Table III—Assay Results [a] on Hydrocortisone Stored at 65°

Vehicle	Initial Concentration	Number of Days Stored	Percent Retained by HPLC	Percent Retained by USP Method
Water	20 μg/ml	14	54.9	—[b]
Water with 50% alcohol	20 μg/ml	14	71.2	—[b]
Alcohol	20 μg/ml	14	92.6	93.7
Water with 10% (v/v) glycerin	20 μg/ml	14	84.8	—[b]
Polyethylene glycol	10 mg/g	9	59.2	—
Polyethylene glycol	10 mg/g	14	13.6[c]	76.8[c]
Polyethylene glycol	5 mg/g	9	47.2	
Under basic conditions[d]	1 mg/ml	3	25.1	24.7

[a] Some other results are presented in Figs. 2 and 3. [b] It was not possible to assay by the USP method since the system must be essentially free of water. [c] There was no interference from the base even after storing at 65° for 10 days. [d] See text for details.

It is apparent that the USP method is not a stability-indicating assay method. In fact, it proved to be stability indicating only under abnormal conditions of a highly basic pH, which is rarely found in practice. The data on Lot A were further analyzed using the following relationship:

$$\frac{X_1}{C_0} = \frac{k_1}{k}(1 - e^{-Kt}) \qquad \text{(Eq. 2)}$$

where C_0 is the initial concentration of hydrocortisone in Lot A; $X_1 = (C_0 - C)$ and is the concentration of the decomposition products according to Pathway 1 since C at time t could be determined by the USP method; k_1 is the decomposition constant according to Pathway 1; and $K = k_1 + k_2$, where k_2 is the decomposition constant according to Pathway 2. The K value was determined from Fig. 4 to be 0.0988/day. By using Eq. 2, the values for k_1 and k_2 in Lot A at higher temperature were estimated to be 0.0908 and 0.008/day, respectively.

The chromatogram of Lot H at room temperature (Fig. 5B) did not indicate much decomposition because of the small peaks from the decomposition products. The low percent of hydrocortisone (55%) found may be the result of its adsorption onto the plastic container in which it was compounded. The other possibility is the adsorption of decomposition products onto the container or the other ingredients (talc and zinc oxide). None of these observations was confirmed. The ointments were compounded in a stainless steel mixer and stored in ointment jars made of opal glass.

REFERENCES

(1) H. L. Mason, *Proc. Staff Meet. Mayo Clin.*, **13**, 235 (1938).

(2) L. Velluz, A. Petit, and R. Berret, *Bull. Soc. Chim. Fr.*, **1947**, 123.

(3) D. E. Guttman and P. D. Meister, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 773 (1958).

(4) J. W. Mauger, A. N. Paruta, and R. J. Gerraughty, *J. Pharm. Sci.*, **58**, 574 (1969).

(5) T. Chulski and A. A. Forist, *J. Am. Pharm. Assoc., Sci. Ed.*, **47**, 553 (1958).

(6) A. O. Weis-Fogh and C. F. Wiese, *Arch. Pharm. Chemi*, **71**, 835 (1964).

(7) T. Takubo, T. Tadaoka, and T. Sawai, *Yakuzaigaku*, **22**, 66 (1962).

(8) A. E. Allen and V. D. Gupta, *J. Pharm. Sci.*, **63**, 107 (1974).

(9) W. C. Landgraf and E. C. Jennings, *ibid.*, **62**, 278 (1973).

(10) M. C. Olson, *ibid.*, **62**, 2001 (1973).

(11) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 566.

(12) *Ibid.*, p. 103.

(13) V. D. Gupta and A. N. Deleon, *Indian J. Hosp. Pharm.*, **10**, 141 (1973).

(14) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 238.

Demonstration of Penicillamine as a Product in Benzylpenicillenic Acid Degradation in Neutral Media Using Differential Pulse Polarography

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Received February 28, 1977, from the **Department of Medicinal Chemistry and Pharmacognosy and the* ‡*Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN 47907.* Accepted for publication May 25, 1977.

