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Electron Transfer Mechanism for β-Lactam Antibiotic Action via Side-Chain Imine[1]

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Evidence has been previously presented for an electron transfer (ET) component associated with the mechanism of action of β-lactam antibiotics, in addition to enzyme inactivation. For the fused-ring types, apparently the ET entity is a conjugated iminium group formed as a result of ring-opening. We now report on the feasibility of ET associated with several monocyclic and cephalosporin β-lactams that contain conjugated imine in the acyl side chain. The side chain assumes increased importance for the monobactams and nocardicins since ring scission does not generate iminium. The monocyclic agents generally reduced in the favorable range of −0.5 to −0.6 V at pH 4.1. The cephalosporin drugs potentially contain two electroactive sites, iminiums from ring-opening and from generation in the side chain. Electroreduction involving the side-chain oxime occurred at about −0.4 to −0.7 V (pH 4.1). Model compounds are used to provide additional mechanistic insight. There are various sources of hydrogen ions needed for iminium formation at the active site. Comparisons are made between reduction potential and antibiotic activity. The mode of bactericidal action is discussed with focus on ET by iminium.

1. INTRODUCTION

Antibacterial activity can be manifested by a variety of routes. One of the most widespread and generally accepted is interference with DNA replication or synthesis (*1*). Cell membranes and enzymes have been designated as targets (*1–3*). Recently a broadly unifying proposal was advanced implicating electron transfer (ET) and oxidative stress (*2*). In relation to the important β-lactam category, we have provided evidence to support the contention that ET plays an important role in the antibiotic activity (*3*). Thus, on binding to cell wall enzyme, β-lactams form precursors of conjugated inimium species that apparently possess favorable reduction potentials (−0.18 to −0.37 V) based on studies with model compounds **1** and **2.** A good example is cephalosporin iminium **3** derived from attack by enzyme (EOH) at the binding site. In the case of penicillin, **4** would represent an analogous derived structure. ET by iminium could conceivably interfere with normal electro-

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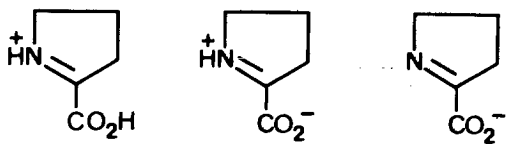
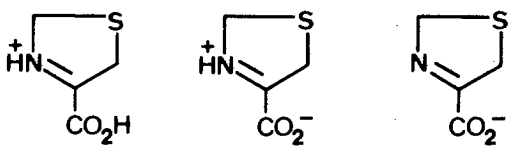


a b c

SCHEMES **1a–1c**



a b c

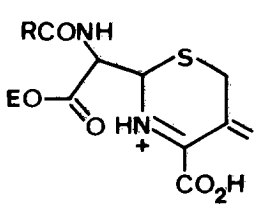
SCHEMES **2a–2c**

physiological processes, resulting in bacterial cell death. From this viewpoint, site binding not only inactivates cell wall enzyme but also provides a destructive ET entity at a sensitive locale.

Additional support for this thesis is furnished by studies on cephalosporin nephrotoxicity (*4–9*). Various types of activated oxygen species are generated, giving rise to the usual consequences of oxidative stress. The adverse effects can be alleviated by antioxidants, catalase, and superoxide dismutase. Cephalosporin iminium **3** is a likely agent responsible for the catalytic redox cycling (*3*).

The drugs that readily fit into this unifying theme contain a ring fused to the β-lactam. If the assumption is valid that all β-lactams display part of their activity via ET, then the monocyclic types present a challenge since they cannot generate iminium on ring opening. In a prior report (*3*), we pointed out that this class has functionalities in the side chains that can conceivably act as precursors of ET structures. Based on this general approach, essentially all of the β-lactams might then be accommodated within the dual mechanistic concept of enzymatic binding and formation of an electroactive site.

