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A Novel Approach to β-Lactam Chemistry *in Vivo*: Electron Transfer and Oxy Radical Formation by Iminium[1]

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On binding to cell-wall enzyme, β-lactams form precursors of conjugated iminium species that apparently possess favorable reduction potentials based on studies with model compounds. The models used were iminium salts of Δ^1-pyrroline-2-carboxylic acid and Δ^3-thiazoline-4-carboxylic acid. Reduction potentials of −0.76 to −0.92 V increased to −0.18 to −0.37 V with decrease in pH. The potentials of the iminium species are similar to those of well-known electron transfer (ET) agents, such as quinones, nitroheterocycles, and metal complexes. Catalytic ET by these cations is discussed in relation to nephrotoxicity, antibiotic action, and cell culture redox potential. Reactions of penicillin at the binding site are addressed. We propose that the bactericidal effect involves various modes of action, including inactivation of cell-wall enzyme and electrochemical interference with normal electron transfer processes.

INTRODUCTION

There is increasing support for the view that electron transfer (ET) and oxidative stress play important roles in medicinal chemistry (*1, 2*). Four main types of ET agents are known: quinones, $ArNO_2$, iminium ions, and metal complexes (*3–17*). The iminium types have been the least investigated systematically. The active form functions catalytically by accepting electrons from a donor, e.g., protein or DNA, and then conveying them to an acceptor, such as oxygen or a cellular constituent. In our laboratory, the theory has been applied to carcinogens (3), anticancer agents (*4, 5, 15–17*), bactericides (*6, 7, 13*), antimalarials (*8*), mesoionics (9), benzodiazepines (*10*), spermine (*11*), phencyclidine (*11*), nicotine (*11*), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (*12*), and amebicides (*14*).

Using the hypothetical framework, we now address the β-lactam antibiotics. The conjecture has been advanced that the iminium ion participates importantly in the biological chemistry of this class (*3, 18, 19*). Almost three score years have elapsed since the appearance of Alexander Fleming's initial publication on penicil-

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lin (*20*). Subsequently, a vast amount of research has been done on β-lactams, including elegant mechanistic studies.

There is widespread acceptance in the life science community of the notion that the antibacterial action is satisfactorily rationalized by the sole event involving inactivation of cell-wall enzyme. Interestingly, questioning of this oversimplified view is found mainly in the writings of experts in the area (*21a,b, 22–25*). They point out that there are many unanswered questions, inconsistencies, and other serious weaknesses in that approach. The relationship of the various end results (loss of viability, lytic death, nonlytic death, tolerance, and morphological alterations) to binding remains obscure. Evidence indicates that loss of viability is more complicated than the simple inactivation of one or more crucial enzymes, and that lysis and death are secondary, indirect responses to interaction of β-lactam with the enzyme. The existence of diverse binding sites raises the possibility of multiple mechanisms. Furthermore, interaction with enzyme protein does not automatically result in lysis and lethality. Hence, the mechanism of the irreversible effects must involve events in addition to the binding step. It is realistic to widen one's focus from inactivated bacterial enzymes to an entire inhibitory pathway of multiple steps and factors (*22*).

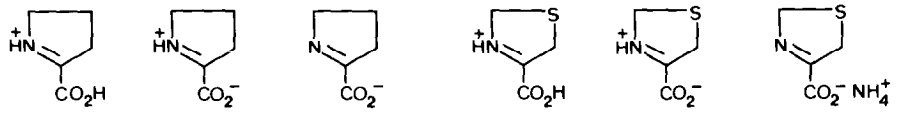
Quite remarkably the first hints of an additional unifying theme were advanced a number of years ago (*21a, 26a*). A common basis of unknown nature was proposed for the diverse, secondary responses of various bacteria to the same stimulus. More concretely, evidence showed that inhibition of early, intermediate, or late stages of peptidoglycan assembly can all result in the same end result, namely, growth inhibition or destruction by lytic or nonlytic means.

Our objective was to investigate the iminium hypothesis of β-lactam action. We demonstrate that essentially all of the agents can potentially generate this cationic functionality. The principal experimental approach entailed electrochemical studies on simple models of the drug metabolites formed at the active site. Data on energetics provide information on the feasibility of ET in the biological milieu. Literature findings on nephrotoxicity, antibiotic action, bacterial cell potential, and site-binding chemistry are addressed based on the unified concept.

EXPERIMENTAL PROCEDURES

The ethyl ester of 2,3-dioxopiperidine-4-carboxylic acid, mp 147–148°C, lit. mp 148°C, mass spectrum *m/z* (CI)(rel (int): 185 ($M^+ + 1$) (69), 168 (14), 140 ($M^+ + 1 - C_2H_5OH$)(100), was used to prepare α-keto-δ-aminovaleric acid hydrochloride, mp 109–110°C (dec.), lit. mp 113°C (dec.) (Ref. (*27*)); mass spectrum *m/z* (CI)(rel int): 114($M^+ + 1 - HCl - H_2O$(100), 96 ($M^+ + 1$-HCl–$2H_2O$)(44.4), 70($M^+ + 1 - HCl - CO_2 - H_2O$)(13.1); elemental analysis for $C_5H_9NO_3 \cdot HCl$: Calc. C, 35.83; H, 6.01; N, 8.36. Found C, 36.94; H, 5.97; N, 8.52. The high carbon content may be due to dehydration of the sample on standing over P_2O_5, as proposed in the case of the next higher homolog (*28*). The infrared spectrum in nujol reproduced that of the reported one (*29*). The 2,4-dinitrophenylhydrazone melted at 221–222°C (dec.), lit. mp 223°C (dec.) (*30*); elemental analysis was satisfactory. The product was

cyclized in solution (*27, 31*) to the various forms of **1** (**a**, **b**, or **c**) by variation in pH.



1a **1b** **1c** **2a** **2b** **2c**

Compound **2c** (*32*), mp 167°C (dec.), lit. mp 172°C (dec.) was prepared from ammonium 3-mercaptopyruvate, mp 182°C, lit. mp 182°C (*33–35*), which was obtained from chloropyruvic acid (*36*). Compound **2c** was characterized by: IR(KBr) 1600 cm^{-1} (carboxylate) and 3300–3000 cm^{-1}, broad (ammonium); mass spectrum *m/z* (CI) (rel int): 132 (M^+ + 1–NH_3)(100), 88 (M^+ + 1–NH_3–CO_2)(40.1); ^1H NMR (D_2O)(TMS): δ 4.09 (t, 2H, from HC-5), and 5.23 (t, 2H, from HC-2) in an AA′XX′ pattern, close to an A_2X_2; ^{13}C NMR (D_2O)(TMS): δ 43.70, 67.65, 170.21, 172.71; elemental analysis for $C_4H_8N_2O_2S$: Calc.: C, 32.42; H, 5.44; N, 18.91; S, 21.64. Found: C, 32.44; H, 5.58; N, 18.69; S, 21.49.