Abstract □ During the study of the temporal changes of benzylpenicillenic acid in aqueous buffers using differential pulse polarography, penicillamine was found to be a degradation product at neutral pH. Since this result was not previously reported, the effects of pH and buffer concentration on penicillamine formation were investigated. The amount of penicillamine produced was greatest under conditions producing maximum benzylpenicillenic acid stability. Penicillamine was not obtained from benzylpenicilloic acid, the reported degradation product of benzylpenicillenic acid at neutral pH. Penicillamine also was detected in penicillin G solutions of neutral pH. Therefore, it is suggested that penicillamine found in penicillin G solutions arises from benzylpenicillenic acid degradation which, in turn, is produced from penicillin G isomerization. A pathway is proposed to show that penicillamine originates from the UV-absorbing isomer of benzylpenicillenic acid.

Keyphrases □ Benzylpenicillenic acid—degradation in neutral media, differential pulse polarographic study, penicillamine demonstrated as product □ Degradation—benzylpenicillenic acid in neutral media, differential pulse polarographic study, penicillamine demonstrated as product □ Differential pulse polarography—study of benzylpenicillenic acid degradation in neutral media, penicillamine demonstrated as product □ Penicillamine—demonstrated as product of benzylpenicillenic acid degradation in neutral media, differential pulse polarographic study □ Antibacterials—benzylpenicillenic acid degradation in neutral media, differential pulse polarographic study, penicillamine demonstrated as product

Benzylpenicillenic acid, which forms spontaneously from penicillin G (benzylpenicillin), has been implicated in penicillin allergy (1). Benzylpenicillenic acid reacts with nucleophiles and has been proposed as the intermediate in the reaction of penicillin G with proteins (2, 3). The penicillamine moiety is an antigenic determinant in penicillin allergy, and penicillamine cross reacts with both penicillin G and benzylpenicillenic acid (1, 4).

It has been suggested (5–8) that benzylpenicillenic acid (I) in aqueous solutions is in equilibrium with a thiazoli-

dinyloxazolone isomer (II) that does not possess absorbance at λ_{max} 322 nm as does I (Scheme I, reaction 1). The hydrolysis of I at various pH conditions was studied, and the hydrolysis products were reported (6). Benzylpenicilloic acid (III) was the main I hydrolysis product in 0.1 *M* phosphate buffer, pH 7.5 (9).

During the study of the electrochemical behavior of I using differential pulse polarography in buffers of different pH values, a new polarographic wave was observed as the wave height of I decreased with time. The appearance of this new wave in solutions of neutral pH was of special interest since most parenteral penicillin solutions are prepared at neutral pH. The new wave that developed as the result of I degradation is penicillamine (IV). A pathway for the formation of IV from I near neutral pH is proposed.

EXPERIMENTAL

Materials—Benzylpenicillenic acid (I) was obtained commercially[1] as prepared from penicillin G by Levine's method (9).

Benzylpenicilloic acid (III) was prepared fresh as needed (10). For a 0.004 *M* benzylpenicilloic acid solution, exactly 0.143 g of penicillin G sodium was added to a 100-ml volumetric flask containing 2 ml of water. Exactly 0.8 ml of 1.0 *N* NaOH was added, and the solution was allowed to stand for 2 hr at room temperature. Then 0.8 ml of 1.0 *N* HCl was added, and the solution was diluted to volume with Sorensen pH 7.0 phosphate buffer.

Benzylpenaldic acid (V) was prepared from benzylpenicilloic acid (11). Fifty milliliters of 4×10^{-3} *M* benzylpenicilloic acid in Sorensen pH 7.0 phosphate buffer was mixed with 50 ml of 2×10^{-3} *M* mercuric chloride. The reaction was followed by UV spectrophotometry. The absorption at 277 nm decreased steadily, and benzylpenaldic acid production was taken to be complete when the absorption had disappeared completely. Only fresh solutions of benzylpenaldic acid were used.

DL-Penicillamine[2] was obtained commercially and was labeled 99+%.

Apparatus—The polarographic analyzer[3] was used in conjunction with a drop timer[4]. The polarographic cell bottom[5] was provided with a cell top[6]. A three-electrode system was used with dropping mercury as the working electrode, saturated calomel electrode[7] (SCE) as the reference electrode, and platinum wire as the counter electrode. The mercury capillary had the following characteristics. In 0.056 *M* citrate buffer, pH 6.45 (with 5% alcohol USP), at open circuit, the mercury flow rate was 2.57 mg/sec and the natural drop time was 3.0 sec at a mercury reservoir height of 78.6 cm.

