SUMMARY

The study of micelle–solute interactions by means of partial ultracentrifugal separations of the micellar components offers some possible advantages over other procedures. It permits relatively rapid separations, which are advantageous when solutes are unstable chemically, and avoids the use of membranes or other added materials for the purpose of micelle isolation. Moreover, flexibility in the choice of systems is essentially limited only by the need to assay both the micellar and secondary solute components. While the data were treated in terms of a nonspecific partitioning model in the present paper, the general approach is equally compatible with various adsorption, specific site, or other models of micelle–solute interactions.

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Physicochemical Properties of β-Lactam Antibacterials: Deuterium Solvent Isotope Effect on Penicillin G Degradation Rate

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Abstract □ To obtain kinetic evidence on the degradation mechanism of penicillin in aqueous solution, degradation rates of penicillin G in water and deuterium oxide were measured in the pH (pD) range of 4–10. The solvent isotope effect (k^{H_2O}/k^{D_2O}) of 1.53 below pH (pD) 6 supports the mechanism of water-catalyzed rearrangement of undissociated penicillin G to benzylpenicillenic acid. The spontaneous degradation at neutral pH (pD) and the hydroxide-ion-catalyzed degradation in the alkaline pH (pD) range progress with a deuterium solvent isotope effect (k^{H_2O}/k^{D_2O}) of 4.5 and 0.59, respectively. This finding indicates the mechanisms of general base-catalyzed hydrolysis by water in the neutral pH range and of nucleophilic attack of the hydroxide ion on the β-lactam in the alkaline pH range. No significant side-chain dependency was observed in the reaction of penicillins with bases. The solvent isotope studies led to the conclusion that penicillin degradation is catalyzed by a series of bases *via* general base-catalyzed and nucleophilic mechanisms, depending on their basicity.

Keyphrases □ Penicillin G—degradation in aqueous solution, deuterium solvent isotope effect, pH 4–10 □ Degradation—penicillin G in aqueous solution, deuterium solvent isotope effect, pH 4–10 □ Deuterium oxide—solvent isotope effect on penicillin G degradation in aqueous solution, pH 4–10 □ Antibacterials—penicillin G, degradation in aqueous solution, deuterium solvent isotope effect, pH 4–10

Although the stability of penicillins in aqueous solution has been studied (1–14), little work has been done on their mechanism of degradation.

Interest in the degradation of penicillin in acid and neutral solutions has been stimulated by the suggestion that its degradative product, penicillenic acid, rather than penicillin itself, may be responsible for allergic reactions[1]. During the degradation of penicillin G at a physiological pH of 7.5, the solution slowly gave rise to both benzylpenicilloic acid and benzylpenicillenic acid (15, 16). Levine (15, 16) suggested that the formation of benzylpenicilloic acid proceeded by way of benzylpenicillenic acid as an intermediate, because the latter compound was converted rapidly into the former with a half-life of 6.5 min at pH 7.5 and 37° (17).

Furthermore, some investigators (18, 19) assumed that higher sensitivity of the β-lactam ring in penicillin molecules relative to that of simple β-lactams to hydroxide-ion-catalyzed degradation can be attributed in part to the intramolecular attack of the side-chain amide on the β-lactam moiety. These two hypotheses (15–19) recently were criticized (13, 20) on the basis of kinetic observations, although the mechanism of degradation in neutral and alkaline solutions is not completely understood.

The present study was undertaken to obtain kinetic

[1] The chemical aspects involved in penicillin allergy were reviewed by M. A. Schwartz, *J. Pharm. Sci.*, **58**, 643 (1969).

Table I—Rate Constants [a] and pKa Values Determined for the Degradation of Penicillin G in Water and Deuterium Oxide at 0.5 Ionic Strength

Solvent	Temperature	k_0, hr^{-1}	$10^3 k_0'$, hr^{-1}	$10^{-3} k'_{OH}$ [b], $M^{-1} hr^{-1}$	pKa
Water	35°	3.41	0.90	1.19	2.80[c]
Water	60°	37.4	7.20	5.39	2.78[d]
Deuterium oxide	60°	24.5	1.60	9.12	3.24[c]

[a] Based on the kinetic expression of Eq. 1. [b] Calculated using the autoprotolytic constant: $K_w = 2.09 \times 10^{-14}$ at 35° and $K_w = 9.61 \times 10^{-14}$ at 60° (31); $K_{D2O} = 1.36 \times 10^{-14}$ at 60°, which was extrapolated from the data of Corington *et al.* (32) by the least-squares curve-fitting method. [c] Determined potentiometrically. [d] Reference 7.

evidence on the degradation mechanism of penicillin and to consider possible ways of stabilizing penicillins in aqueous solution. The rates of degradation of penicillin G as a model penicillin were measured in buffered and unbuffered deuterium oxide solutions.