Our objective was to ascertain at various pH values the electrochemical characteristics of monolactams incorporating the imino functionality commonly found in the side chain. In addition, studies were made of fused ring β-lactams that possess side chain imine. The reduction potential was determined for imine-containing



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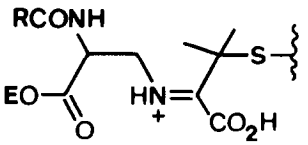
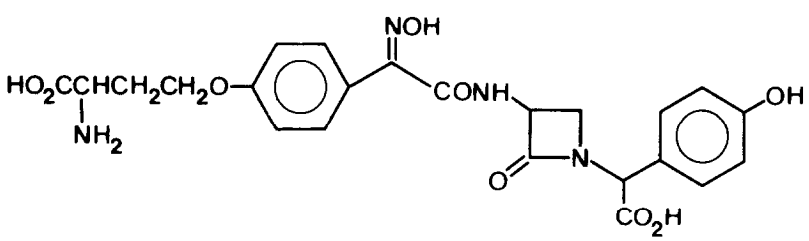


SCHEME 4

side chain devoid of the lactam nucleus. In some cases, comparison is made with reference antibiotics lacking the oximino group. The physiological roles of the imine moiety are discussed with focus on the ET mechanism of bactericidal action involving iminium.

2. EXPERIMENTAL PROCEDURES

Samples were obtained from the following sources: nocardicin A, **5,** Fujisawa Pharmaceutical Co.; carumonam, **6,** Hoffmann–La Roche; aztreonam, **7,** Squibb Institute for Medical Research; cefuroxime, **8,** Glaxo Research Laboratories; cefotaxime, **9,** Roussel Laboratories; cephalothin, **10,** penicillin G, **11,** and compounds **13b** and **13c,** Aldrich or Sigma Chemical Co.; clavulanic acid, **12,** Beecham Laboratories. Compound **13a** was prepared by a literature method (*10*), mp 197°C (dec.), lit. mp 199°C (dec.); the NMR spectra were essentially identical. Oximes of benzophenone, mp 142–143°C, lit. mp 144°C (*11*), and of acetophenone, mp 58–59°C, lit. mp 59°C (*11*), were prepared by standard reactions.



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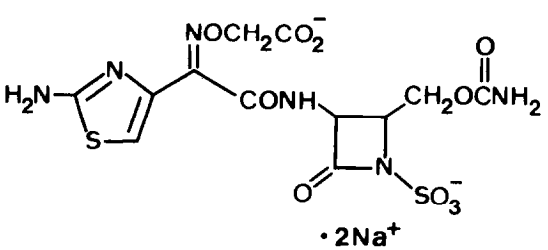

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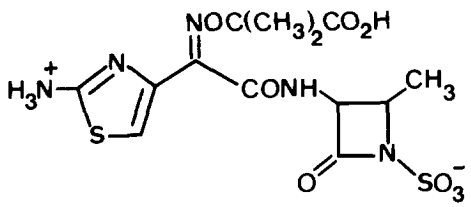

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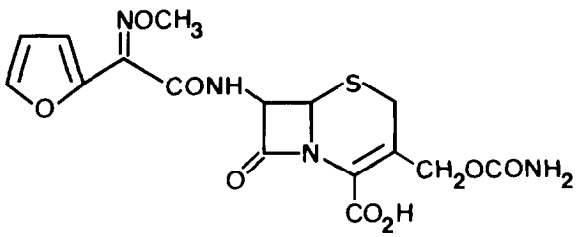

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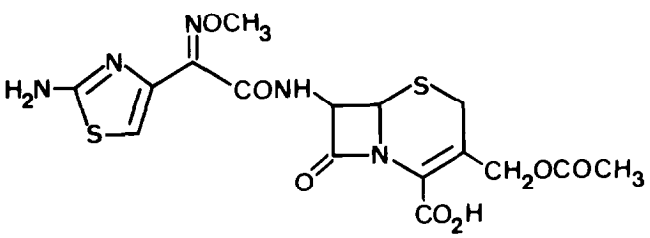

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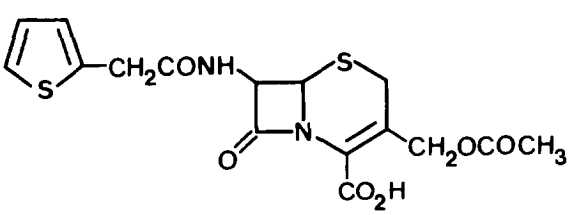