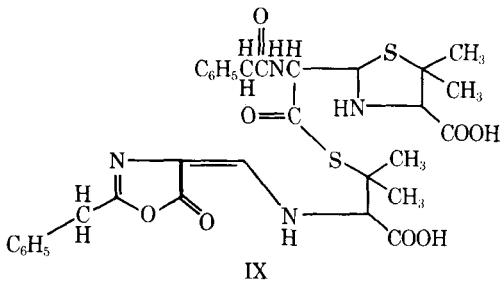
Prepurified nitrogen was used to remove oxygen from the polarographic solutions and to provide a blanket against the intrusion of air. An *x–y* recorder[8] was used to record polarographic waves.

Procedure—Common buffers were made in a standard way (12). A 0.056 *M* citrate buffer, pH 6.45, was prepared using 16.0 g of trisodium citrate dihydrate and 0.51 g of citric acid monohydrate/liter.

Because I is unstable in aqueous solutions (6, 7), stock solutions were prepared in absolute alcohol. Such stock solutions degraded slowly. Their concentrations were checked before use by measuring UV absorbance at 322 nm. Stock solutions of 5×10^{-3} *M* deteriorated at the rate of about 1%/hr at room temperature.

Penicillamine (IV) stock solutions were prepared in double-distilled water. Their concentrations changed upon aging and were checked before use by differential pulse polarography in 0.1 *M* sodium potassium phosphate buffer, pH 6.8. Penicillamine solutions of 5×10^{-3} *M* deteriorated at the rate of 0.5%/hr at room temperature.

All polarographic and UV data were obtained at 22 ± 1°. The polarographic data were secured as follows. The appropriate amount of I stock solution was transferred quantitatively to a 10-ml volumetric flask.



IX

Sufficient alcohol was added to make the final solution 5% in alcohol. Then the appropriate buffer was added to volume, and the time of buffer addition was recorded. The solution was immediately transferred to a polarographic cell, and a stream of prepurified nitrogen was bubbled through it for about 5 min. Nitrogen flow was then directed so that it formed a blanket over the solution, and recording of the polarogram was begun immediately. Recording was repeated as required, with the solution kept under a nitrogen blanket.

To estimate the amount of IV produced from the I solution in the polarographic cell, a known concentration of IV was added to the polarographic cell solution while gently bubbling with nitrogen. Bubbling was continued for about 2 min before recording of the IV wave was initiated.

Solutions of I for UV[9] studies were prepared in 10-ml volumetric flasks under a nitrogen atmosphere. Deaerated buffer was mixed with sufficient deaerated alcohol to produce a solution 5% in alcohol, and a sample of deaerated I stock solution was added to the mixture. The resulting solution was immediately transferred under a nitrogen atmosphere to a UV cell[10] provided with ground-glass stoppers.

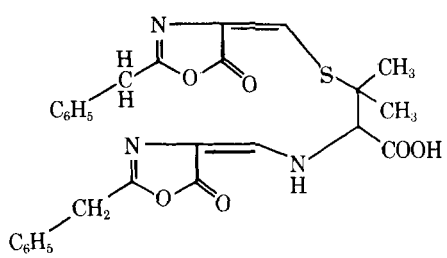
RESULTS AND DISCUSSION

The commercial I used gave a molar absorptivity of 21,000 at λ_{max} of 322 nm, which is lower than the 30,500 value reported for the pure compound (7). The low value was attributed in part to the presence of water of hydration. A trace amount of III was detected in the product using TLC [silica gel[11]: 1-butanol–water–acetic acid (66:17:17); with R_f values of 0.39 for III and 0.70 for I]. Compound IV, however, was not detected in fresh samples using either TLC or differential pulse polarography.

Derivatives of I such as the benzylpenicilloylpenicillenic acid derivative (IX), absorbing at λ_{max} 322 nm, have been proposed (7). Because these derivatives do not possess the sulfhydryl group, they are more stable than I in solution (5–7). To determine if these compounds were present in the samples of I, solutions were prepared in pH 2.32 McIlvaine buffer and studied using UV spectrophotometry. The UV absorbance decreased rapidly due to I degradation. A stable absorbance at λ_{max} 350 nm was observed, however, and this absorbance was attributed to a compound (X) proposed previously (6). The absorbance of this compound in the product used in this research was established at 6% of the total absorbance at 322 nm.