EXPERIMENTAL

Materials—Penicillin G potassium[2], cloxacillin sodium[2], ampicillin sodium[3], and cyclacillin[3] were used as supplied. All other chemicals were the highest commercial grades available. Double-distilled water was used.

Kinetic Procedures—Degradation was initiated by the addition of a known weight of penicillin to a buffer solution preheated to the desired temperature. Aliquots of the solution were withdrawn at suitable time intervals. Unless otherwise stated, the residual penicillin concentration was analyzed by the modified iodometric method (7). In most cases, the initial concentration of penicillin was 5×10^{-3} *M*. The pseudo-first-order rate constants, k_{obs}, were calculated by linear regression analysis of a plot of the logarithm of the penicillin concentration against time.

The rate constants of penicillin G corresponding to those in unbuffered solution of water (k_{pH}) and deuterium oxide (k_{pD}) were determined by the two methods: (*a*) by extrapolation of a plot of k_{obs} *versus* buffer total concentration to zero concentration at constant pH (pD) or by direct determination from analysis of the solution whose pH was maintained constant with the aid of a pH-stat[4], and (*b*) by following the rate of acid formation on a pH-stat. The rate constants were calculated from Guggenheim plots of recorded curves of the volume of titrant [standard 0.01–0.1 *N* NaOH (NaOD) solution whose ionic strength was adjusted to 0.5 with potassium chloride] as a function of time.

In the pH-stat experiments, edetate disodium was added to a final concentration of 5×10^{-4} *M* to avoid occasional erratic results, apparently due to heavy metal-catalyzed decomposition. The concentration of penicillin G was in the range of $5–10 \times 10^{-3}$ *M*.

Unless otherwise stated, kinetic experiments were conducted at 35 or 60° and at a constant ionic strength of 0.5 adjusted with potassium chloride. The pH (pD) of a kinetic solution was measured[5] both before and at the end of the experiments. Where the buffer capacity was too low, constant pH was maintained by a pH-stat. When using deuterium oxide as a solvent, the pD values were taken as the pH meter reading plus the electrode correction of 0.25 at 60° (21).

pKa Determination—The pKa of penicillin G in deuterium oxide at 60° and $\mu = 0.5$ was determined in the manner described by Finholt *et al.* (7). The pKa values of conjugated acids of oxygen bases and amines were determined by the method of half-neutralization.

RESULTS AND DISCUSSION

Degradation Pathway and Mechanism—Figure 1 shows the rate constants, k_{pH}, of penicillin G degradation at 35 and 60° as a function of pH in water. Above pH 4, this degradation is known to obey the following rate law (5, 7, 13):

$$k_{pH} = k_0 \frac{a_{H^+}}{K_a + a_{H^+}} + (k_0' + k'_{OH}\text{-}K_w/a_{H^+}) \frac{K_a}{K_a + a_{H^+}} \quad \text{(Eq. 1)}$$

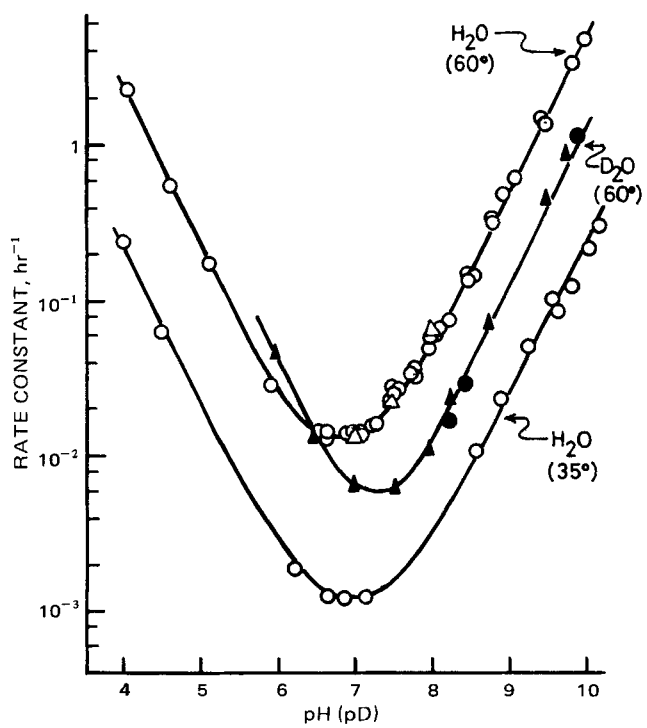


Figure 1—*Log k–pH (pD) profiles for the degradation of penicillin G in water (○, △) and in deuterium oxide (●, ▲) at 35 and 60° and 0.5 ionic strength. Triangles and circles refer to the data determined by the pH-stat alkalimetric titration and iodometric methods, respectively.*