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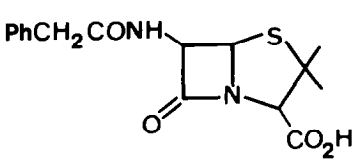

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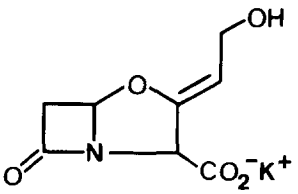

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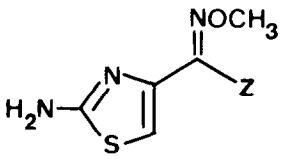


Z=
a) $CONHC_2H_5$
b) $CO_2C_2H_5$
c) CO_2H

SCHEMES **13a–13c**

Buffer solutions, prepared according to prescribed methods (*12*), constituted the carrier electrolyte in the electrochemical measurements (KOH was substituted for NaOH); pH 3.6 to 4.8, potassium biphthalate; pH 6.2 and 7.6, KH_2PO_4. A Sargent Model LS pH meter was used to monitor the acidity. Solutions for **9** and benzophenone oxime contained 10% dimethylformamide (DMF, Aldrich); those for **13** were 5% DMF, and acetophenone oxime was studied in 5% ethanol (U.S. Industrial Chemicals Co.) in the designated buffer. Cyclic voltammetric measurements were performed as previously described (*3*) in the absence of artificial light. The reported values are the average of two or more separate determinations and are reported versus the normal hydrogen electrode (NHE).

3. RESULTS AND DISCUSSION

3.1. Electrochemistry

A. Monocyclic β-lactams. In this section, attention will be centered on two major types of monocyclic agents, namely, the nocardicins and monobactams. Data for these antibiotics are summarized in Table 1 and Figs. 2 and 3. At pH 4.1, diffusion-controlled currents, with current function (CF: $A/(V/s)^{1/2}M$) of 0.37, were observed in the electroreduction of nocardicin A, **5**, with E_p at −0.47 V, and $E_{pp/2}$ ($E_p - E_{p/2}$) of 55 mV at a sweep rate of 0.1 V/s (Fig. 1). However, converse reoxidation currents were not observed at this sweep rate. The peak potential

TABLE 1

Cyclic Voltammetry of Monocyclic β-Lactams[a]

Compound	$-E_p$[b] (V)	$E_{pp/2}$ (mV)
5	0.47	55
6	0.61[c]	85
7[d]	0.64[c]	90

[a] 100 mV/s, HMDE versus NHE, pH 4.1, 0.5 m*M*.
[b] Irreversible.
[c] ±0.01 V.
[d] At pH 6.2, no reduction before −1.0 V.

shifted 30 mV (Fig. 2) per decade change in sweep rate, characteristic of an electron transfer step followed by fast follow-up chemistry (EC mechanism). Potential sweeps of less than 100 mV/s were accompanied by slight adsorption for the anodic scan. More acidic conditions produced positive shifts in E_p (Fig. 3), whereas increasing the pH from 4.1 resulted in broadening of the reduction peaks; $E_{pp/2}$: 70 mV at pH 4.8, 110 mV at 6.2, and 95 mV at 7.6, accompanied by a decrease in cathodic potentials (Fig. 3) and currents. The linear plot shown in Fig. 3 symbolizes a similar reduction process at all pH values studied yielding

$$E_p(V) = -0.115-0.087 \text{ pH (pH 3.6–7.6)}. \quad [1]$$

The monobactam **6,** carumonam, differs structurally from lactam **5** in several ways. First, the oximino group is O-alkylated by an acetate residue and is conjugated with an aminothiazole group, rather than a phenolic ether entity. Electrochemically, carumonam behaved similarly to **5** in certain respects. A −0.030-V change in potential versus sweep rate was realized at pH 4.1 (Fig. 2); the voltammograms increased in width ($E_{pp/2}$ of 185 mV at pH 4.7) and decreased in height as

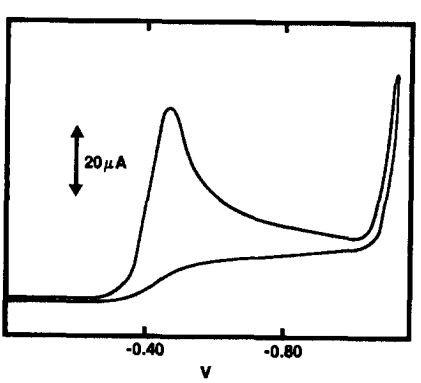


FIG. 1. Cyclic voltammogram of **5** at pH 4.1, 0.1 V/s, HMDE versus NHE.