The I concentrations reported are corrected values. All were calculated on the basis that the molar absorptivity of pure I was 30,500. Correction for the presence of X was made by subtracting 6% of the total absorbance at 322 nm in absolute alcohol.

Compound I gave an anodic wave due to its sulfhydryl group in buffers at various pH values. The electrode reaction of a sulfhydryl group involves oxidation of mercury in the presence of the sulfhydryl into mercury mercaptide (13, 14). Compound I was studied using three modes of po-



X

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] PAR model 174A, Princeton Applied Research Corp., Princeton, N.J.
[4] PAR model 174/70.
[5] PAR model 9301.
[6] PAR model 9300.
[7] Model 3-712, Coleman Instruments, Oak Brook, Ill.
[8] Model 2000, Omnigraphic, Houston Instruments, Bellaire, Tex.
[9] Cary 17 spectrophotometer, Cary Instruments, Monrovia, Calif.
[10] Model 193-QS Hellma cells, Hellma Cells Inc., Jamaica, N.Y.
[11] Precoated TLC silica gel plates, E. M. Laboratories, Elmsford, N.Y.

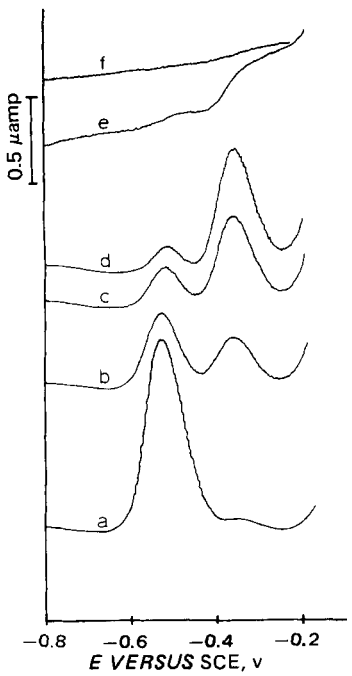


Figure 1—*Polarograms of 7.10 × 10^{-5} M I in 0.1 M sodium potassium phosphate buffer, pH 6.80. The age of solution when recording started (in seconds) was: a, 900; b, 2700; c, 4500; d, 6300; e, 6750; and f, 7150. Polarograms a–d were recorded in the differential pulse mode, e was recorded in the pulse mode, and f was recorded in the dc mode. The drop time was 2 sec, the scan rate was 2 mv/sec, and the pulse amplitude was 25 mv. The low-pass filter was at off for differential pulse and pulse modes but was at 3.0 for the dc mode.*

larography: dc polarography, pulse polarography, and differential pulse polarography. Well-defined waves were observed in all three modes, but differential pulse polarography was the most sensitive. One or two waves were observed depending on the concentration of I taken, the mercury electrode capillary, the height of the mercury reservoir, and the drop time used (with the drop timer used, various automatic drop times could be used for the same capillary and the same mercury reservoir height).

The objective of this study was not to present a detailed account of the electrochemical properties of I but rather to investigate changes in the polarograms of I solutions during aging in aqueous buffer preparations and to report on the development of a new polarographic wave that appeared as the wave of I decreased. To follow the development of the new wave with time, it was necessary to select concentrations of I giving only a single wave because the second wave of I and the IV wave were not resolvable, as will be shown.

When a I polarogram at pH 6.80 was followed during aging, the wave due to I decreased with time and a new wave developed on the anodic side of the I wave. Typical polarograms of a I solution during aging are shown in Fig. 1; polarograms e and f demonstrate that the differential pulse mode is a more sensitive technique than either the pulse mode or the direct current mode.

The polarographic peak that appeared during the aging of I was due to the appearance of IV and not to the formation of III, the reported degradation product of I at neutral pH (6, 9). Polarographic analyses of III solutions did not exhibit a wave at the potential of the I wave or at the potential of the second increasing wave. When the polarography of a penicillin G solution at neutral pH was undertaken, two waves were seen immediately after solution preparation: one at the potential of the I wave and the other at the potential of the second wave of the I polarogram. In the penicillin G solution polarograms, both polarographic peak heights increased with time.