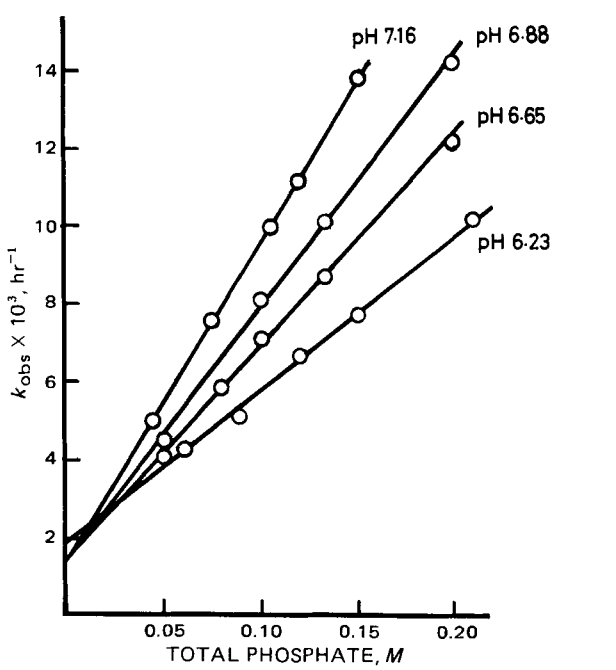


Figure 2—*Plots of pseudo-first-order rate constants* versus *total phosphate concentration for the degradation of penicillin G in aqueous solution at 35° and 0.5 ionic strength.*

[2] Meiji Seika Kaisha, Ltd., Tokyo, Japan.
[3] Takeda Chemical Ind., Ltd., Osaka, Japan.

[4] Radiometer pH-stat titrimeter assembly consisting of TTT2 titrator, SBR3 titrigraph, and ABU12b autoburet or TTT11 titrator, PHM26 pH meter, SBR3 titrigraph, and ABU12b autoburet.
[5] Radiometer PHM26 pH meter or TTT2 titrator.

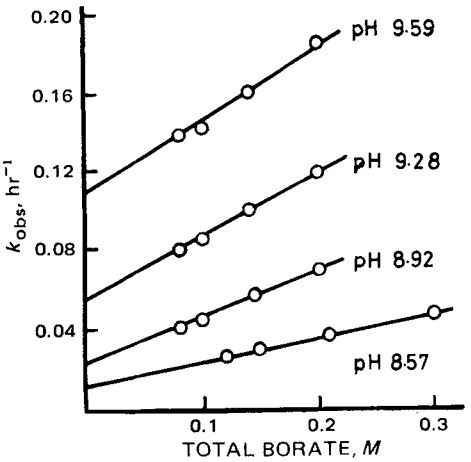


Figure 3—*Plots of pseudo-first-order rate constants* versus *total borate concentration for the degradation of penicillin G in aqueous solution at 35° and 0.5 ionic strength.*

where K_a is the dissociation constant of penicillin G; K_w is the autoprotolysis constant for water; a_{H^+} is the hydrogen-ion activity as determined by the glass electrode; k_0 and k_0' represent the spontaneous or water-catalyzed degradation rate of undissociated and ionized penicillin G, respectively; and k'_{OH^-} is the second-order rate constant for the hydroxide-ion-catalyzed degradation of ionized penicillin G. In the experimental pH region, acid-catalyzed degradation of undissociated penicillin G can be neglected, since such a reaction is only significant below pH 2 (1, 5). The specific rate constants and the dissociation constant are listed in Table I. These values at 60° are in good agreement with those reported by Finholt *et al.* (7).

The rates in deuterium oxide were also determined in the same way as in water. The log k_{pD}–pD profile at 60° is shown in Fig. 1, and the various specific rate constants corresponding to those in water and the pKa value are given in Table I. Above pH (pD) 7, the rates in water were about five times faster than those in deuterium oxide; below pH (pD) 6, the rates in deuterium oxide were about twice those in water.

The k'_{OH^-}/k'_{OD^-} ratio (Table I) was 0.59, consistent with the expected kinetic isotope effect for the reaction involving nucleophilic attack of OH^- and OD^- on the carbonyl carbon of carboxylic acid derivatives (22). The lack of a significant side-chain dependency on k'_{OH^-} (13, 23–25) and the present results led to the conclusion that the 10^4-fold higher reactivity of penicillins toward hydroxide-ion attack compared with that of monocyclic β-lactam and ordinary amides is not primarily due to an intramolecular participation effect as suggested (18, 19) but exclusively due to the strained fused-ring β-lactam and/or to a suppression of the usual amide resonance resulting from the nonplanarity of the β-lactam nitrogen atom.

