solvent front, but we have not been able to resolve this into a discrete chromatographic locus. The metabolites that can be recovered from the plasma, the diastereoisomers of reduced warfarin and 7-hydroxy warfarin, were described at the June 1970 conference on Drug Metabolism in Man (4). Metabolites of warfarin were identified in human urine as well (5).

Second, these authors (1) contend that an assay of warfarin based on chromatographic isolation of unchanged drug offers no advantage over methods relying solely on differential extraction. The interested reader should make reference to our formal report on the assay of warfarin (3). The most pertinent aspects of this paper noted consistent (five of five subjects) differences when data from a fluorimetric assay (6) or a spectrophotometric assay (7) were used to determine apparent plasma clearance rates. When the spectrophotometric method was compared to the TLC method as we described it, the former indicated slower clearance rates in two of four subjects: $T_{1/2}$ of 57.5 hr. *versus* 43.1 hr. and $T_{1/2}$ of 36.2 hr. *versus* 30.5 hr. were determined by the least-squares best fit line relating log warfarin concentration to time.

Since we have no experience with the TLC assay of Welling *et al.* (1), it is impossible to assess adequately the possible sources of error. In common with these investigators, we noted that outdated blood bank plasma gives higher blank values in the spectrophotometric assay than does freshly drawn citrated plasma. For this reason, we prepare all plasma ourselves. The high blank values may have contributed to the discrepancy in their results. In Table I of their report (1), the slope of the line relating net absorbance to warfarin concentration is 0.182. Blank plasma in their hands resulted in a net absorbance of 0.216 ± 0.056 or 0.261 ± 0.024, the equivalent of 1.2 ± 0.3 or 1.4 ± 0.1 mcg. "warfarin"/ml. plasma. Since warfarin has an apparent volume of distribution of 8–9 l. calculations from the 25-mg. dose would indicate that the maximum plasma concentration of their samples would be about 3 mcg./ml. at T_0. Subsequent values would be much less. Thus, these investigators were working at plasma levels at most only 2–3 times their blank. In our studies, we employed a dose of 1.5 mg. warfarin/kg. body weight, which produced T_0 concentrations of about 12 mcg./ml. Our values for blank plasma were the equivalent of 0.15 mcg./ml. plasma.

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Assay of Warfarin: A Rebuttal

Keyphrases ☐ Warfarin—small, single oral dosage plasma levels, TLC and spectrophotometric assays ☐ Plasma levels—warfarin, small oral dosage ☐ TLC—assay, warfarin

Sir:

Dr. Lewis' communication (1) refers to the warfarin report of Welling *et al.* (2) and his own published reports (3, 4). Concerning the application of our methods, Dr. Lewis and I are not nearly so far apart as his communication tends to indicate. We clearly indicated that our results were obtained following oral administration of 10- and 25-mg. single doses of sodium warfarin. We clearly stated that our results may not apply after administration of large single doses nor after multiple doses of sodium warfarin. However, under the conditions of our studies, metabolites of warfarin were not present in detectable amounts in plasma. Furthermore, the modified O'Reilly assay and TLC did give equivalent results, as shown in Fig. 1 of our paper (2). I have no doubt that following large single doses, such as 1.5 mg./kg. as administered by Lewis and Trager (5), or when assaying plasma samples obtained after multiple doses of warfarin, warfarin metabolites are detectable as shown by Lewis *et al.* (3, 4).

The TLC procedure that we used was almost identical to the method used by Lewis *et al.* (3, 4). The adsorbent in both cases was silica gel, but they used Eastman No. 6061 silica gel G TLC sheets, while we used our own 20 × 20-cm. plates with a 250-μ thickness of silica gel GF 254. The solvent was 9:1 (v/v) 1,2-dichloroethene–acetone in both cases. Our silica gel contained a fluorescent indicator, while theirs did not. However, both we and they reported essentially the same R_f value (0.50–0.54) for unchanged warfarin. We reported clean separations of the 4′-OH, 6-OH, and 7-OH metabolites of warfarin, with R_f values of 0.22, 0.18, and 0.23, respectively. Lewis and Ilnicki (3), following an unspecified dose of warfarin, reported separation of two metabolites from plasma (R_f 0.23 and 0.19). Lewis *et al.* (4) displayed a photograph showing separation of a mixture of diasterisomeric warfarin alcohols from warfarin on their plates. The evidence suggests strongly that after 10- and 25-mg. doses of warfarin, metabolites are not detectable in plasma as we concluded, whereas after higher doses the metabolites are obviously detectable as Lewis *et al.* (4) showed. The discrepancy appears not to lie in any basic difference in the TLC procedures employed but rather in the doses administered. The reason why our blank plasma samples gave spots on the thin-layer plates, whereas Lewis *et al.* (4) never observed such spots, is unknown. The difference may or may not be due to the fluorescent indicator.