Mass spectral data were obtained with a Hewlett–Packard 5985B (GC/MS) instrument; IR spectra were obtained with a Mattson Instruments Polaris FT-IR spectrometer connected to a Hewlett–Packard 7470A plotter; NMR spectra were obtained with a Bruker WM 250 instrument (^1H, 250 MHZ; ^{13}C 62.9 MHz).

Acid and buffer solutions were prepared in accord with the methods reported (*37*) (pH 2.0 HCl/KCl, pH 2.8–5.3 potassium hydrogen phthalate buffer, pH 6.2–8.0 phosphate buffer). The pH was checked prior to each measurement with a Sargent Model LS pH meter.

Cyclic voltammetry (CV) was performed with a PARC Model 174A polarographic analyzer connected to a Hewlett–Packard Model 7035B X-Y recorder. All solutions were deaerated for 15 min with prepurified nitrogen. A hanging mercury drop electrode (HMDE) was used for CV; a fresh drop was used for each scan. The reference and counter electrodes for all solutions were a Corning aqueous saturated calomel electrode (SCE) and a platinum wire, respectively. All potentials were converted to NHE electrode by addition of 0.24 V to the SCE values. Bulk electrolysis was performed with an ECO Model 553 potentiostat connected to a PARC Model 379 digital coulometer; a mercury pool was the working electrode.

RESULTS AND DISCUSSION

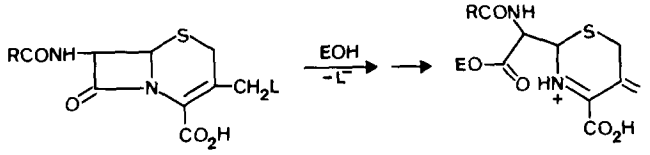
Pathways to Conjugated Iminium

In this section we provide reasonable reaction sequences which can lead to conjugated iminium species for essentially all of the main types of β-lactams (*23, 24, 26b*). The possibility of iminium involvement in the chemistry of these agents has generally not been considered. A rare exception is found in kinetic studies of ring cleavage (*38*). In almost every case, the lactam ring opening generates a basic

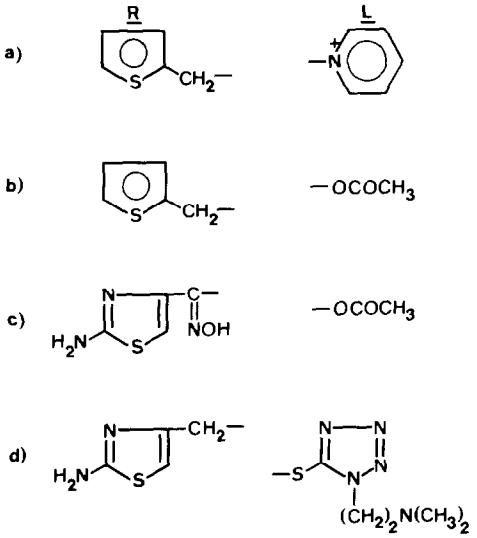
imine that can form iminium by protonation. Nearby acidic hydrogen is available for salt formation. In relation to the role of the ring fused to the β-lactam, prior rationale usually invoked conformational and strain effects that enhanced lactam reactivity (*22–24, 26b, 39, 40a*). From our viewpoint, an additional unifying feature is the ability of the second ring to generate a conjugated imine after scission of the four-membered moiety by enzyme (EOH).

Cephalosporins (23, 24, 26b)

Δ^3-*Derivatives with leaving groups in the 3 position.* Apparently a good leaving group (L) exerts a beneficial influence on *in vitro* activity of cephalosporins **3** and analogs (*21b*). When this type of elimination (Eq. [1]) is prohibited by moving the

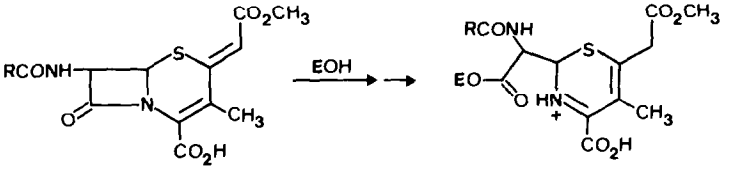


3 4 [1]



double bond to the Δ^2-position, the compound is virtually inactive (*21b*). On the other hand there is little adverse sensitivity to replacement in the 1 or 2 position (*24*).

2-[(Methoxycarbonyl)methylene]cephalosporins (41). In the first step (Eq. [2])

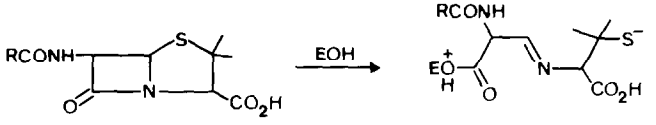


[2]

the resulting carbanion is stabilized by the methyl ester group. Alternatively, opening of the six-membered ring might occur at the C–S bond, analogous to clavulanic acid (*vide infra*).

Penicillin (23, 24, 26b) (**5**)

The thiazolidine ring can readily reform by sulfide anion attack on imine or especially on the derived salt. The iminium salt of **6** may undergo rearrangement (Eq. [3]) to the more stable structure **7** containing conjugation with carboxyl.



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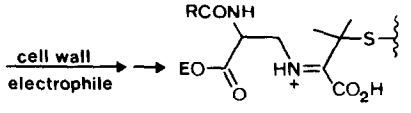
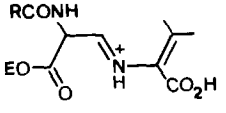


[3]

7

Imine isomerization (transamination) is well documented. Plausible pathways for the sulfide ion **6** are conversion to thiol (less likely, *vide infra*) or covalent attachment to an appropriate group in the receptor chamber (more likely, *vide infra*). Acid-catalyzed 1,2-elimination of sulfur would generate **8**. A similar reaction with **7** yields a conjugated species related to **4**. Admittedly, no evidence exists for the postulated transformations.



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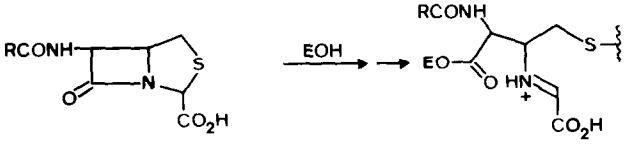
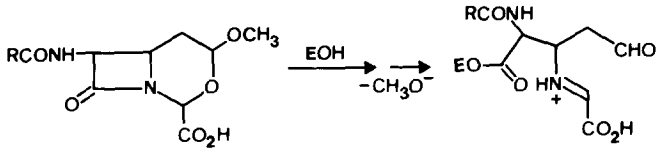
Bisnorisopenicillin (42)

Chemical behavior (Eq. [4]) is predicted to be somewhat analogous to the penicillin case.



