BENZYLPENICILLIN MIGRATES IRREVERSIBLY INTO HUMAN ERYTHROCYTES*

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Benzylpenicillin, 5.2% ^{14}C-enriched, was incubated with human whole blood. The erythrocyte component was washed and exhaustively dialyzed and found to contain 6.3% of the original radioactivity. None of the penicillin was found to bind to the cell membrane. Dialysis of the cell contents indicated that none of the penicillin binds irreversibly to the proteins within the cell. Infrared examination of the contents of the cell leads to the conclusion that benzylpenicillin (a monovalent anion) enters the erythrocyte and is hydrolyzed to benzylpenicilloic acid (a divalent anion). This structural change prevents the antibiotic from migrating out of the cell.

WATSON[1], measuring microbiological activity, found that human erythrocytes take up benzylpenicillin. This work also suggested that benzylpenicillin was not covalently bound to the erythrocyte membrane but was instead associated with the intracellular contents. More recent work[2,3] has shown both uptake and release of antibiotics by human erythrocytes. The present study describes the migration of benzylpenicillin into human erythrocytes, its hydrolysis to benzylpenicilloic acid and the subsequent entrapment of this hydrolysis product in the erythrocytes.

Materials and Methods

Four milliliter samples of whole normal human blood obtained by venipuncture *via* a Vacutainer system (Becton-Dickinson) containing EDTA were incubated with 2.0 ml isomolar saline-sodium phosphate buffer (pH 7.0) containing 4 μCi/ml of the potassium salt of benzylpenicillin (1.0 mg benzylpenicillin/ml; 0.65 mg benzylpenicillin[^{14}C] (Amersham-Searle) containing 50 μCi of activity was combined with 11.85 mg benzylpenicillin[^{12}C] (Eli Lilly Laboratories); the mixture was dissolved in 12.5 ml buffered saline solution) in a shaking water bath for 9 hours at 37°C. The whole blood was centrifuged at 1,300 *g* for 5 minutes and the separated erythrocytes were washed with buffered saline five times. They were then transferred into dialysis casings (molecular weight cutoff—3,500) and exhaustively dialyzed against 50 ml saline solution at 4°C. Suitable aliquots were withdrawn from the incubation mixture, supernatant solution, washes, pooled dialysates of erythrocytes, and erythrocytes for radioactive analysis in a Beckman Model LS-100 liquid scintillation spectrometer. Hemolysis of the erythrocytes was accomplished with both distilled water and Triton X-100 aqueous solution[4]. Following separation of the stroma, the cell contents were exhaustively dialyzed against 0.0085% NaCl solution. Suitable aliquots were withdrawn from the stroma, dialyzed intracellular material, and pooled dialysates of the intracellular material for liquid scintillation counting. These pooled dialysates were lyophilized and the infrared spectrum of an aliquot of the resulting residue in the form of a micro KBr pellet 1.5 mm in diameter was recorded with a Beckman 4260 spectrophotometer with the pellet placed at the focus (Fig. 1). The infrared spectum of the pooled dialysates of the erythrocyte washes was also recorded in a similar manner (Fig. 1). This technique is capable of

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distinguishing between benzylpenicillin and benzylpenicilloic acid at the 100 nanogram level[5].

Results and Discussion

Several studies[2,3,6,7,8] of the interaction between the components of human blood and antibiotics have been reported. KUNIN *et al.*[6] have demonstrated binding of antibiotics to serum proteins. NISHIDA *et al.*[7] studied the uptake by human erythrocytes of six penicillin analogues and found that the uptake of these drugs by erythrocytes was proportional to the degree of binding of that drug to serum proteins. BRATLID[8] has reported that the binding of bilirubin to erythrocytes is inhibited in the presence of albumin but is increased when the bilirubin is displaced from the protein by highly bound antibiotics. KORNGUTH and KUNIN[2] have described both the uptake and release of antibiotics from the cells. They have also presented evidence[3] that these drugs are bound to hemoglobin and carbonic anhydrase molecules within the erythrocytes. This study was an attempt to elucidate the nature of the association of benzylpenicillin with intraerythrocytic proteins such as hemoglobin and carbonic anhydrase.

Table 1 lists the distribution of ^{14}C-enriched benzylpenicillin in the supernatant solution (76.6 %) and in the washed and exhaustively dialyzed erythrocyte fraction (6.3 %). Further work by TALBERT[9] involving exhaustive dialysis of the supernatant solution (plasma fraction in saline), showed that 7.1 % of the initial antibiotic was irreversibly bound to plasma protein(s).

