaminidase and α-galactosidase. It largely dissociates from those of alkaline phosphodiesterase (pericellular membrane) and cytochrome oxidase (mitochondria). In sucrose gradients a large proportion of [^{14}C]ABP, however, was found in the first fractions of the gradients corresponding to the volume of the sample deposited at the top of the gradient. In separate experiments free [^{14}C]ABP was placed at the top of a sucrose gradient, and centrifugation was carried out as described above for the fractionation of the cell homogenates. This added [^{14}C]ABP was almost entirely soluble; i.e., it was collected in the first three fractions of the sucrose gradient (Fig. 3A). Thus, we may assume that [^{14}C]ABP accumulated by the cells is partly associated with lysosomes. Following an approach previously used by our laboratory to assign constituents that display a bimodal distribution to two distinct subcellular entities (10, 21, 37), we write the following equation for each fraction: $100c = ax + by$, where a and b are the fraction contents in lysosomal enzymes and added [^{14}C]ABP (Fig. 3A and B), respectively; c is the fraction content in [^{14}C]ABP accumulated by cells (Fig. 3A and B); and x and y give the percentages of [^{14}C]ABP accumulated by cells incubated with the drug which can be assigned to the lysosomes and to the soluble fraction, respectively. A similar approach was used for Percoll gradients, for which the distribution of the soluble [^{14}C]ABP was determined by mixing the free drug throughout the gradient before centrifugation (Fig. 4A). The best-fitting values of x and y obtained by a least-squares method (5), using the data of all fractions for the sucrose and the Percoll gradient, respectively, are shown in Table 2. From 49 to 62% of ABP could be assigned to lysosomes. To check for the consistency of our observations, these experiments were repeated with a different batch of [^{14}C]ABP. Results were similar to those presented in Fig. 3A and 4A (data not shown).

Figures 3B and 4B show the distribution of [^{14}C]penicillin G found in homogenates of cells that were incubated with this antibiotic and fractionated through sucrose or Percoll gradients. [^{14}C]penicillin G was found to be entirely soluble, and no difference was noticed between these patterns and those of added [^{14}C]penicillin G (Fig. 3B and 4B). Thus, the pattern of [^{14}C]penicillin G was completely dissociated from those of the lysosomal hydrolases, cytochrome oxidase, and alkaline phosphodiesterase (data not shown). Accordingly, almost all [^{14}C]penicillin G could be assigned to the soluble fraction (Table 2).