[4]

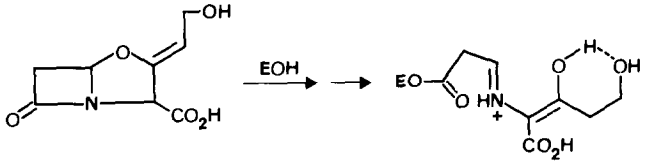
3-Oxa-1-azabicyclo[4.2.0]octan-8-one-2-carboxylic Acid (40a)



[5]

There is structural similarity to bisnorisopenicillin. After cleavage of the β-lactam (Eq. [5]), the hemiacetal anion should expel methoxide.

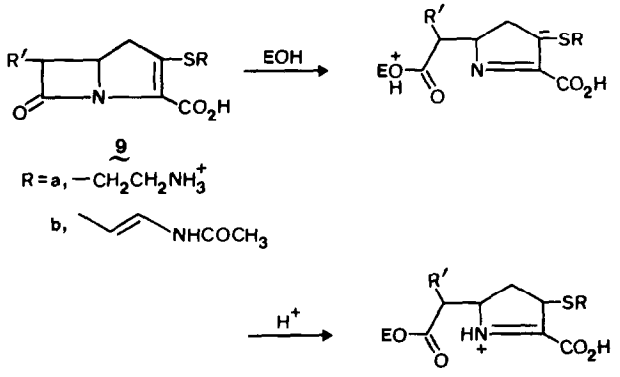
Clavulanic Acid (24, 26b)



[6]

The literature contains discussion of ring opening with formation of the keto (enol)-containing chain (Eq. [6]) (*40b, 43, 44a*). Isomerization to the enamine in conjugation with ester has been observed (*44a*). Decarboxylation, characteristic of α,β- or β,γ-unsaturated acids, can occur (*40b*). The compound, a relatively weak antibiotic, is most useful as an irreversible inhibitor of bacterial β-lactamases (*40a*).

Thienamycins and Olivanic Acid (24, 26b, 44b)



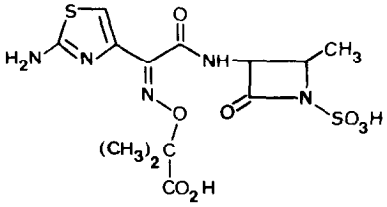
[7]

A favorable driving force consists of intramolecular protonation of the developing carbanion which is stabilized by adjacent sulfur (Eq. [7]) (*45a*). Both **9a** and **9b** possess acidic-type hydrogens appropriately situated stereochemically in the R group for donation. Some thienamycins contain sulfur in sulfone or sulfoxide form, functionalities also known to stabilize adjacent carbanion (*45a*). On the other hand, a proton-donating side chain is not essential. Replacement by hydrogen or phenyl results in activity equivalent to that of natural analogs with *N*-acyl

side chains (*44b*). In these cases, carboxyl may provide a proton intramolecularly to the incipient carbanion in the transition state.

Monocyclic β-Lactams

In relation to the theoretical framework, there is greater divergence for this category, which is not surprising since the usual fused ring structure is absent and imines are not expected from ring opening. Two principal types are monobactams (**10**) (*44b*) and nocardicin (**11**) (*24, 44b*). Quite remarkably, this class also possesses the possibility for ET. The side chains contain precursors of electroactive functionalities, namely, conjugated imine for iminium and phenol (*24*) for quinone (*46*). The imino group is known to increase activity (*41*), and evidence exists for metabolic activation (possibly quinonemethine (**12**) formation) in the case of **11** (*47*). Our preliminary data (unpublished work) indicate favorable E values for the imine drugs without ring opening.



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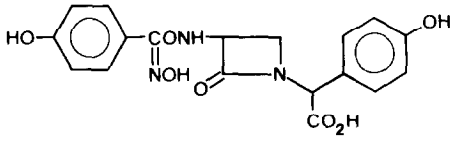

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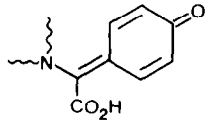

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It is noteworthy that for the fused ring β-lactams, a prior report suggested further cleavage of the ring system following β-lactam scission as an important event for activity (*40a*). The various intermediate anions and imines can accept protons from diverse sources: intramolecularly from carboxyl or the conjugate acid of the ester, or intermolecularly from an acidic donor at the binding site.

Electrochemistry

In all cases, electron reduction of iminium is facilitated by conjugation. 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 the iminium site.

Although an analog of **4** can be generated *in vitro*, electrochemical studies were not possible due to the known extreme instability in solution at acidic pH (*38, 48*). Also, emulating formation of **7** under *in vitro* conditions may present difficulties. As a result we resorted to simple models, **1** and **2**. Compound **1** is spontaneously

TABLE 1

Cyclic Voltammetry of **1** and **2**[a]

Compound	pH	$-E_p$(V)[b]	$E_{pp/2}$	Intercept[c]
1	2.0	0.37, 0.54	50	1.8
1	2.8	0.45, 0.80[d]	60	—
1	3.7	0.48	50	—
1	5.3	0.60, 0.84	50	2.7
1	6.2	0.64, 0.90	50	2.2
1	8.0	0.92, 1.13	60	0.73
2	2.0	0.18	50	1.84
2	2.8	0.30	50	5.24
2	3.7	0.39	55	1.69
2	5.3	0.61	105	5.9
2	6.2	0.76	85	1.24
2	8.0	NR[e]	—	—

[a] 100 mV/s, HMDE versus NHE.
[b] Irreversible.
[c] Intercept (μA) from the plot of the peak current versus the square root of the sweep rate.
[d] Second peak is sometimes absent or very small.
[e] No reduction.

formed from the straight-chain aminoketone precursor above pH 2 (*27, 31*). Both of these models can be converted to iminium containing carboxyl attached to unsaturated carbon; the minimum requirement for antibacterial activity is β-lactam with accompanying carboxyl (*40a, 44b*).

Cyclic voltammetry in aqueous solution is summarized in Table 1. The discussion will generally focus on the first reduction wave. Compound **1** exhibited multiple waves with the most positive at -0.37 V at pH 2.0 (Fig. 1). There was no indication of reoxidation. A linear plot was obtained for the peak current versus the square root of the sweep rate with intercept of 1.8 μA. E_p was independent of sweep rate. The $E_{pp/2}$ ($E_p - E_{p/2}$) value at 100 mV/s was 50 mV. In pH 2.8 buffer a single wave was obtained with E_p of -0.45 V. When the sweep rate was lowered (20 or 50 mV/s) the potential became more negative by about 90 mV. Although the

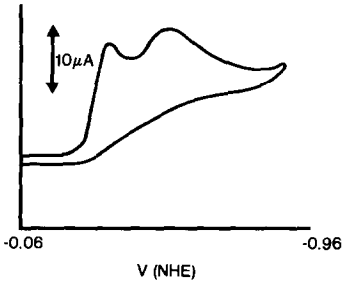


FIG. 1. Cyclic voltammogram of **1** at pH 2.0; scan rate 100 mV/s.