The reaction below pH 6 is attributed to the spontaneous degradation of unionized penicillin G or the kinetically equivalent hydrogen-ion-

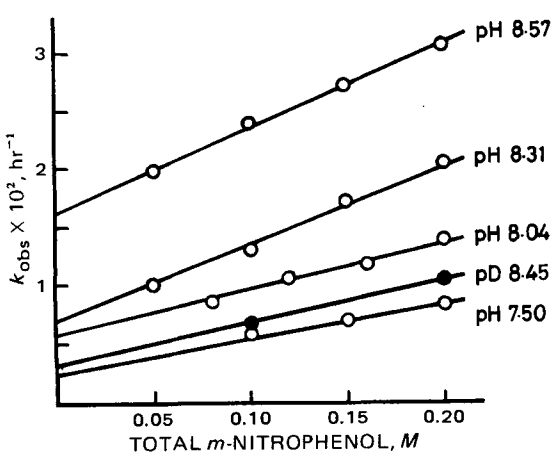
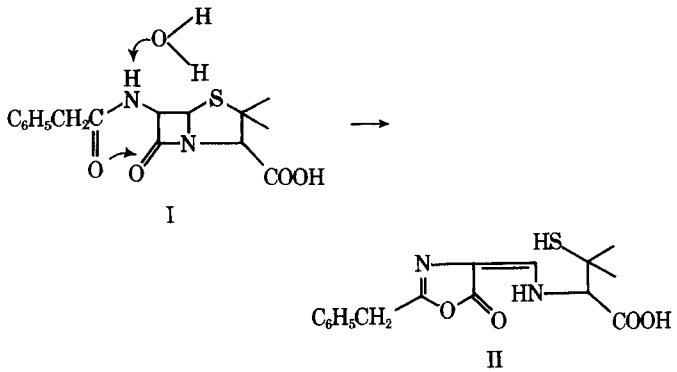


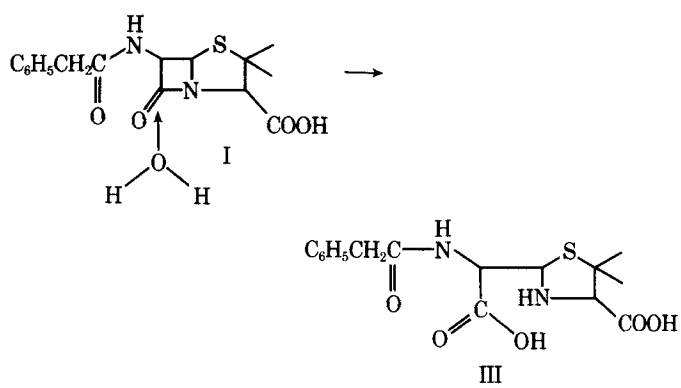
Figure 4—*Plots of pseudo-first-order rate constants* versus *total m-nitrophenol concentration for the degradation of penicillin G in water (○) and deuterium oxide (●, 50% free base) at 60° and 0.5 ionic strength.*



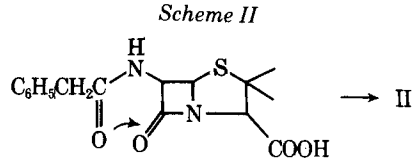
Scheme I

catalyzed degradation of its ionized form. The observed solvent isotope effect of 1.53 for $k_0^{H_2O}/k_0^{D_2O}$ is consistent with the former reaction mechanism favored by Finholt *et al.* (7) and Yamana *et al.* (13) on the basis of the kinetic observation of little ionic strength effect in the pH 4–6 region. However, a more plausible mechanism would be water-catalyzed rearrangement of undissociated penicillin G (I) to benzylpenicillenic acid (II) (Scheme I).

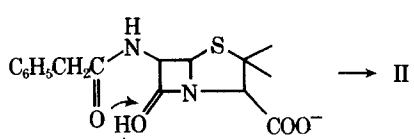
The proposed mechanism should give the expected solvent isotope effect of $1 < k^{H_2O}/k^{D_2O} < 2.5$. A value of 1.53 is not totally consistent with water attack on the β-lactam ring to afford the penicilloic acid (III) (Scheme II, k^{H_2O}/k^{D_2O} expected to be >2.2) or with direct attack of the neighboring acylamide oxygen on the β-lactam ring to produce the penicillenic acid (II) (Scheme III, expected isotope effect of 1.0). The other two kinetically undistinguishable mechanisms, hydrogen-ion-catalyzed rearrangement of ionized penicillin G to benzylpenicillenic acid (Scheme IV) and attack of water on the protonated β-lactam moiety (Scheme V),