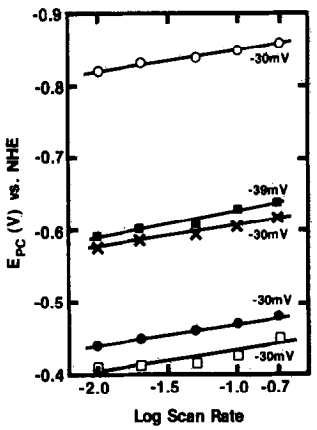


FIG. 2. Plot of E_{pc} versus scan rate for **5** (●); **6** (×); **7** (■); **8** (□); acetophenone oxime (○) at pH 4.1.

the pH increased. However, there were significant differences, e.g., reduction potentials were slightly more negative (Table 1 and Fig. 3). At pH 4.1 the antibiotic reduced at −0.61 V with $E_{pp/2}$ of 85 mV. The reaction was diffusion-limited as evidenced by the independence of CF (0.39) and the scan rate. As the hydrogen ion concentration decreased a marked deviation from linearity was observed for E_p (Fig. 3), suggesting a change in the reduction process from that at lower pH. The compound was not examined at pH values greater than 4.8.

Aztreonam **7** gave results comparable to **6** (Table 1 and Fig. 3) with CF of 0.39. The peak potential was −0.64 V with a difference of 90 mV between E_p and $E_{p/2}$ at pH 4.1 and 100-mV/s sweep rate. A slope of −39 mV was obtained from the plot of E_p versus scan rate (Fig. 2), in line with a reduction process analogous to that of **5** and **6.** A larger deviation from linearity was observed for this drug compared to **6** (Fig. 3); a different reductive transformation from that in more acidic media is again indicated. Values of pH greater than 4.8, e.g., 6.2, gave no distinct wave

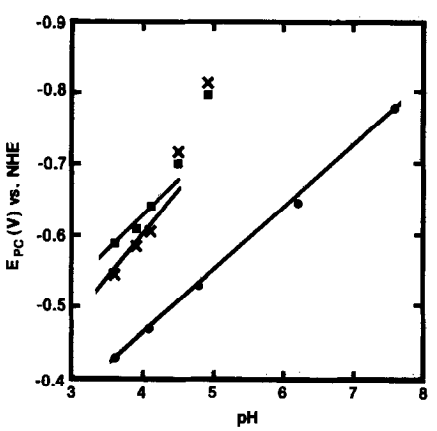


FIG. 3. Plot of E_{pc} versus pH for compounds **5** through **7** at 0.1 V/s; see Fig. 2 for symbol designations.

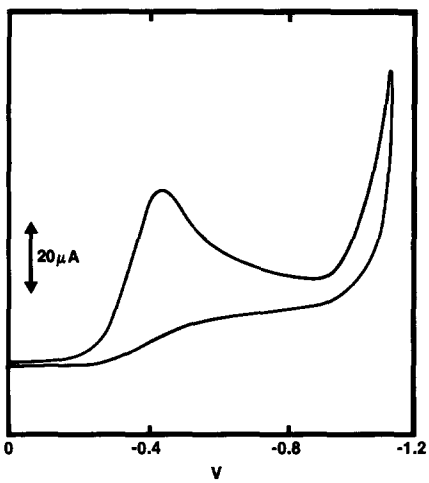


FIG. 4. Cyclic voltammogram of **8**, pH 4.1, 100 mV/s, HMDE versus NHE.

before the buffer discharge, possibly pointing to the absence of iminium formation.