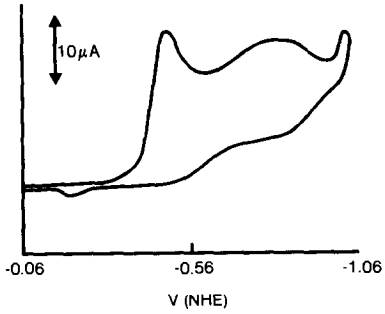


FIG. 2. Cyclic voltammogram of **1** at pH 3.7; scan rate 100 mV/s.

exact reason for this behavior is unknown, it may be a surface phenomenon. However, when the concentration was lowered to 0.12 m*M* the same results were realized. The $E_{pp/2}$ value at 0.1 V/s was 60 mV. Similar findings were obtained at pH 3.7 (Fig. 2), although two waves were observed at scan rates of 200 and 100 mV/s. Rates of 50 and 20 mV/s produced single waves with more negative potentials (20 mV/s, E_p −0.59 V). The first E_p at 100 mV/s was −0.48 V with difference in peak potential and half-peak potential of 50 mV. Of the peaks generated at pH 5.3 (Fig. 3), the first occurred at −0.60 V and the second at −0.84 V, both of about equal height, unaccompanied by reoxidation. The E_p changed about 20 mV for each decade change in sweep rate. A plot of the first peak current versus the square root of the sweep rate gave a correlation of 0.998 and an intercept of 2.7 μA. $E_{pp/2}$ values were constant, about 50 mV, indicating an αn value of about 0.95. Bulk electrolysis at the first peak resulted in the uptake of 2.3 electrons. From this the transfer coefficient (α) was found to be about 0.48. As the pH was increased to 6.2 the reduction became more negative, −0.64 V; a smaller peak at −0.90 V was also present. The intercept was 3.6 μA for the line derived from i_{pc} versus the sweep-rate square root. The $E_{pp/2}$ values were 50 mV for the scan rates employed. At pH 8.0 the initial reduction peak was at −0.92 V (Fig. 4). The reaction was diffusion controlled and irreversible with $E_{pp/2}$ of 60 mV, accompanied by minor reduction at −1.13 V.

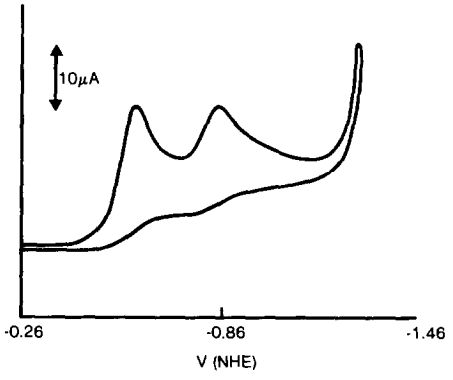


FIG. 3. Cyclic voltammogram of **1** at pH 5.3; scan rate 100 mV/s.

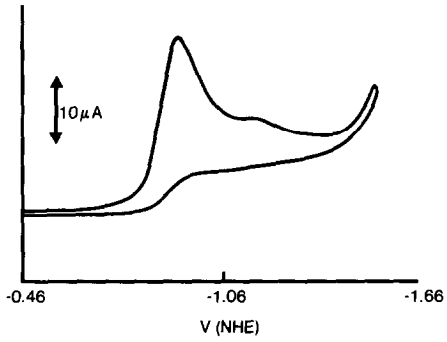
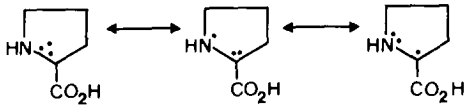


FIG. 4. Cyclic voltammogram of **1** at pH 8.0; scan rate 100 mV/s.

As the pH decreased the reduction potential became more positive in agreement with progression from **1c** to **1b** to **1a**. The predominant species present in solution between pH 2 and 6 are expected to be **1a** and **1b** (p*K*: COOH, 1.8; pyrrolinium, 6.0) (*29, 49*). More basic conditions result in **1c**. One-electron reduction of **1a** yields the resonance-stabilized radical **13**. Carboxyl can facilitate the process in



13a **13b** **13c**

several ways. In **13b** there is delocalization of α carbanion by carbonyl. A radical **13c** adjacent to carbonyl is also stabilized (*50, 51*). The increase in reduction potential (about 0.5 V) by fixation of carboxyl to iminium (*11*) is similar to the enhancement (0.64 V) reported (*6*) for 2-carboxyquinoxaline di-*N*-oxide versus the parent. According to prior thinking, the principal role of carboxyl is involvement in site binding (*23*). Our data point to an additional important function entailing facilitation of electron uptake by iminium. Although the exact state of the carboxyl group at the active site is unknown, with a large number of agents the unionized molecule is much more active than the ion (*52*). The greater permeability of the cell to the undissociated form may be a factor.

Compound **2** showed similarities to **1** in its electrochemical characteristics; e.g., all reductions were irreversible and gave linear plots for the peak current versus square root of the sweep rate (Table 1). However, there were significant differences; e.g., only one reduction peak was observed in all cases. At pH 2.0, **2** reduced with E_p of -0.18 V. The $E_{pp/2}$ value was 50 mV at the 100-mV sweep rate, possibly indicating absorption of two electrons. Upon a decade change in rate of sweep the potential changed 20 mV. Taking the pH to 2.8 reduced the potential by 120 mV. A 25-mV change in peak potential with a 10-fold increase in sweep rate and an $E_{pp/2}$ value of 50 mV characterized the irreversible reduction. A further decrease in reduction potential was observed at pH 3.7, E_p -0.39 V (Fig. 5). The parameters included ΔE_p of 20 mV per decade increase in sweep rate and constant

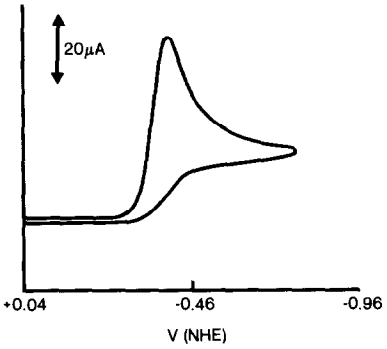
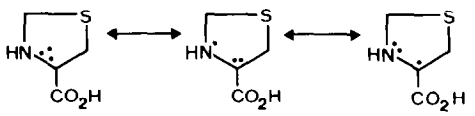


FIG. 5. Cyclic voltammogram of **2** at pH 3.7; scan rate 100 mV/s.