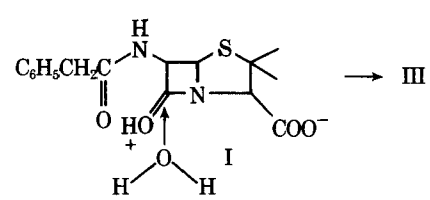
Scheme II



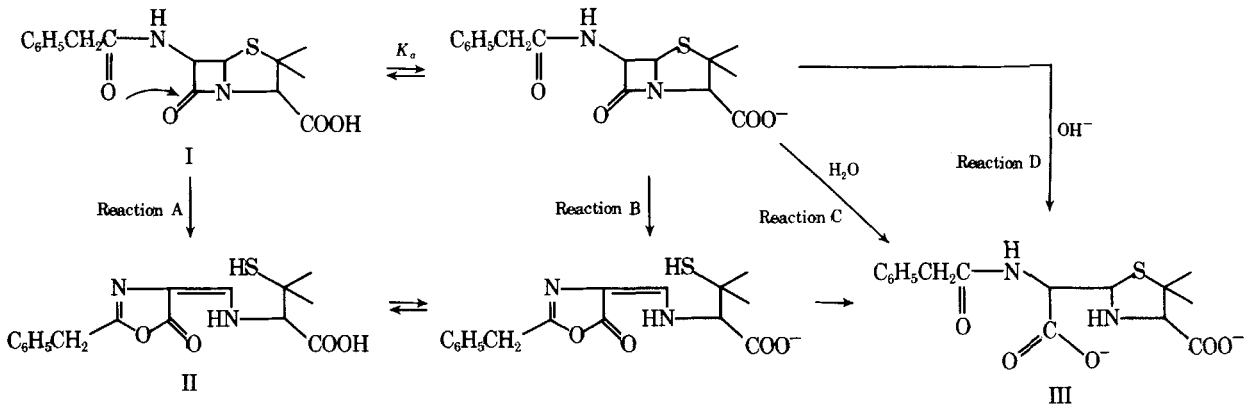
Scheme III



Scheme IV



Scheme V



Scheme VI

can probably both be excluded because k^{H_2O}/k^{D_2O} should be[6] <1.

The production of unstable benzylpenicillenic acid as the reaction intermediate under the conditions below pH (pD) 6 was confirmed by the increase in absorbance at 325 nm of the reaction solution in both water and deuterium oxide. Based on these facts, Scheme I would be the most reasonable to account for the k_0 reaction.

The unique feature of the pH–rate profile for the degradation of penicillin G is the plateau in the neutral pH region. Remarkably broad plateaus have been observed in the degradation of penicillins such as ampicillin (9), cyclacillin (13), phenethicillin (4), and cloxacillin (10), which have characteristically lower k_0 values than penicillin G. The spontaneous rate, k_0', may be due to (*a*) intramolecular attack of the side-chain amide carbonyl on the β-lactam of ionized penicillin (Scheme VI) to produce the penicillenic acid (II) (Reaction B) and/or (*b*) a water-catalyzed β-lactam cleavage to give the penicilloic acid (III) (Reaction C).

Levine (15, 16) postulated that the degradation of penicillin G at pH 7.5 proceeds through benzylpenicillenic acid (II) (*via* Reaction B, Scheme VI), which can be rapidly converted to benzylpenicilloic acid (III) (17). The intramolecularly catalyzed mechanism is an unlikely explanation for the plateau rates, since the large deuterium solvent isotope effect on k_0' ($k_0'^{H_2O}/k_0'^{D_2O}$ = 4.5, Table I) and largely negative entropies of activation ($\Delta S^{\ddagger}$ = −30 eu)[7] are inconsistent with Reaction B (Scheme VI). The most logical possibility for the pH-independent degradation (k_0' reaction) of penicillin G at neutral pH is spontaneous hydrolysis involving nucleophilic attack at the β-lactam ring by a water molecule facilitated (general base) by neighboring water molecules to produce benzylpenicilloic acid (III) directly (Reaction C, Scheme VI). Two possible transition states (I*a* and I*b*) for Reaction C might explain the extremely large solvent isotope effect of 4.5.