B. Fused-ring β-lactams. Although some of the agents in this class contain an imine moiety in the side chain, similar to the monocyclic β-lactams, the nucleus is of the fused-ring type. There are differences in the side chain within the cephalosporin category; e.g., cefuroxime **8** incorporates a furan entity, while cefotaxime **9** has an aminothiazole group. Both aromatic nuclei are in conjugation with the oxime.

Figure 4 exemplifies the reduction observed with **8.** At pH 4.1 E_p was −0.44 V (CF = 0.32) with a half peak width of 85 mV (Table 2). This value became larger as the pH increased, e.g., 130 mV at pH 4.8. The same reduction process was

TABLE 2

Cyclic Voltammetry of Fused-Ring β-Lactams and **13**[a]

Compound	$-E_p$ (V)	$E_{pp/2}$ (mV)
8	0.44	85
9	0.71	160
10	NR[b]	—
11	NR	—
12	NR	—
13a	0.77	145
13b	0.76	240
13c	1.02	105

[a] 100 mV/s, substrate 0.5 mM, HMDE versus NHE, pH 4.1, irreversible.

[b] NR denotes no reduction before −1.0 V.

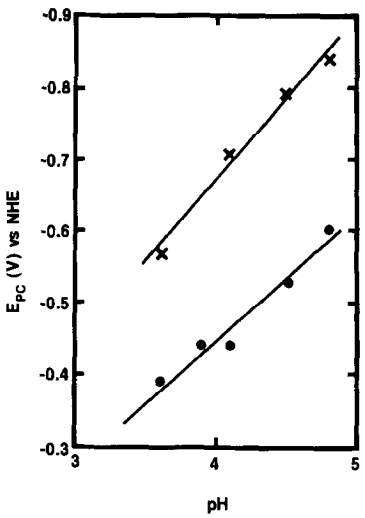


FIG. 5. Plot of E_{pc} versus pH for **8** (●); **9** (×); 0.1 V/s.

indicated by the linear plot realized for E_p versus pH (Fig. 5). Equation [2] was derived for the indicated pH range. A slope of −30 mV was observed for the plot of

$$E_p(V) = 0.242–0.173 \text{ pH (pH 3.6–4.8)}. \quad [2]$$

E_p versus log scan rate (Fig. 2) is in line with the other oximino-containing compounds.

Cefotaxime reduced irreversibly at −0.71 V with $E_{pp/2}$ of 160 mV (Table 2) with diffusion-controlled currents (CF = 0.31); no reoxidation currents were observed at a scan rate of 100 mV/s. The E_p value became 20 mV more positive as the sweep rate increased from 10 to 200 mV/s, possibly due to a surface phenomenon. The equation for the peak reduction potential derived from Fig. 5 is shown

$$E_p(V) = -0.225 \text{ pH} + 0.230 \text{ (pH 3.6–4.8)}. \quad [3]$$

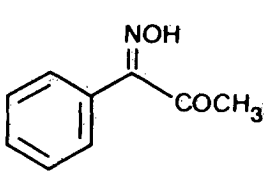
There have been several electroanalytical techniques developed for cephalosporins **8** and **9.** The studies, carried out in very acidic solutions, yielded values of −0.38 and −0.54 V (reference electrode not given, probably SCE) for cefuroxime and cefotaxime, respectively, at pH 2.0 (*13*), and −0.15 V for **8,** at pH 2.5 (*14*). The azomethine group is apparently reduced under these conditions (*13*). Our data are in general accord with the prior work.

Cephalothin **10,** devoid of side-chain oxime, was used as a control. Antibiotic **10** gave no reduction before −1.0 V (Table 2), similar to earlier studies (*14, 15*). This result verifies the conclusion that the reduction obtained for the cephalosporin types is due to the side-chain iminium species generated under acidic conditions. Several other bactericides in this category were examined electrochemically as controls. Penicillin G **11** and clavulanic acid **12** did not reduce before −1.0 V (Table 2). The findings demonstrate that prior to active-site fixation these antibiotics are unable to act as ET agents. After binding, i.e., generation of an iminium ET entity, the reduction potential is believed to be appreciably enhanced (*3*).