$E_{pp/2}$ value of 55–60 mV for the scan rates employed (20–200 mV/s). The E_p at pH 5.3 was similar to that for **1**, −0.61 V; however, the $E_{pp/2}$ value was 105 mV. The broadness of the peak and change of 50 mV upon decade change in sweep rate are strong indicators of irreversibility. Electrolysis at this pH resulted in the uptake of 2.02 electrons. Reduction at pH 6.2 (E_p −0.76 V) was characterized by $E_{pp/2}$ of 85 mV at a scan rate of 0.1 V/s and an E_p change of 20 mV per decade change in sweep rate. No electrochemical reaction was observed at pH 8.0, perhaps due to degradation or resistance to reduction.

Compound **2** represents a model closer to both **4** and **7** since sulfur is present β to iminium. The cyclic structure makes it particularly akin to **4**. Again, as with **1**, lowering the pH had a favorable influence on the reduction potential. However, in the case of the sulfur analog **2**, there is a striking effect on electron uptake that is associated with variation in pH. In comparison with **1**, the reduction potential is more negative at higher pH values, but more positive at lower ones. Two principal factors that influence ease of reduction are electrophilicity of iminium (*12, 53*) and delocalization of the generated radical. Sulfur is known to stabilize carbocations when in the β position (*45b*). At higher pH the positive character on nitrogen in **2b** is diminished both by sulfur and the carboxylate anion. At lower values, carboxylate is converted to unionized carboxyl. Furthermore, the data for **2** show that carboxyl exerts a more favorable effect on reduction than does carboxylate. Since sulfur is known to stabilize radicals in the β position (*45c*), favorable interaction of this type is expected in **14** (product from one-electron reduction). It is intriguing that the various atoms and groups in the nuclei of the β-lactams efficiently assume a variety of biochemical roles.



14a **14b** **14c**

Although the reactions observed in this study were all irreversible and electrolysis indicated the uptake of two electrons, 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 (*6, 9, 11*). Biologically active agents known to effect ET which display similar potentials (about −0.4 V) include quinones (*4*), nitroheterocycles (*13*), triarylmethane dyes (*13*) (iminium), and metal complexes (*5, 8, 14*). Although a direct correlation between reduction potential and physiological activity would not be expected in all cases, appreciable numbers of examples are known which demonstrate such a relationship (*5, 13, 14*).

It is highly significant that evidence, both recent and long standing, exists for participation of ET in various physiological responses of β-lactams. These supportive observations are treated in the following sections.

Cephalosporin Nephrotoxicity

Most of this work has been done with cephaloridine **3a**. A surge of activity in recent years has resulted in considerable elucidation of the basic chemistry (*54–59*). Various types of activated oxygen species are apparently generated including superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen. The usual consequences of oxidative stress, namely lipid peroxidation, conjugated diene formation, and malondialdehyde generation, were observed. The endogenous thiol, glutathione, which protects against the toxic effects of oxy radicals, was depleted by drug administration, with concomitant increase in the level of oxidized glutathione. Removal of vitamin E and selenium (radical scavengers) from the diet potentiated nephrotoxicity. In keeping with this scenario, antioxidants (α-tocopherol, *N,N'*-diphenyl-*p*-phenylenediamine, promethazine, mannitol) hindered lipid peroxidation. Furthermore, inhibitory action was displayed by the combination of superoxide dismutase and catalase, as well as by histidine, a singlet oxygen trap.

On the basis of the evidence, it was quite reasonable to suggest an electron-transfer mechanism for generation of activated oxy entities via superoxide (*54, 55*). The proposal was advanced that NADPH–cytochrome *P*-450 reductase catalyzes the reduction of cephaloridine with NADPH serving as an electron donor (*54*). Subsequent ET to oxygen occurs with superoxide formation. Several groups have focused attention on the pyridinium moiety and have drawn analogy with the same process involving paraquat (*54, 55*). Although there is some structural similarity, the comparison neglects the important aspect of energetics associated with electron uptake. Reduction potentials (V, DMF) for relevant ions are paraquat (*60*), −0.19; 1-methyl-4-phenylpyridinium (cyperquat) (*12*), −0.90; 1-methylpyridinium (*12*), −1.08. Therefore, the significantly more negative potential for *N*-methylpyridinium makes cephaloridine per se an unlikely candidate for that type of role.

There exists additional compelling evidence against the crucial involvement of pyridinium in ET. Cephalothin **3b**, which possesses an acetate leaving group, also yields malondialdehyde (*59*). On this basis it was suggested that moieties common to the cephalosporins, rather than (L) in **3**, may contribute to the generation of radicals (*57*). The most obvious structural correspondence is the fused ring sys-

tem. As discussed in a prior section, both types of cephalosporins **3a** and **3b** can serve as precursors of conjugated iminium **4**. It is reasonable to regard this ionic functionality as the catalyst for ET leading to activated oxygen. The reduction potentials for **1** and **2** are of the same order of magnitude as for the antitumor antibiotic quinones (*4*) that are known to undergo redox cycling with formation of oxy radicals. The overall mechanistic analysis assumes that kidney tissue binding is chemically akin to the bacterial case.

As often occurs *in vivo* there is specificity involving other important variables, since cefotaxime **3c** (related to cephalothin) does not produce significant amounts of superoxide and hydrogen peroxide (*54*). Even with cephaloridine, nephrotoxicity can vary widely depending upon experimental conditions including host age and the animal used (*56, 61*). Cefotiam **3d**, which incorporates a conjugated sulfide leaving group, also produces kidney toxicity (*62*).

Antibacterial Action

Almost all of the principal categories of synthetic antibacterial agents, in addition to a number of the naturally occurring ones, can be accommodated within our theoretical framework based on ET and oxy radicals (*13*). Despite the behavior in nephrotoxicity, evidence for oxidative stress during antibiotic action of β-lactams is essentially lacking. In fact, there are reports of no influence by antioxidants on penicillin activity (*63, 64*). Furthermore, this class is effective against both aerobic and anaerobic bacteria (*26d*). Various categories of drugs can apparently function by ET without involvement of superoxide, including radiosensitizers (*65*), CNS agents (*9–12, 66*), and quinoxaline di-*N*-oxide (antibacterial) (*6*). Thus, in relation to the β-lactams, ET by iminium could conceivably interfere with normal electrophysiological processes, in line with a prior proposal of such competition (*67*). A catalytic mechanism is consistent with effectiveness at low concentrations (*23*). Observations show that penicillin action resembles that of some other classes of antibiotics (*68*, cf. *13*).