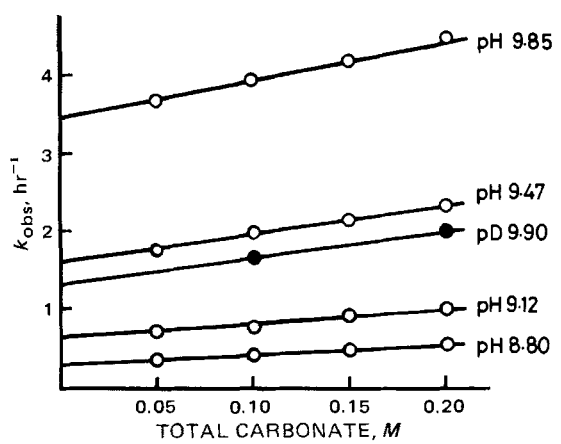
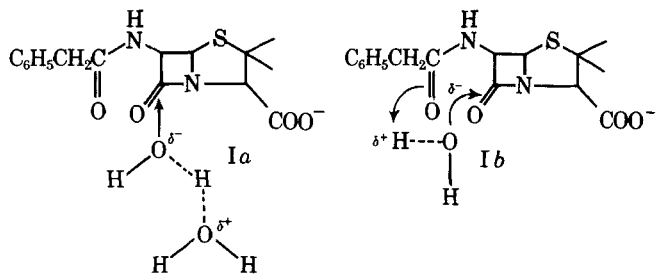


Figure 5—*Plots of pseudo-first-order rate constants* versus *total carbonate concentration for the degradation of penicillin G in water* (○) *and deuterium oxide* (●, *50% free base) at 60° and 0.5 ionic strength.*



Although Reaction C rather than Reaction B has widely been believed to proceed in neutral degradation of penicillins, no reliable evidence sufficiently supports Reaction C and differentiates these reactions. Yamana *et al.* (13) and Butler *et al.* (20) reached the same conclusion from the absence of significant side-chain dependency on k_0' values and by following the benzylpenicillenic acid formation rate, respectively.

Reaction Mechanism with General Base—Previous work on penicillin aminolysis (26, 27) showed that the reaction of penicillin G with various primary and secondary amines proceeds exclusively through a nucleophilic attack pathway to produce the corresponding benzylpenicilloylamides. In the present work, the reactions of penicillin G with oxygen bases were studied. A linear relationship between k_{obs} and total oxygen-compound concentration was observed (Figs. 2–5).

The second-order rate constants, k_B, thus determined at 35 and 60° from the relationship between ($k_{obs} - k_{pH}$) values and base concentrations are recorded in Table II, together with values reported by Finholt *et al.* (7).

Figure 6 shows a plot of log k_B against pKa of the conjugated acid of an oxygen base. The solid line corresponds to the Brønsted equation, log k_B = 0.56pKa − 4.41, for the unassisted nucleophilic reaction of an amine with penicillin G at 60° and μ = 0.5 (26).

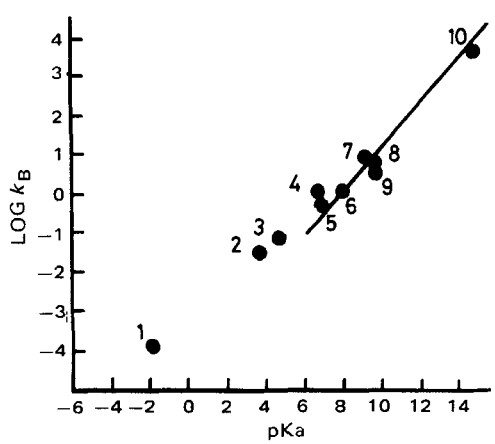


Figure 6—*Brønsted plots for the reaction of penicillin G with oxygen bases in aqueous solution at 60° and 0.5 ionic strength. The numbers refer to the bases in Table II. The solid line corresponds to the Brønsted equation, log* k_B = 0.56pKa − 4.41, *for the unassisted nucleophilic reaction of amines with penicillin G at 60° and 0.5 ionic strength (26).*

[6] R. L. Schowen University of Kansas, Lawrence, Kans., personal communication.

[7] Where $\Delta S^{\ddagger} = 2.303R[\log k_0' - \log(\kappa T/h)] + \Delta H^{\ddagger}/T$ at 35°. The k_0' value in Table I and that at 45° from Ref. 12 gave a $\Delta H^{\ddagger}$ value of 16.8 kcal/mole at 35°.

Table II—Second-Order Rate Constants for the Reactions of Penicillin G, Cloxacillin, Ampicillin, and Cyclacillin with Oxygen Bases and Nitrogen Bases at 35 or 60° and 0.5 Ionic Strength [a]