C. Electrochemical correlations. Compound **13a,** in which the β-lactam is absent, was scrutinized as a control for both the monocyclic and fused-ring antibiotics in order to gain further insight into the involvement of side-chain imine. It reduced in an irreversible manner, $E_{pp/2}$ of 0.145 V, at −0.77 V, pH 4.1 (Table 2), close to the value for **9.** Ester **13b** behaved similarly (−0.76 V), with $E_{pp/2}$ of 240 mV in pH 4.1 buffer (Table 2). The acid **13c** reduced at −1.02 V at pH 4.1 (Table 2), in an irreversible manner ($E_{pp/2}$ = 105 mV), more negative than the antibacterial samples with comparable structures. The magnitude may be rationalized as follows. At pH 4 it is reasonable to expect the carboxyl group to be in anionic form and the imine to be protonated. The zwitterionic nature would have an adverse effect on reduction, as a result of the overall neutral charge. Electrophilicity is an important factor in reduction (*16*). An analogous situation was observed for **1** and **2** (*3*). The zwitterion **1b** is reduced at values about 0.3 V more negative than the cationic species **1a.** A 300-mV increase for **13c** gives −0.7 V, similar to that observed for **9.** A series analogous to **13a–13c** has been investigated (4-substituted quinolines) (*17*).

Additional simple oximes were examined as model compounds. Oximes and their O-alkylated derivatives are readily reduced only in the protonated form (*18*); therefore, oximes in Tables 1 and 2 should reduce with difficulty in the absence of acid. Acetophenone oxime reduced irreversibly at −0.85 V at pH 4.1. A previous study furnished a value of about −0.83 V (*19*). A slope of −30 mV was realized from comparison of E_p versus scan rate (Fig. 2). Increasing the conjugation shifted the potential to −0.69 V for benzophenone oxime, cf. −0.62 V (*19*), closer to the values for the antibiotics in Tables 1 and 2. The increase in E_p is in line with previous investigations involving substitution of methyl by phenyl (*20*). As the sweep rate was decreased from 100 to 20 mV/s, two peaks developed, one at −0.61 and the other at −0.74 V, probably due to a two-step reduction (*18*). Further alteration in the oxime structure to give **14,** closer to the principal substances in this study, produced reduction at −0.54 V (pH 4) (*19*). This value is in general accord with the results in Tables 1 and 2. The enhanced potential reflects the favorable ability of carbonyl to stabilize radical and anion character in the intermediate from electroreduction. These simple examples clearly illustrate the influence of conjugation on electron uptake by iminium. There is also the possibility for additional stabilization of the resulting radical by ester, amide, or other oxygen- or sulfur-containing functionalities that lie in close proximity to iminium at the binding site, as shown in studies with the model compound **2** (*3*).

Next the plots of E_p versus scan rate and pH will be discussed. The comparable slopes for E_p versus the log of the scan rate at pH 4.1 (Fig. 2) indicate that a similar reduction mechanism is involved for these compounds. The overall nature of the



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slopes of pH versus E_p (Figs. 3 and 5) may be rationalized by the type of imino substitution present. The linear plots obtained at low pH for all compounds denote the same process at these acidities, namely, reduction of the protonated azomethine. The deviations found for compounds **6** and **7** at pH values greater than 4.1 suggest an appreciable chemical change. The difference is likely due to the nature of the oxime, i.e., whether or not carboxyl is present. At low pH values carboxyl will be in the acid form and the imine will be protonated. Thus, reduction is due to the iminium species. The acetate ion formed as the pH is increased has an influence on the potential in several ways. The group is in a stereochemically favorable position to compete intramolecularly with the azomethine group for the proton, thus reducing the positive charge associated with the imine. Also, the presence of carboxylate anion would result in no net overall charge, similar to **13c** (vide supra). Antibiotics **5, 8,** and **9** have no carboxyl group attached to the oxime to influence the reduction process; thus no deviation from linearity is observed.

Although the reactions in this study were all irreversible, behavior *in vivo* may be different for the drug immobilized at the active site. Also, the energetics may be more favorable. These aspects are treated in greater detail elsewhere (*21–23*).