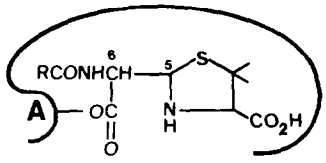
It is remarkable that 40 years ago the proposal was advanced suggesting crucial involvement of electrochemical phenomena in the reaction mechanism of certain antibiotics, including penicillin (*67*). The hypothesis attracted little attention, probably because of difficulties in interpreting the data, as well as eclipsing by the subsequent enzyme-inactivation mechanism. The highly significant observation of a dramatic increase in the reduction potential of bacterial cell cultures on addition of the agent (*67, 69, 70*) was made. No change occurred in the case of resistant strains. Our approach involving catalytic ET by penicillin iminium is in accord with the prior conclusion (*67*) that the medium undergoes oxidation during drug action. As a result there is competition with normal electrophysiological processes, resulting in bacterial cell destruction. Hence, a multipronged assault may pertain to the mode of action, similar to that of some other drugs (*3–17*). Also, penicillin inhibits amino acid deamination, but only when redox processes are involved (*71*).

A parallel situation may pertain in the anticancer domain in relation to the mode

of action for the enzyme inhibitors, methotrexate and α-difluoromethylornithine (*4*).

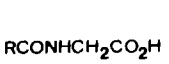
Penicillin Chemistry at the Active Site

Prior studies have been carried out which shed considerable light on reactions that take place during and after site binding. Enzyme acylation by penicillin is thought to entail formation of an ester group derived from a serine residue of the protein, **15** (region A) (*21b, 23, 39, 44a*). Nucleophilic ring opening is usually



15

depicted as leading to the structure in **15**. An unusual transformation which furnishes chemical insight is cleavage of the C_5–C_6 bond, which is apparently required for deacylation (*26e*). The end products are regenerated enzyme, acylglycine **16**, and *N*-formyl D-penicillamine **17** (*21b, 39*). The process appears to



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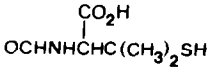
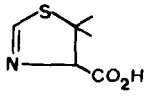


17

proceed via formation of an unknown (X) followed by rate-limiting conversion to enzyme-bound **16** and an unknown (Z) that is a forerunner of **17**. Initially the thiazoline **18** was thought to be the first-formed fission product which served as the precursor of **17** (*72*). However, subsequent kinetic investigations led to the conclusion that this pathway was unlikely (*73*).



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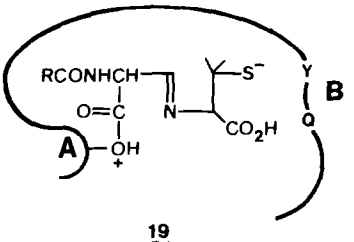
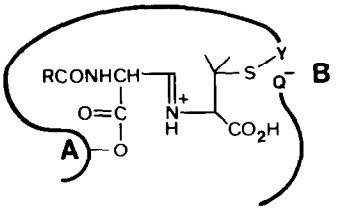
From our standpoint, nucleophilic attack on the lactam ring by enzyme leads to **19**. At this stage it is important to recognize that formation of **17** is delayed when



19

compared with release of **16**. The mercaptide may form (Z) which then, after C_5–C_6 cleavage, generates **17** fairly slowly by reversion. Conceivably, the sulfide anion reversibly attacks a nearby electrophilic site (Y–Q) at region B in **19**. Studies of cell wall composition, including covalently attached materials, revealed the presence of amide, ester, and phosphate functionalities (*26f, 44a*). The ester linkage is susceptible to nucleophilic attack by sulfide ion (*74*). A subsequent step comprises conversion of imine to iminium by either of two acidic hydrogens (from carboxyl or region A) that are favorably situated geometrically. Unknown (X) **20** is considered to exist at this stage of the reaction sequence.



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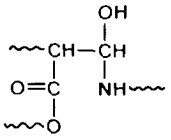
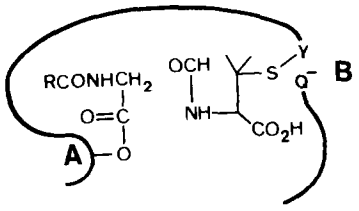
In the ensuing step, water attacks the iminium cation. Scission of the resulting labile carbinolamine **21** at the C_5–C_6 bond proceeds with expulsion of C_6 in carban-



21

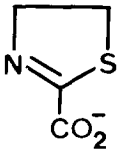
ion form (resonance stabilized). This is in accord with the finding of deuterium incorporation onto the α carbon of acylglycine when D_2O was used (*21b*). The cavity contents are now represented in **22**. Rapid nucleophilic attack at region A provides **16** and the serine residue. We propose that (Z) is the –S–Y– derivative (region B) **22** which slowly cleaves to **17**. Quite significantly, prior investigators have theorized that comparatively strong interaction of the thiazolidine moiety with region B (perhaps another enzyme binding site) is responsible for the stability associated with (Z) (*26e, 39*). There is relevancy in the finding that (Z) contains no detectable thiol group (*26e*).



22

Other Considerations

Iminium carboxylates related to **1b** are believed to play a role in biochemical systems, e.g., reductive amination of α-ketoglutarate catalyzed by glutamate dehydrogenase (*31, 75*). In view of the present findings, involvement of electrochemical pathways should be considered. A similar situation may pertain to the sulfur analog **2**. The parent dihydro form, which can undergo oxidation to imine, inhibits cell division in growing cultures of *Escherichia coli* (*76*). The isomer **23** also fits in



23

this category. There has been discussion of possibilities for participation in various biochemical processes, e.g., as a metabolic regulator, intracellular mediator of certain hormones, reversible effector of enzymic reactions, or ligand (cf. **1c** and **2c**) for metal ions (*77–81*).

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REFERENCES

1. HALLIWELL, B., AND GUTTERIDGE, J. M. C. (1985) Free Radicals in Biology and Medicine, Clarendon Press, Oxford.
2. SIES, H. (Ed.) (1985) Oxidative Stress, Academic Press, New York.
3. KOVACIC, P., CRAWFORD, P. W., RYAN, M. D., AND NELSON, V. (1986) *Bioelectrochem. Bioenerg.* **15,** 305 (and references therein).
4. KOVACIC, P., AMES, J. R., LUMME, P., ELO, H., COX, O., JACKSON, H., RIVERA, L. A., RAMIREZ, L., AND RYAN, M. D. (1986) *Anticancer Drug Des.* **1,** 197 (and references therein).
5. CRAWFORD, P. W., LUMME, P., ELO, H., RYAN, M. D., AND KOVACIC, P. (1987) *Free Radical Res. Commun.* **3,** 347.
6. CRAWFORD, P. W., SCAMEHORN, R. G., HOLLSTEIN, U., RYAN, M. D., AND KOVACIC, P. (1986) *Chem.-Biol. Interact.* **60,** 67.
7. RYAN, M. D., SCAMEHORN, R. G., AND KOVACIC, P. (1985) *J. Pharm. Sci.* **74,** 492.
8. AMES, J. R., RYAN, M. D., KLAYMAN, D. L., AND KOVACIC, P. (1985) *J. Free Radicals Biol. Med.* **1,** 353.
9. AMES, J. R., POTTS, K. T., RYAN, M. D., AND KOVACIC, P. (1986) *Life Sci.* **39,** 1085 (and references therein).
10. CRAWFORD, P. W., KOVACIC, P., GILMAN, N. W., AND RYAN, M. D. (1986) *Bioelectrochem. Bioenerg.* **16,** 407.