Table 1. Uptake of ^{14}C-enriched (5.2 %) benzylpenicillin by whole blood and relative concentration of drug detected in each fraction of the erythrocyte preparation.

Preparation	% of initial amount (0.89 μmole/ml) in each fraction
Incubation mixture*	100
Supernatant solution	76.6
Wash 1	11.3
Wash 2	4.6
Wash 3	0.8
Wash 4	0.3
Wash 5	0.2
Pooled dialysates after exhaustive dialysis of erythrocytes	0.1
Erythrocytes**	6.3

* Benzylpenicillin[^{14}C] - 8 μCi - activity - 6,332,456 cpm (counts per min)

** Activity - 399,250 cpm

Lysis of the erythrocytes and separation of the stroma from the intracellular material showed that none of the penicillin was bound to the stroma (Table 2). In addition, virtually all the ^{14}C-enriched benzylpenicillin was removed from the intracellular material by dialysis (Table 2). This indicates that irreversible binding to either the cell membrane or protein contents of the erythrocytes is negligible. Thus, benzylpenicillin appears to migrate into the erythrocyte, is trapped, and is released only after hemolysis of the cell. Since there appears to be no irreversible binding of benzylpenicillin to erythrocyte cell components, what prevents this antibiotic from migrating out of the cell? A possible explanation could be that benzylpenicillin undergoes a structural change after migration into the erythrocyte.

Table 2. Association of ^{14}C-enriched benzylpenicillin with erythrocyte components

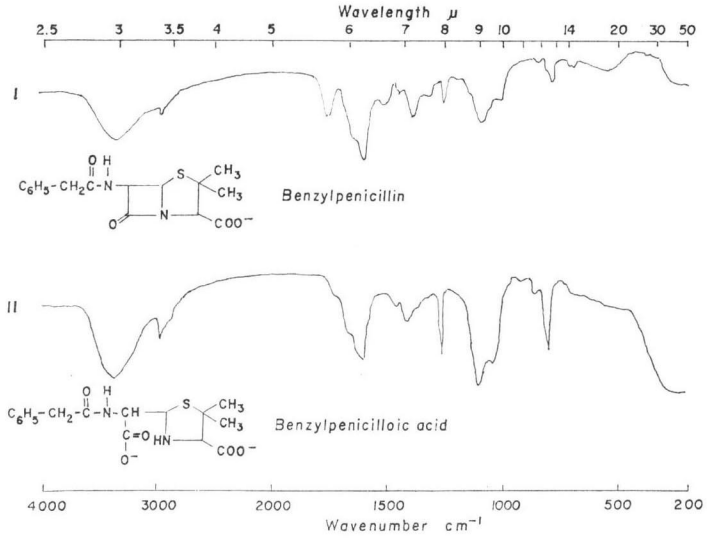
Preparation	% of initial amount (0.89 μmole/ml) in each fraction
Intracellular material	<0.1
Stroma	<0.1
Pooled dialysates of intracellular material*	5.9

* Activity - 375,418 cpm

Lyophilization of the exhaustively dialyzed erythrocyte washes and the pooled dialysates of the intracellular material followed by IR analysis of the resulting residues (Fig. 1) allowed the determination

Fig. 1. Spectra of residues isolated from pooled dialysates of erythrocyte washes[1] and of erythrocyte intracellular material[2]

[1] KBr pellet −22.48 μg sample +125.30 μg KBr
[2] KBr pellet −25.57 μg sample +125.83 μg KBr



of the molecular form of the penicillin found outside and inside the erythrocytes, respectively. The spectrum of the residue isolated from the pooled dialysates of erythrocyte washes was identical to that of benzylpenicillin. The spectrum of the residue isolated from the pooled dialysates of the erythrocyte intracellular material is identical to that of benzylpenicilloic acid. Note the β-lactam absorption band[10] at 1763 cm^{-1} in spectrum I compared to the absence of β-lactam absorption at 1763 cm^{-1} in spectrum II. The IR examination of the contents of the erythrocytes leads to the conclusion that benzylpenicillin (a monovalent anion) enters the erythrocyte and is hydrolyzed to benzylpenicilloic acid (a divalent anion) and that this structural change prevents the hydrolyzed benzylpenicillin from migrating out of the cell.

Studies are in progress to identify the intraerythrocytic protein(s) responsible for the hydrolysis of benzylpenicillin and to determine the degree to which erythrocyte membrane permeability is dependent on the charge distribution on migrating molecules.

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