Although there is some correlation between reduction potential (**6** and **7**) and antimicrobial activity, (*24–26*), analogous comparisons for the cephalosporin group (**8** and **9**) are less consistent (*27–30*). Prior reports demonstrate a relationship between biological activity and reduction potential for various agents (*2, 31–33*). There are numerous other factors that influence antibacterial activity. Biologically active substances known to affect ET, which display similar potentials (about −0.4 V) to the compounds in this study, include quinones (*34*), nitroheterocycles (*2*), triarylmethane dyes (iminium) (*2*), and metal complexes (*32, 33, 35*).

3.2. Oxime Protonation

Various sources of acidic hydrogen are available at the site containing the bound drug, which might serve to protonate basic imine functionalities that are initially present or generated during ring scission. One is from the carboxyl group of the antibiotic, and another is from enzyme serine after attack on the β-lactam. Some agents, e.g., **7**, contain carboxyl in the oxime portion, which could generate iminium intramolecularly. Evidence was presented for the existence of positive charges associated with protein near carboxyl of the bound drug (*36, 37*). It should be recognized that availability of protons at the binding locale may not correspond to medium acidity *in vitro*. Alternately, salt formation involving the oxime might occur prior to site binding.

It is reasonable to adopt the proposition that imine basicity in the living system may not be identical to that *in vitro*. The homogeneous solution used experimentally differs from the heterogeneous environment *in vivo*. Also, theoretical calculations reveal that competition of imine and ammonia for proton (iminium–ammonium equilibrium) is importantly influenced not only by inherent basicity but also by geometrical considerations as would pertain at the active site in a biological system (*38*). In an arrangement in which the lone pairs of the two bases point toward one another, the proton prefers the Schiff base. Coincidentally, this theoretical model corresponds closely to functionalities present in the site cavity after

binding of the β-lactam. In addition, donation of the proton from $-NH_3^+$ of lysine to the nitrogen of bound drug has been suggested (*37*).

Ligands adjacent to the oxime would be expected to enhance iminium stability as a result of intramolecular coordination involving hydrogen. The complexing groups consist of a heterocyclic nucleus, e.g., thiazole, and side-chain amide. The *E* or *Z* form of the oxime, which can differ in antibacterial activity (*39, 40*), would determine which ligand participates.

3.3. Alternative Routes to ET Entities

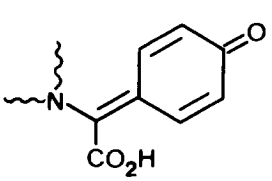
Various structural modifications in active analogues of nocardicin can provide imine precursors in the amide side chain (*41*). For example, pri-amine ($ArCHNH_2$ CONH–) might undergo dehydrogenation, or imine formation by condensation with carbonyl, followed by rearrangement to the conjugated form. Drug carbonyl (ArCOCONH–) can condense with pri-amine of amino acid or protein. In other cases, a methoxyl substituent on β-lactam of monobactam, e.g., sulfazecin (*41*), may lead to conjugated imine after ring cleavage, expulsion of methanol, and isomerization. Imine formation has been mentioned in connection with eliminations involving *N*-hydroxy β-lactams (*42*). The imine portion of the various agents could serve as part of a bidentate ligand for metal coordination.

Alternatively, phenols are metabolically converted to ET quinones (*43*). In the case of nocardicin, metabolism is reported to generate the quinonemethine **15** (*44a–46*).

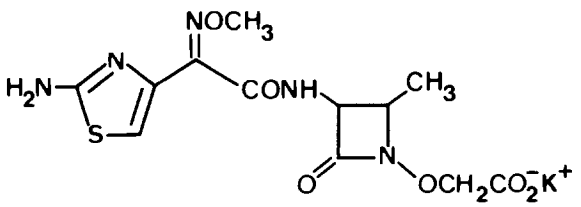
3.4. Role of the Imine Group

The literature contains discussions of several principal roles for the oximino functionality (*40*). Greater stability is provided toward most β-lactamases. Also, a wider spectrum of antibacterial activity and greater potency have been reported which will be the center of our attention.