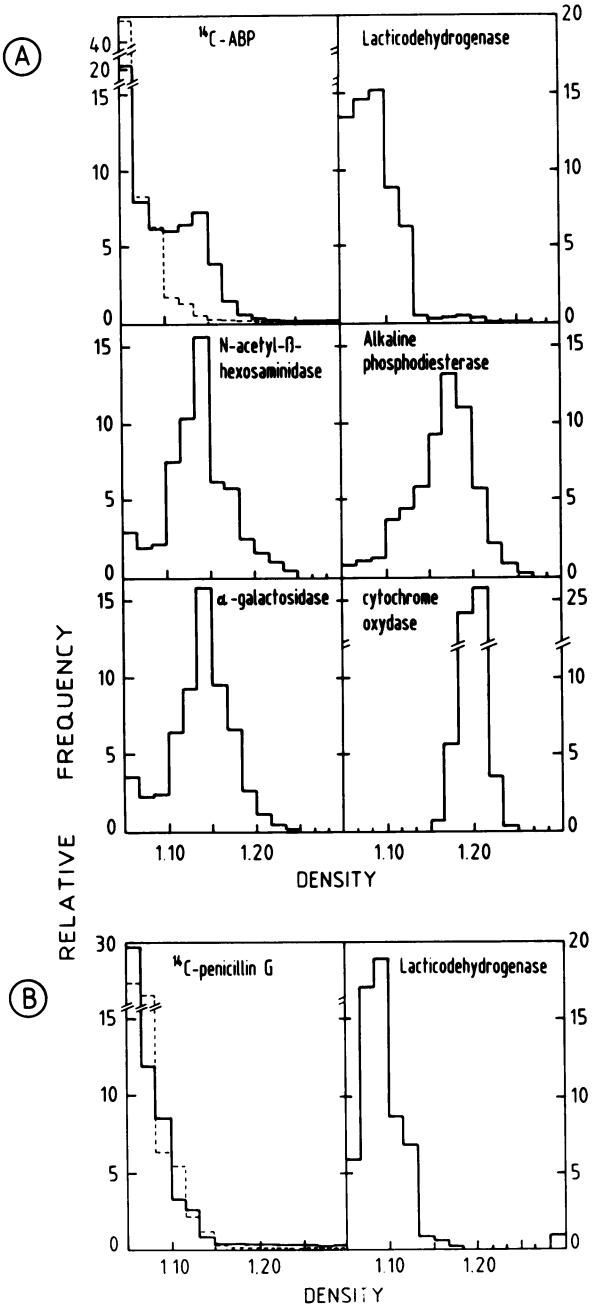

FIG. 3. Density distribution patterns of [^{14}C]ABP (A), [^{14}C] penicillin G (B), and marker enzymes after fractionation of cytoplasmic extracts by isopycnic centrifugation in linear sucrose gradients. For [^{14}C]ABP (A), and [^{14}C]penicillin G (B), the solid line refers to the distribution of the drug found in homogenates of cells incubated with the corresponding drug (3 mM, 1 h, 37°C). The dotted line refers to the distribution of free drug placed on the top of the gradient before centrifugation. Results are presented as normalized density distribution histograms (see reference 3 for a complete description of the mathematical procedures and advantages of such a mode of representation). Briefly, the abcissa is the density scale of the gradient divided into 15 sections of equal density increments. The ordinate shows the relative frequency of each constituent in each fraction (i.e., $Q_i/(\Delta\rho \times \sum_i^n Q_i)$, where Q_i is the activity or amount found in the fraction i, $\sum_i^n Q_i$ is the sum of activities or amount found in all fractions, and $\Delta\rho$ is the constant density increment). The total area of each histogram is thus equal to 1. Note that the absolute amount of [^{14}C]ABP found in the cell homogenates was approximately fivefold larger than that of [^{14}C]penicillin G (Fig. 2A).

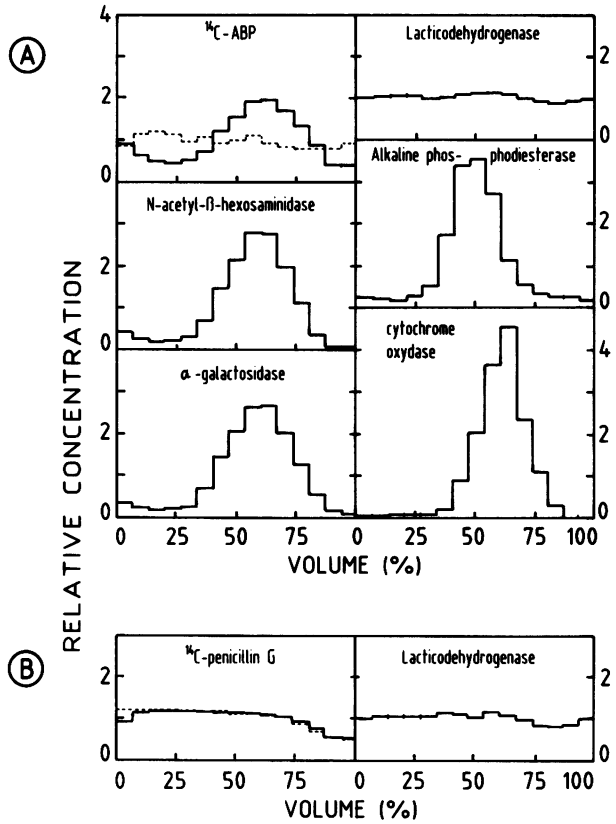

FIG. 4. Volume distribution patterns of [^{14}C]ABP (A), [^{14}C]penicillin G (B), and enzymes after fractionation of cytoplasmic extracts by isopycnic centrifugation in isoosmotic Percoll gradients. For [^{14}C]ABP (A) and [^{14}C]penicillin G (B), the solid line refers to the distribution of the drug found in homogenates of cells incubated with the corresponding drug (3 mM, 1 h, 37°C). The dotted line refers to free drug mixed throughout the tube content before centrifugation. Results are presented as normalized volume distribution histograms, where the abcissa is the fractional cumulative volume (divided into 15 sections of equal value), and the ordinate is the relative concentration [i.e., $Q_i/(\Delta V \times \sum_i^n Q_i)$, where Q_i is the activity or amount found in fraction i, $\sum_i^n Q_i$ is the sum of activities or amount found in all fractions, and ΔV is the constant fractional volume]. The total area of each histogram is thus equal to 1. Note that the absolute amount of [^{14}C]ABP found in the cell homogenate was approximately fivefold larger than that of [^{14}C]penicillin G (Fig. 2A).