11. AMES, J. R., BRANDÄNGE, S., RODRIGUEZ, B., CASTAGNOLI, N., JR., RYAN, M. D., AND KOVACIC, P. (1986) *Bioorg. Chem.* **14,** 228 (and references therein).
12. AMES, J. R., CASTAGNOLI, N., JR., RYAN, M. D., AND KOVACIC, P. (1986) *Free Radical Res. Commun.* **2,** 107 (and references therein).
13. AMES, J. R., RYAN, M. D., AND KOVACIC, P. (1986) *J. Free Radicals Biol. Med.*, **2,** 377 (and references therein).
14. AMES, J. R., HOLLSTEIN, U., GAGNEUX, A. R., AND KOVACIC, P. (1987) *Free Radicals Biol. Med.*, **3,** 85 (and references therein).
15. KOVACIC, P., AMES, J. R., AND RYAN, M. D. (1987) *Anticancer Drug Des.* **2,** 37.
16. KOVACIC, P., AMES, J. R., AND RYAN, M. D. (1987) *in* Proceedings of the Third International Symposium of Redox Mechanisms and Interfacial Behaviors of Molecules of Biological Importance (Dryhurst, G., and Niki, K., Eds.), Plenum Press, New York (in press).
17. CRAWFORD, P. W., FOYE, W. O., RYAN, M. D., AND KOVACIC, P. (1987) *J. Pharm. Sci.*, **76,** 481.
18. KOVACIC, P. (1984) *Kem. Ind.* **33,** 473 (and references therein).
19. KOVACIC, P. (1983) Fundamental Chemistry of Life and Death, University of Wisconsin-Milwaukee Bookstore, Milwaukee.
20. FLEMING, A. (1929) *Brit. J. Exp. Pathol.* **10,** 226.
21. (a) SHOCKMAN, G. D., DANEO-MOORE, L., MCDOWELL, T. D., AND WONG, W., pp. 303–338; (b) WAXMAN, D. J., AND STROMINGER, J. L., pp. 209–285; (c) KOSTER, W. H., CIMARUSTI, C. M., AND SYKES, R. B., pp. 339–375 (1982) *in* Chemistry and Biology of β-Lactam Antibiotics. The Biology of β-Lactam Antibiotics (Morin, R. B., and Gorman, M., Eds.), Vol. 3, Academic Press, New York.
22. TOMASZ, A. (1983) Handbook of Experimental Pharmacology, Antibiotics Containing the β-Lactam Structure (Demain, A. L., and Solomon, N. A., Eds.), Vol. 67, Part I, Chap. 2, Springer-Verlag, New York.
23. GALE, E. F., CUNDLIFFE, E., REYNOLDS, P. E., RICHMOND, M. H., AND WARING, M. J. (Eds.) (1981) The Molecular Basis of Antibiotic Action, Wiley, New York.
24. HOOVER, J. R. E., AND DUNN, G. L. (1979) *in* Burger's Medicinal Chemistry (Wolff, M. E., Ed.), Part II, Chapt. 15, Wiley, New York.
25. BETINA, V. (1983) The Chemistry and Biology of Antibiotics, Chapt. 11, Elsevier, Amsterdam/New York.
26. (a) SHOCKMAN, G. D., DANEO-MOORE, L., MCDOWELL, T. D., AND WONG, W., pp. 31–65; (b) CHRISTENSEN, B. G., pp. 101–122; (c) KUNUGITA, K., AND TAMAKI, S., pp. 185–197; (d) GORBACH, S. L., pp. 461–473; (e) GHUYSEN, J.-M., FRÉRE, J.-M., LEYH-BOUILLE, M., AND DIDEBERG, O., pp. 127–152; (f) BADDILEY, J., pp. 13–30 (1981) *in* β-Lactam Antibiotics. Mode of Action, New Developments and Future Prospects (Salton, M. R. J., and Shockman, G. D., Eds.), Academic Press, New York.
27. HASSE, K., AND WIELAND, A. (1960) *Chem. Ber.* **93,** 1686.
28. SKURSKY, L., AND MACHOLAN, L. (1958) *Coll. Czech. Chem. Commun.* **23,** 150.
29. MACHOLAN, L., AND VENCALKOVA, J. (1963) *Chem. Ber.* **96,** 237.
30. MACHOLAN, L. (1957) *Coll. Czech. Chem. Commun.* **22,** 479.
31. SRINIVASAN, R., AND FISHER, H. F. (1985) *J. Amer. Chem. Soc.* **107,** 4301 (and references therein).
32. ASINGER, F., THIEL, M., SEDLAK, H., HAMPEL, O., AND SOWADA, R. (1958) *Liebigs Ann. Chem.* **615,** 84.
33. PARROD, J. (1942) *Compt. Rend.* **215,** 146.
34. PARROD, J. (1947) *Bull. Soc. Chim. Fr.* 109.
35. SCHNEIDER, F. (1947) *Biochem. Z.* **318,** 329.
36. CRAGOE, E. J., JR., AND ROBB, C. M. (1960) *Org. Synth.* **40,** 54.
37. WEAST, R. C. (Ed.) (1983) CRC Handbook of Chemistry and Physics, 64th ed., p. D150, CRC Press, Boca Raton, FL.
38. DAVIS, A. M., AND PAGE, M. I. (1985) *Chem. Commun.*, 1702.
39. GHUYSEN, J.-M., FRÉRE, J.-M., LEYH-BOUILLE, M., COYETTE, J., DUSART, J., AND NGUYEN-DISTÈCHE, M. (1979) *Annu. Rev. Biochem.* **48,** 73.
40. (a) HOLDEN, K. G., Chapt. 2, (b) CHERRY, P. C., AND NEWALL, C. E., Chapt. 6 (1982) *in*