When IV was added to either the I or the penicillin G solution, the IV wave appeared at exactly the same peak potential as the second wave of I or penicillin G. The peak potential of IV in a given buffer changed slightly with its concentration and also with the presence of adsorbents. The peak potential of IV shifted in the anodic direction when it was added to a I or penicillin G solution, probably because of some adsorbing impurities in the preparations. The I used was prepared from penicillin G, and common penicillin products contain impurities of protein origin that act as adsorbents (15). Thus, to compare the peak potential of an authentic sample of IV and the peak potential of the second wave, IV must be added to the I or penicillin G solution.

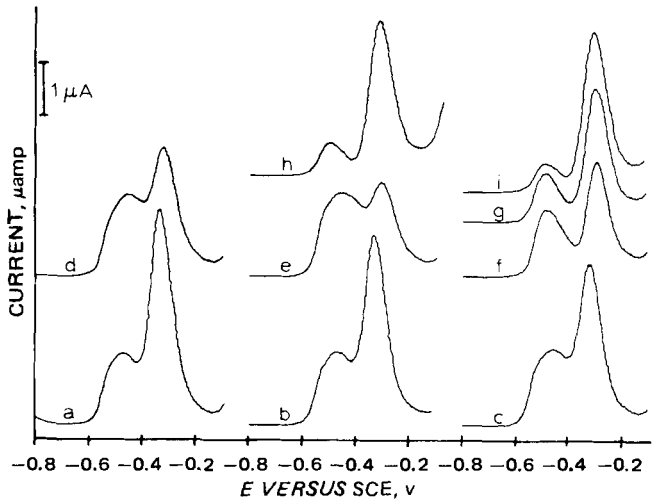


Figure 2—*Change of differential pulse polarogram of 2.0 × 10^{-4} M I in 0.056 M citrate buffer, pH 6.45, giving two peaks with time. The age of solution when recording started (in seconds) was: a, 400; b, 900; c, 1600; d, 2600; e, 4600; f, 7500; g, 9400; h, 11,700; and i, 12,300. Instrument settings were as in Fig. 1.*

When TLC was used with I solutions that showed the second wave, no IV spot could be detected. However, IV spots on TLC could be seen with concentrated penicillin G solutions (about 3 × 10^{-1} *M*) aged for several days and showing IV waves. TLC of such penicillin G solutions also showed V [silica gel[12], 1-butanol–water–acetic acid (66:17:17); R_f 0.48 for IV and 0.31 for V]. The I solutions used for polarography were too dilute to show IV by TLC. It was not possible to achieve concentrated I solutions because of the limited solubility of the compound in aqueous buffers of neutral pH. Based on the polarographic and TLC results, the second wave seen in I solutions and also in penicillin G solutions is believed to be due to IV.

When a sufficiently high concentration (above 1.0 × 10^{-4} *M*) of I was employed, two waves were obtained for I. The first wave was the one seen in low concentrations, and the second wave was due to the additional I. Once the second wave started to show, the height of the first wave remained unchanged with increases in concentration. The potential of the second wave of I was very close to the potential of IV, and the two could not be resolved. Therefore, it was difficult to follow the production of IV in such concentrated I solutions (Fig. 2).

In polarogram a of Fig. 2, the two waves belong to I. From polarograms a through e, the second wave decreased while the first remained unchanged. From f through i, the first wave of I started to decrease, indicating that the second wave of I had all disappeared. The second peak seen in this case was due to IV produced from I and, thus, was increasing while the I was decreasing. Up to the time that the first wave of I started to decrease, the second wave was due to both IV and I; it would be difficult to measure the amount of IV in this period.

It was known from the literature that phosphate buffer species accelerate I degradation (6, 7). Hence, the effect of acceleration of I degradation of IV production was studied. The production of IV from I in pH 6.8 sodium potassium phosphate buffers of concentrations ranging from 0.1 to 1.0 *M* was determined. The amount of IV produced increased with time and then leveled off. As expected from the difference in the rate of I degradation, IV production in the 1.0 *M* phosphate buffer leveled off earlier than in the 0.1 *M* buffer.