TABLE 2. Assignment of [^{14}C]ABP and [^{14}C]penicillin G to lysosomes and soluble fraction after fractionation of homogenates by isopycnic centrifugation

Gradient and drug	Percentage of drug assigned to[a]:		
	Lysosomes	Soluble fraction	Unassigned
Sucrose			
[^{14}C]ABP	48	46	6
[^{14}C]penicillin G	0	98	2
Percoll			
[^{14}C]ABP	60	38	2
[^{14}C]penicillin G	9	88	3

[a] Assignment of [^{14}C]ABP or [^{14}C]penicillin G present in homogenates of cells incubated with the drugs was made with respect to the lysosomes and the soluble fraction using the distribution of *N*-acetyl-β-hexosaminidase and α-galactosidase, on the one hand, and added [^{14}C]ABP or [^{14}C]benzylpenicillin, respectively, on the other hand. For [^{14}C]ABP the distribution patterns used are those obtained from the experiments shown in Fig. 3A and 4A. For [^{14}C]benzylpenicillin the distribution patterns are those obtained from the experiments shown in Fig. 3B and 4B (patterns of the lysosomal enzymes are not illustrated).

DISCUSSION

Results of the experiments reported here indicate that conversion of penicillin G, an acidic antibiotic, into a basic derivative markedly enhances its intracellular accumulation and results in its partial localization in the lysosomes. The ^{1}H NMR and IR spectra of our product and the mode of synthesis ensure that the expected derivative, namely, ABP, was obtained. The radioactive material accumulated by the cells had the same radiopurity as the product that was synthesized, ruling out preferential accumulation of an impurity or retention of a degradation product. Localization of sedimentable ABP in lysosomes (50 to 60% of the total amount of drug accumulated by the cells [Table 1]) seems most likely because the drug distribution pattern largely followed that of marker enzymes of these organelles and was dissociated from that of the other main sedimentable components of the cells. This behavior was observed when lysosomes were separated on the basis of their wet (isoosmotic Percoll) or dried (hypertonic sucrose gradients) buoyant density (see reference 3 for a discussion). In this respect the intracellular fate of ABP resembles that of several other drugs that have a weak basic character but that are otherwise structurally unrelated, such as chloroquine, daunorubicin, amantidine, propranolol, or erythromycin. These drugs also accumulate in cells, and tissue fractionation or morphological studies have demonstrated their accumulation, at least partially, in the lysosomes (28, 31, 47; M. B. Carlier, A. Zenebergh, and P. M. Tulkens, Program Abstr. 26th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 929, 1986). Assuming that lysosomes represent approximately 2.5% of the cell volume of J774 macrophages as in peritoneal macrophages (41), it can be calculated that

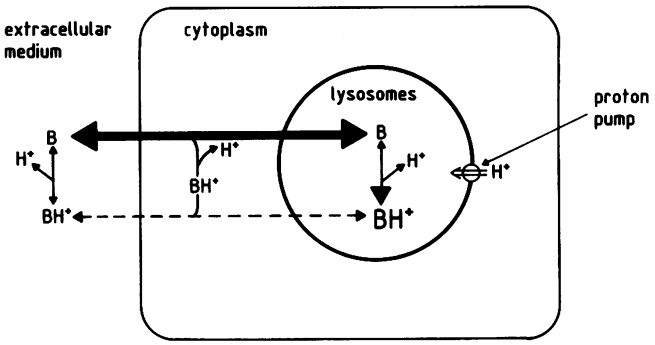

FIG. 5. Proposed mechanism of accumulation of ABP in cells and lysosomes. Biological membranes are more permeable to the unprotonated form of weak, organic bases (B) than to their protonated form (BH^+). The cytoplasmic pH is usually 0.5, and the lysosomal pH is 2, which is lower than that of the extracellular milieu (30, 34, 46). Lysosomes contain an ATP-driven H^+ pump (29). Thus, weak organic bases tend to become accumulated in lysosomes, and to some extent in cytoplasm, due to the pH gradient. Conversely, weak organic acids, which are more lipid soluble under their protonated form (—COOH) than under their unprotonated form ($—COO^-$) tend to be excluded from acid, membrane-bound compartments (for a general discussion, see references 9 and 27).