Chemistry and Biology of β-Lactam Antibiotics. Nontraditional β-Lactam Antibiotics (Morin, R. B., and Gorman, M., Eds.), Vol. 2, Academic Press, New York.
41. KIM, C. U., MISCO, P. F., HAYNES, U. J., AND MCGREGOR, D. N. (1984) *J. Med. Chem.* **27,** 1225.
42. HUFFMAN, W. F., HALL, R. F., GRANT, J. A., AND HOLDEN, K. G. (1978) *J. Med. Chem.* **21,** 413.
43. BROOKS, G., BRUTON, G., FINN, M. J., HARBRIDGE, J. B., HARRIS, M. A., HOWARTH, T. J., HUNT, E., STIRLING, I., AND ZOMAYA, I. I. (1985) *in* Recent Advances in the Chemistry of β-Lactam Antibiotics (Brown, A. G., and Roberts, S. M., Eds.), Chapt. 15, Burlington House, London.
44. (a) GEORGOPAPADAKOU, N. H., AND SYKES, R. B., Chapt. 12; (b) HOOVER, J. R. E., Chapt. 14; (c) BROWNING, M. C., AND TUNE, B. M., Chapt. 16 (1983) *in* Handbook of Experimental Pharmacology. Antibiotics Containing the Beta-Lactam Structure (Demain, A. L., and Solomon, N. A., Eds.), Vol. 67, Part II, Springer-Verlag, New York.
45. BLOCK, E. (1978) Reactions of Organosulfur Compounds (a) Chapt. 2, (b) Chapt. 4, (c) Chapt. 5, Academic Press, New York.
46. IRONS, R. D., AND SAWAHATA, T. (1985) *in* Bioactivation of Foreign Compounds (Anders, M. W., Ed.) pp. 259–281, Academic Press, New York.
47. HAKIMELAHI, G. H., AND JUST, G. (1979) *Canad. J. Chem.* **57,** 1932.
48. HAMILTON-MILLER, J. M. T., NEWTON, G. G. F., AND ABRAHAM, E. P. (1970) *Biochem. J.* **116,** 371.
49. CABELLO, J., LEON, B., PRAJOUX, V., AND PLAZA, M. (1964) *Arch. Biochem. Biophys.* **107,** 51.
50. PRYOR, W. A. (1966) Free Radicals, p. 171, McGraw–Hill, New York.
51. ZAWALSKI, R. C., LISIAK, M., KOVACIC, P., LUEDTKE, A., AND TIMBERLAKE, J. W. (1980) *Tetrahedron Lett.* **21,** 425.
52. EAGLE, H., LEVY, M., AND FLEISCHMAN, R. (1952) *Antibiot. Chemother.* **2,** 563.
53. KADYSH, V. P., STRADYN, Y. P., LAVRINOVICH, E. S., AND ZARIN, P. P. (1975) *Chem. Heterocycl. Comp.* **11,** 588.
54. COJOCEL, C., HANNEMANN, J., AND BAUMANN, K. (1985) *Biochim. Biophys. Acta* **834,** 402.
55. KUO, C.-H., MAITA, K., SLEIGHT, S. D., AND HOOK, J. B. (1983) *Toxicol. Appl. Pharmacol.* **67,** 78.
56. KUO, C.-H., AND HOOK, J. B. (1982) *Toxicol. Appl. Pharmacol.* **63,** 292.
57. GOLDSTEIN, R. S., PASINO, D. A., HEWITT, W. R., AND HOOK, J. B. (1986) *Toxicol. Appl. Pharmacol.* **83,** 261.
58. COJOCEL, C., LAESCHKE, K. H., INSELMANN, G., AND BAUMANN, K. (1985) *Toxicology* **35,** 295 (and references therein).
59. COJOCEL, C., HANNEMANN, J., INSELMANN, G., LAESCHKE, K. H., AND BAUMANN, K. (1984) *Pflügers Arch.* **400** (Suppl.), R19.
60. HUNIG, S., GROSS, J., AND SCHNEK, W. (1973) *Liebigs Ann. Chem.* 324.
61. RUSH, G. F., SMITH, J. H., NEWTON, J. F., AND HOOK, J. B. (1984) *Crit. Rev. Toxicol.* **13,** 99.
62. WACHSMUTH, E. D. (1981) *Arch. Toxicol.* **48,** 135.
63. LEONARD, C. S. (1946) *Science* **104,** 501.
64. ROSSO, C. (1954) *Patol. Sper.* **42,** 166; (1955) *Chem. Abstr.* **49,** 3302.
65. NARAYANAN, V. L., AND LEE, W. W. (1982) *Adv. Pharmacol. Chemother.* **19,** 155.
66. KARREMAN, G., ISENBERG, I., AND SZENT-GYÖRGYI, A. (1959) *Science* **130,** 1191.
67. MULÉ, F. (1947) *Int. Cong. Microbiol. Rep. Proc.* **4,** 119.
68. HERRELL, W. E. (1946) Penicillin and Other Antibiotic Agents, Saunders, Philadelphia.
69. KRÁMLI, A., STUR, J., AND TURAY, P. (1954) *Acta Physiol. Hung.* **5,** 549.
70. DROUHET, E. (1949) *Ann. Inst. Pasteur Paris* **76,** 168.
71. RAYNAUD, M., NISMAN, B., AND COHEN, G. N. (1947) *Ann. Inst. Pasteur Paris* **73,** 1012.
72. HAMMARSTRÖM, S., AND STROMINGER, J. L. (1976) *J. Biol. Chem.* **251,** 7947.
73. ADRIAENS, P., MEESSCHAERT, B., FRÉRE, J.-M., VANDERHAEGHE, H., DEGELAEN, J., GHUYSEN, J.-M., AND EYSSEN, H. (1978) *J. Biol. Chem.* **253,** 3660.
74. REID, E. E. (1962) Organic Chemistry of Bivalent Sulfur, Vol. 4, p. 28, Chem. Pub. Co., New York.
75. SRINIVASAN, R., MEDARY, R. T., FISHER, H. F., NORRIS, D. J., AND STEWART, R. (1982) *J. Amer. Chem. Soc.* **104,** 807.

76. COCCIA, R., BLARZINO, C., FOPPOLI, C., AND CINI, C. (1979) *Ital. J. Biochem.* **28,** 252 (and references therein).
77. BURNS, C. L., MAIN, D. E., BUCKTHAL, D. J., AND HAMILTON, G. A. (1984) *Biochem. Biophys. Res. Commun.* **125,** 1039.
78. NABER, N., VENKATESAN, P. P., AND HAMILTON, G. A. (1982) *Biochem. Biophys. Res. Commun.* **107,** 374.
79. VENKATESAN, P. P., AND HAMILTON, G. A. (1986) *Bioorg. Chem.* **14,** 392.
80. HAMILTON, G. A., BUCKTHAL, D. J., MORTENSEN, R. M., AND ZERBY, K. W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 2625.
81. HAMILTON, G. A. (1985) *Adv. Enzymol. Relat. Subj. Biochem.* **57,** 85.