				k_B, M^{-1} hr^{-1}				
		pKa[b]		Penicillin G				
Number	Base	60°	35°	60°	35°	Cloxacillin, 35°	Ampicillin, 35°	Cyclacillin, 35°
Oxygen bases								
1	Water[c]	−1.74	−1.74	1.30×10^{-4}	1.62×10^{-5}	1.69×10^{-5} [d]	1.35×10^{-5} [e]	4.49×10^{-5}
2	HCO_2^- [f]	3.60	—	3.20×10^{-2}	—	—	—	—
3	$CH_3CO_2^-$ [f]	4.58	—	8.00×10^{2}	—	—	—	—
4	HPO_4^{2-}	6.54	6.59	0.87	7.80×10^{-2}	9.20×10^{-2} [d]	0.17[e]	—
5	p-$NO_2C_6H_4O^-$	6.80	—	0.50	—	—	—	—
6	m-$NO_2C_6H_4O^-$	8.04	—	1.00	—	—	—	—
	in deuterium oxide	8.45	—	0.75	—	—	—	—
7	$H_2BO_3^-$	8.75[g]	8.92	9.30[g]	0.47	0.92[d]	—	—
8	CO_3^{2-}	9.47	9.67	7.00	0.90	0.59	—	1.50
	in deuterium oxide	9.90	—	7.00	—	—	—	—
9	$C_6H_5O^-$	9.51	—	3.45	—	—	—	—
10	OH^-	14.76	15.42	5.39×10^{3}	1.19×10^{3}	1.34×10^{3} [d]	2.57×10^{3} [e]	1.10×10^{3}
Nitrogen bases								
11	Imidazole	6.65	6.96	0.25[h]	3.50×10^{-2} [h]	6.00×10^{-2}	0.10	3.00×10^{-2}
12	Glycine	8.87	9.41	2.50[h]	0.40	—	0.40	0.40

[a] All rates were determined by the iodometric titration method. Each reaction was carried out at three or more different buffer concentrations at each of three or more different pH values. [b] Determined by the half-neutralization method. [c] $k_0'/55.5$. [d] Reference 10. [e] Reference 9. [f] The reactions were carried out at pH 7.0 and 7.5. The pH of the reaction solution was maintained by means of a pH-stat. [g] Reference 7. [h] Reference 26.

The reactions of penicillin G with oxygen bases, except acetate, formate, and water, appear to represent a nucleophilic displacement reaction rather than a general base-catalyzed degradation, because the rate constants, k_B, for m-nitrophenol (Fig. 4) and carbonate (Fig. 5) showed only a small deuterium solvent isotope effect, with a $k_B^{H_2O}/k_B^{D_2O}$ ratio of approximately 1.0 in both cases. Nucleophilic reaction did not lead to a deuterium solvent isotope effect appreciably greater than unity (28). Water, however, probably acts as a general base catalyst as discussed on the basis of a large solvent isotope effect. The formate and acetate ions, having relatively low pKa values, also probably participate as general bases in the degradation of penicillin G, in view of the general principle (28) that a change in the mechanism from a general base-catalyzed path to a nucleophilic reaction may occur as the pKa value of the reacting base becomes greater than that for the leaving group (imino group in β-lactam in this case), as was found for an ester (29) and an amide (30).

Table II also includes k_B values from the literature (7, 9, 10, 13) and from the present study for the reaction of other penicillins with some bases. Figure 7 shows a plot of log k_B of penicillin G *versus* log k_B of other penicillins for the reaction of the same series of bases with penicillin G at 35° and $\mu = 0.5$. The solid line drawn has slope 1.0 and passes through zero. All points almost fall on this line, suggesting that alteration of the side chain of penicillins gives no significant change in the rates of their base-catalyzed degradation.

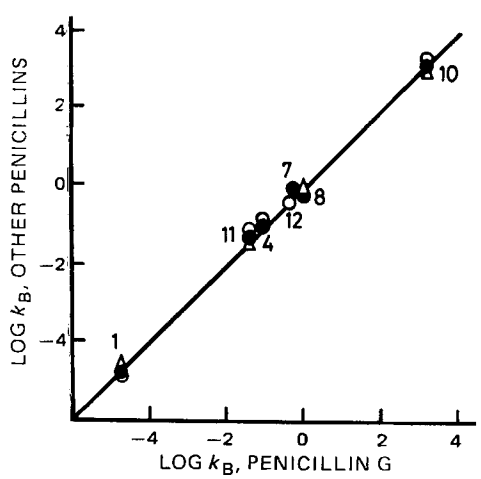


Figure 7—*Double logarithmic plots of second-order rate constants for the reaction of penicillin G with bases* versus *second-order rate constants for the reaction of other penicillins with the same bases. The numbers refer to the bases in Table II.*

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Pharmacokinetics of Methylphenidate in the Rat Using Single-Ion Monitoring GLC–Mass Spectrometry

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Abstract □ A GLC–mass spectrometric assay for methylphenidate in biological fluids was developed using the ethyl ester homolog of the drug as the internal standard. The procedure has a lower level of sensitivity of 1.2 ng/ml and is based on GLC–mass spectrometic monitoring of the *m/e* 180 ion common to the mass spectra of the *N*-trifluoroacetyl derivatives of the drug and internal standard. The brain and plasma levels of methylphenidate in rats were determined after intravenous administration of 0.5 mg/kg of the drug. The two-compartment open pharmacokinetic model fit the data.