the ABP associated with these organelles is concentrated about 100-fold therein. In contrast to [^{14}C]ABP, [^{14}C]benzylpenicillin does not accumulate in J774 macrophages, as has been reported for other cells (12, 19, 22, 32). We have also shown here that intracellular [^{14}C]penicillin G is readily soluble in cell homogenates, which suggests that the antibiotic is free in the cytosol or that it quickly dissociates from a possible storage site.

It is well known that the ionized forms of weak organic acids and bases diffuse much more slowly through biological membranes than their unionized forms. Accordingly, weak bases tend to become concentrated and weak acids become excluded from membrane-bound, acidic compartments (27, 46). Lysosomes are the most acidic compartment in cells, with a pH of about 5 (30, 34, 46), and therefore are expected to concentrate ABP, according to the model depicted in Fig. 5. Because ionized ABP cannot easily leave lysosomes, the concentration ratio of total ABP across the lysosomal membrane could be as high as the H^+ ratio (9), i.e., at least 100-fold. Conversely, the acid character of penicillin G would prevent its accumulation in the lysosomes, although the drug may diffuse through membranes under its unionized form. In this respect it must be pointed out that penicillin G and ABP do not markedly differ in their hydrophobic/hydrophilic ratio, as judged from their chromatographic behavior. The model also explains the rapid release of ABP out of the lysosomes and the cells once the latter are transferred to drug-free medium, because the drug is able to diffuse out under its unionized form. Thus, the rate of entry should be similar to the rate of efflux, which is indeed roughly the case (Fig. 1).

Finally, the model (Fig. 5) also shows that retention of ABP in lysosomes is likely to be energy dependent, because it relies on the capacity of lysosomes to maintain an acid pH through an ATP-driven H^+ pump (29).

The presence of ABP in the soluble fraction may result from leakage out of the lysosomes during the homogenization and fractionation procedures. This interpretation is supported by the fact that more ABP is associated with lysosomes after the Percoll gradient than after the sucrose gradient. Particles indeed move faster in the low-viscosity medium of the Percoll gradient and therefore reach their equilibrium position more rapidly than in the sucrose gradient. The presence of ABP in the soluble fraction could also reflect true partial localization in the cytosol. Yet the maximal concentration of cytosolic ABP would not exceed twofold the extracellular concentration, which is still compatible with the model presented in Fig. 5, assuming a cytoplasmic pH of approximately 0.3 U lower than the extracellular pH (see reference 9 for a further discussion).

As expected (14) the substitution of the free carboxyl group of penicillin G was unfortunately associated with a loss of antimicrobial activity. Thus, exploitation of our observation for the design of β-lactams with enhanced intracellular accumulation would require that derivatives be obtained in which this carboxyl group can be unmasked intracellularly. The amide linkage realized in this study was not hydrolyzed by lysosomal amidases. Substitution of the carboxyl group by amino acids is also unsatisfactory because lysosomal carboxy peptidases do not split peptidic bounds in which the carbon atom adjacent to the carbonyl is of the D-configuration (35), as would be the case for penicillin-amino acid derivatives. Conversely, most ester linkages are quickly hydrolyzed extracellularly by unspecific esterases. In spite of these difficulties, the demonstration that a basic derivative of penicillin G has the potential of accumulating in cells and, more specifically, in the lysosomes is of interest, because these organelles play a key role in the intracellular handling of ingested microorganisms (for reviews, see references 17 and 39). Although intracellular accumulation may not be sufficient for an antibiotic to effectively inhibit the growth of or to kill intracellular organisms (15 and 36), the present data may help in the design of appropriate prodrugs of β-lactam antibiotics in this direction.

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