The amounts of IV produced in different concentrations of pH 6.8 phosphate buffer after practically all I had disappeared are shown in Fig. 3 (the I solutions were followed until about 95% of the I had disappeared; at this time, the variation of IV concentration with time was negligible). For a given concentration of I, the amount of IV produced decreased with an increase in phosphate concentration, which, therefore, means that the amount of IV produced was less under conditions in which I was least stable.

Compound I is most stable at about pH 6.0, and its degradation rate increases as the pH is lowered or raised from this value (6, 7). It was of

[12] Precoated silica gel 1B, J. T. Baker Chemical Co., Phillipsburg, N.J.

Table I—Variation of Penicillamine Produced, Expressed as the Mole Fraction of the Initial Concentration (6.5×10^{-5} *M*) of Benzylpenicillenic Acid, with pH

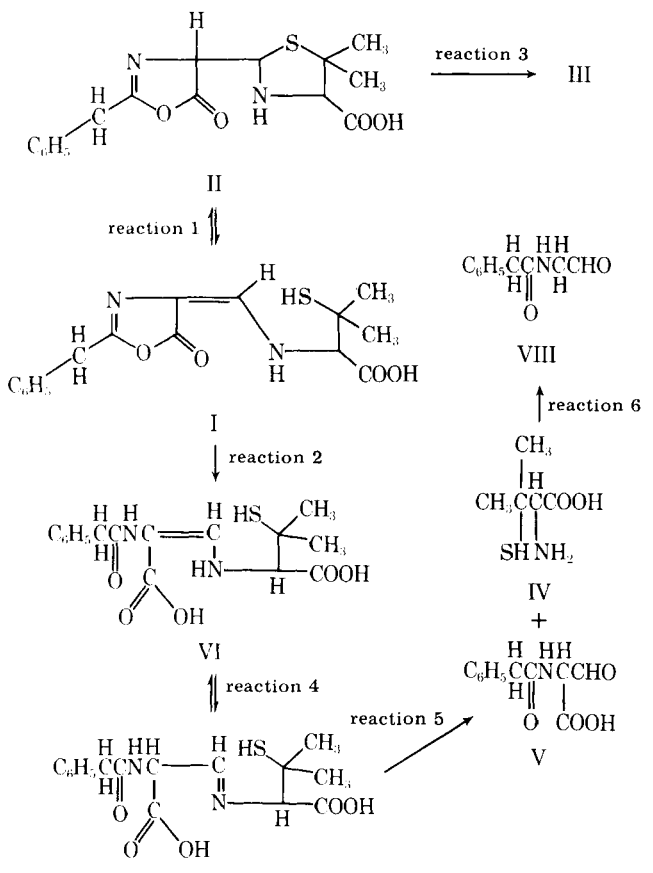
Buffer	pH	Mole Fraction of Penicillamine
Sorensen's glycine	8.7	0
McIlvaine	7.5	0.13
	5.8	0.37
	3.8	0.07
	2.3	0

interest to determine the effect of pH on IV production. Among the buffers listed in Table I, I was most stable in pH 5.8 McIlvaine buffer; the amount of IV produced was the largest in this buffer. The amount decreased as the pH was lowered or raised. These results, coupled with those presented in Fig. 3, show that the conditions favoring I stability also favor IV production.

The finding that IV was produced from I near neutral pH was interesting and was not reported previously (6, 9). Longridge and Timms (6) studied I degradation products in a wide range of pH values and reported III as the only degradation product at neutral pH. Since these workers used TLC for identification of non-UV-absorbing substances, the insensitivity of the detection method probably failed to show the presence of the non-UV-absorbing substance IV.

Levine (9) also reported III as a I degradation product in 0.1 *M* phosphate buffer, pH 7.5, and did not mention the presence of IV. Compound III was identified by converting it to IV and benzylpenilloaldehyde (VIII) (α-decarboxylation product of V, Scheme I) in the presence of mercuric chloride. The yield of III was based on the estimation of VIII. The IV produced directly from I (and also the resulting VIII) would be confounded by that obtained through conversion of III.