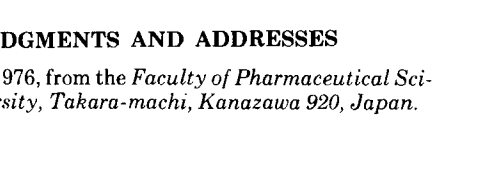
Keyphrases □ Methylphenidate—GLC–mass spectrometric analysis in biological fluids, pharmacokinetics in rats □ GLC–mass spectrometry—analysis, methylphenidate in biological fluids, pharmacokinetic study in rats □ Pharmacokinetics—methylphenidate, GLC–mass spectrometric study in rats □ Stimulants, central—methylphenidate, GLC–mass spectrometric analysis in biological fluids, pharmacokinetics in rats

Methylphenidate [(±)-*threo*-methyl α-phenyl-2-piperidineacetate, I] has pharmacological properties similar to those of dextroamphetamine (1). It is used to treat depressed states of various origins and hyperkinesis in children (2). In addition, I has become a drug of abuse in many countries (3–5).

The metabolic fate of I in humans and several other species was studied by Faraj *et al.* (6), who also reviewed previously published work. The main urinary metabolite in humans, accounting for 30% of the dose, is the hydrolysis product of I, ritalinic acid (II).

BACKGROUND

Studies on the disposition of radioactive I in the rat were reported (7), as was information on plasma I levels in humans (6, 8). While the drug appears to be essentially completely absorbed after oral administration, the concentration of I after a 20-mg oral dose of I hydrochloride was reported not to exceed 20 ng/ml of plasma (6). Plasma I levels in humans were 10–60 ng/ml 1–3 hr after a 0.4-mg/kg oral dose of I hydrochloride (8). Such low levels of I achieved after therapeutic doses may partially explain the paucity of I pharmacokinetic data.



I: $R_1 = CH_3$, $R_2 = H$
II: $R_1 = R_2 = H$
III: $R_1 = CH_2CH_3$, $R_2 = H$
IV: $R_1 = CH_3$, $R_2 = COCF_3$
V: $R_1 = CH_2CH_3$, $R_2 = COCF_3$

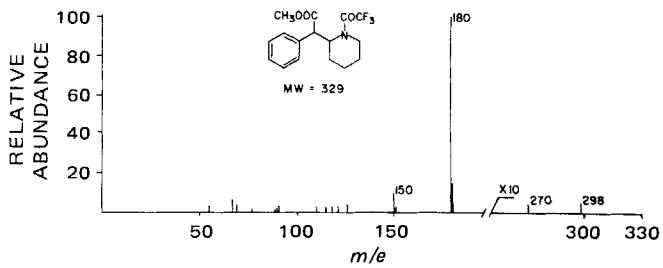


Figure 1—*Mass spectrum of IV obtained by GLC–mass spectrometry under the described conditions.*

Several analytical methods for I were reported (6–10), but none appears capable of measuring I reliably and routinely at the low levels required. *N*-Acylation of I with trichloroacetyl chloride followed by electron-capture GLC gave (9) a lower limit of detection of 9 ng/ml in plasma. Another procedure (10), also using electron-capture GLC, involved reduction of the methoxycarbonyl function of I to $-CH_2OH$ using lithium aluminum hydride, followed by derivatization with pentafluoropropionic anhydride. The sensitivity of the assay was 2 ng of I/ml of plasma. Recently, an analytical procedure for I based on GLC–mass spectrometry, with a sensitivity limit of 1.5 ng/ml in plasma, was described (8).

None of these methods (8–10) included an internal standard. A further difficulty with the published (8) GLC–mass spectrometric assay is that the procedure involved the GLC of underivatized I. Underivatized I may undergo extensive and variable decomposition in the injection port of the gas chromatograph, making quantitative determination difficult at best (11).

A new GLC–mass spectrometric assay for I in biological fluids and tissues is now described. The procedure involves the addition of the ethyl ester homolog of I, Compound III (12), to the biological medium, extraction of the drug and internal standard under mildly basic conditions, concentration of the extract, and derivatization with trifluoroacetic anhydride. GLC–mass spectrometric analysis was then performed with the mass spectrometer focused on an ion common to the spectra of both I and the internal standard. The lower limit of sensitivity of the assay is 1.2 ng of I/ml of plasma. The procedure was applied to the study of the pharmacokinetics of I in the rat, and the data obtained were examined in terms of classical pharmacokinetic models.

EXPERIMENTAL

Materials—Internal standard III was prepared *via* two different methods. A solution of II[1] (0.5 g) in 55 ml of absolute ethanol containing

[1] Ciba-Geigy Corp.