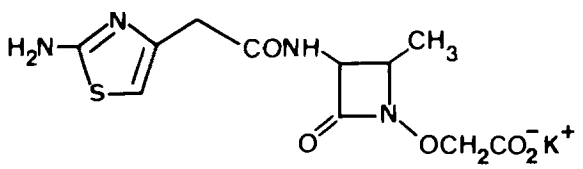
More powerful antibiotic activity was noted for a number of monocyclic β-lactams that incorporated the imino group, including monobactams (*41*) and oxamazins, **16** vs **17** (*47*). Various investigations revealed greater effectiveness bestowed by the oxime group in cephalosporins, particularly against gram-negative bacteria (*40, 48–50*). A similar situation pertained to the bicyclic pyrazolidinones, **18** vs **19** (*51*). Hence, a correlation exists between ease of electroreduction and bioactivity. It is relevant that desthiobenzylpenicillin (a monocyclic type) possesses no activity (*41*). Imine is not generated by ring scission, nor is an ET group present in the side chain. For the fused-ring category, the oxime structure provides an ET site in addition to iminium generated by ring-opening. This could



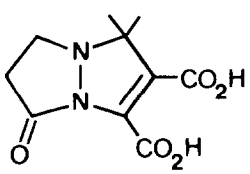
SCHEME 15



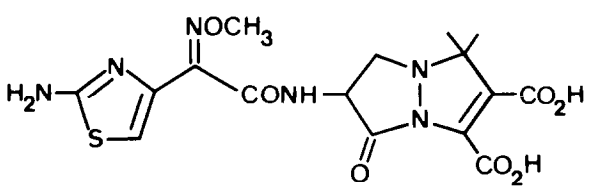
SCHEME 16



SCHEME 17



SCHEME 18



SCHEME 19

account for the properties of enhanced activity and a wider spectrum of activity observed with some of the side-chain imines.

In our initial study, involvement of ET in the mechanism of action was suggested (*3*). Supporting evidence is provided by the observation of a dramatic increase in the reduction potential of bacterial cell cultures on addition of penicillin (*52–54*). Apparently the bacterial medium undergoes oxidation during drug action, thus interfering with normal electrochemical processes and resulting in cell destruction (*52*). As a consequence of mounting evidence for this view, a closer inspection of the implications is now appropriate. The astute, detailed analysis by Tomasz (*55*) of the mode of action furnishes a good foundation for correlation of our novel concept with prior data. Although broadly accepted, the classical mechanistic model involving only enzyme inactivation does not suffice to rationalize the available evidence. Two sequential phases of antibiotic action can be distin-

guished. In the first step, a peptidoglycan enzyme, the binding protein, is inhibited, adversely affecting bacterial growth, followed by a secondary (irreversible) effect. The latter aspect, leading to lysis and lethality, is rarely discussed. There is apparently a premature triggering of murein hydrolase activity leading to scission of covalent bonds in the cell wall, with ensuing exposure of plasma membranes and subsequent rupture (lysis). Furthermore, drug interaction with enzyme protein does not automatically result in lysis and cell death (*44b*). Clearly, evidence points to the indirect nature of bacterial disintegration.

How might electrochemical events translate into drug action after site fixation? During both steps of the sequence, ET could produce adverse effects on vital cellular reactions, including enzyme action. Analogous situations may be cited in the case of radiosensitizers (*56*) and quinoxaline di-*N*-oxide, also an antibacterial agent (*21*). Penicillin inhibits amino acid deamination, but only when redox processes are involved (*57*). It should be noted that evidence has also been advanced for participation of ET in the action of other classes of antibiotics (*2, 52*). Appreciable data indicate that ET can take place over large distances *in vivo* (*23, 58*). Association of ET with enzyme binding may be more widespread than currently recognized (*34*).

3.5. Scope of the ET Theory

It is significant that this theory, usually involving oxy radicals, seems widely applicable to a variety of xenobiotics. In our laboratory, studies have been made of carcinogens (*59*). anticancer agents (*17, 32, 34, 60–62*), bactericides (*2, 3, 21, 63*), antimalarials (*35*), mesoionics (*22*), benzodiazepines (*64*), spermine (*23*), phencyclidine (PCP) (*23*), nicotine (*23*), amebicides (*33*), 1-methyl-4-phenyl-1,2,3, 6-tetrahydropyridine (MPTP) (*65*), antiprotozoan agents (*66*), and cocaine (*67*).

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