To determine whether IV came directly from I or from its major degradation product, III, polarograms of III were studied during aging under



Scheme I

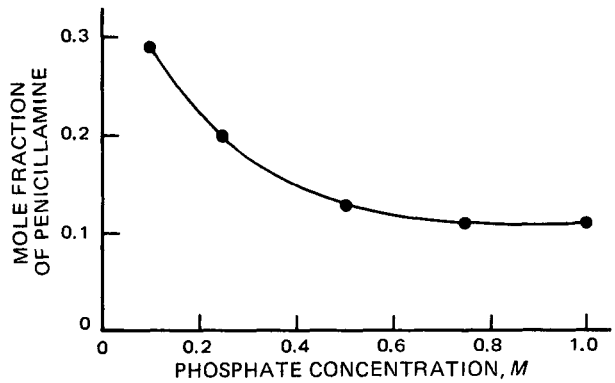


Figure 3—*Penicillamine produced during degradation of* 6.5×10^{-5} M *I in sodium potassium phosphate buffer, pH 6.8, as a function of phosphate concentration. Penicillamine is expressed as a mole fraction of the I concentration at* t = 0.

the same conditions as for I. A IV wave did not develop in III solutions, which means that IV was obtained from I or II. This result suggests that, in addition to the pathway normally proposed for I degradation at neutral pH (6, 7), another pathway involves IV production.

In Scheme I, the main pathway as reported starts with reaction 1. An additional pathway appears to exist; it starts with reaction 2 to produce benzylpenamaldic acid (VI) from the UV-absorbing isomer of I. Reactions 4 and 5 of VI are well known (10, 16). Since development of a UV band at 280 nm due to VI was not observed, it is believed that the rate of reaction 5 is much faster than the rate of reaction 2 and that there is no significant accumulation of VI.

This research has shown that IV found in penicillin G solutions arises from I formed from penicillin G. Parenteral penicillin G solutions containing I also are likely to contain IV. Since IV is reported to have a role in penicillin allergy, it is recommended that quality control procedures of such penicillin preparations include tests not only for the presence of I but also for IV. This testing could be accomplished by using differential pulse polarography.

REFERENCES

(1) M. A. Schwartz, *J. Pharm. Sci.*, **58**, 643 (1969).

(2) B. B. Levine and Z. Ovary, *J. Exp. Med.*, **114**, 875 (1961).

(3) E. S. Wagner, W. W. Davis, and M. Gorman, *J. Med. Chem.*, **12**, 483 (1969).

(4) G. A. Caron, *Immunology*, **6**, 81 (1963).

(5) J. L. Longridge and D. Timms, *J. Chem. Soc. B*, **1971**, 848.

(6) *Ibid.*, **1971**, 852.

(7) K. H. Dudley, T. C. Butler, and D. Johnson, *J. Pharmacol. Exp. Ther.*, **179**, 505 (1971).

(8) H. Bundgaard, *J. Pharm. Sci.*, **60**, 1273 (1971).

(9) B. B. Levine, *Arch. Biochem. Biophys.*, **93**, 50 (1961).

(10) M. A. Schwartz and A. J. Delduce, *J. Pharm. Sci.*, **58**, 1137 (1969).

(11) E. V. Brown, in "The Chemistry of Penicillin," H. T. Clarke, J. R. Johnson, and B. Robinson, Eds., Princeton University Press, Princeton, N.J., 1949, pp. 473–534.

(12) "Scientific Tables," 7th ed., K. Diem and C. Lentner, Eds., Ciba-Geigy Ltd., Basel, Switzerland, 1970, pp. 280–282.

(13) W. Stricks and I. M. Kolthoff, *J. Am. Chem. Soc.*, **74**, 4646 (1952).

(14) F. Peter and R. Rosset, *Anal. Chim. Acta*, **79**, 47 (1975).

(15) G. T. Stewart, *Lancet*, **1**, 1177 (1967).

(16) R. B. Woodward, A. Neuberger, and N. R. Trenner, in "The Chemistry of Penicillin," H. T. Clarke, J. R. Johnson, and B. Robinson, Eds., Princeton University Press, Princeton, N.J., 1949, pp. 415–439.

ACKNOWLEDGMENTS

Supported in part by the African-American Institute and General Research Support Grant 5S01RR05586-07.