Two other comments made by Lewis (1) deserve special attention. Lewis stated: "When the spectrophotometric method was compared to the TLC method as we described it, the former indicated slower clearance rates in two of four subjects: $T_{1/2}$ of 57.5 hr. *versus* 43.1 hr. and $T_{1/2}$ of 36.2 hr. *versus* 30.5 hr. were determined

Table I—Half-Lives of Warfarin in Hours Estimated from Terminal Plasma Concentrations[a] Measured by Modified O'Reilly Assays and TLC Assays on Same Samples[b]

Subject	Treatment A (5 5-mg. Tablets) TLC	O'Reilly	Normalized Difference, %[c]	Treatment B (1 25-mg. Tablet) TLC	O'Reilly	Normalized Difference, %
1	33.6	28.8	−15.4	35.1	34.3	−2.3
2	24.6	27.1	9.7	29.9	29.7	−0.7
3	34.8	47.2	30.2	42.7	55.0	25.2
4	26.8	33.7	22.8	29.5	26.2	−11.8
5	40.8	31.1	−27.0	41.4	56.9	31.5
6	—	—	—	33.7	34.5	2.3
6a	49.5	44.2	−11.3	—	—	—
Averages	35.0[d]	35.4[d]	1.5	35.4[e]	39.4[e]	7.4

	TLC	O'Reilly	Normalized Difference, %
Overall averages	35.2[f]	37.4[f]	4.5

[a] Half-lives estimated by obtaining the slope of line, by the method of least squares, when ln C_p plotted *versus* t and dividing the absolute value of the slope into 0.693. Only plasma concentrations corresponding to times equal to or greater than 24 hr. were employed. [b] Data plotted in Fig. 4 of Welling *et al.* (2). [c] Normalized difference = half-life from O'Reilly assays − half-life from TLC assays/average half-life from O'Reilly and TLC assays × 100. [d] Difference in averages is not significant by paired t-test ($t = 0.097$, $p > 0.25$). [e] Difference in averages is not significant by paired t-test ($t = 1.27$, $p > 0.10$). [f] Difference in averages is not significant by paired t-test ($t = 0.95$, $p > 0.25$).

by the least-squares best fit line relating log warfarin concentration to time." The reader should realize that "two of four subjects" are not sufficient to make a decision that one assay leads to different half-lives than another assay, as was clearly shown in Fig. 4 of our original paper (2). Data plotted in that Fig. 4 are detailed in Table I of this communication. The modified O'Reilly assay gave a longer half-life (slower clearance rate) in six trials, but the TLC assay gave a longer half-life in exactly six other trials. The statistics presented in Table I indicate that the difference in average half-lives obtained by the two methods is not significant ($p > 0.25$). These data also show that the particular tablets administered did not affect the half-lives obtained, as would be expected since the half-lives were estimated after absorption ceased. A measured half-life includes assay error effects and is not an "absolute number" as many scientists would like it to be. Table I clearly shows this. One must have a much larger sample than Lewis' "two of four" to imply conclusions such as he did.

Lewis, in commenting on our blank values in the assay, also forgot that a blank is a function of not only the concentration of extraneous materials that absorb at the $\lambda_{max.}$ of the warfarin but also of the pathlength of the cell used. One cannot compare on a microgram equivalent of warfarin per milliliter (C) basis only but must compare on a C/L basis, where L is the pathlength of the cell used. This was done in our original paper (2) when we showed that our blank values were really essentially the same as those reported by O'Reilly *et al.* The average net absorbance of our subjects' zero-hour plasma was reported as 0.216, which is equivalent to 1.18 mcg. warfarin/ml., but our pathlength was 7.5 cm. Hence, our $C/L = 1.18/7.5 = 0.157$. Lewis, in his communication, gave a value of $C/L = 0.15/1 = 0.15$. Hence, the figures are essentially the same.

A key paper cited by Lewis (1) (his Reference 4) is still not published and has not been available to us.

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Kinetic Demonstration of a Metastable Intermediate in Isomerization of Penicillin to Penicillenic Acid in Aqueous Solution

Keyphrases □ Benzylpenicillin methyl ester, isomerization—metastable intermediate kinetics □ Penicillin to penicillenic acid isomerization—metastable intermediate demonstrated

Sir:

Although several kinetic stability studies on penicillins have been performed [*e.g.*, benzylpenicillin (1, 2), phenethicillin (3), methicillin (4), ampicillin (5), and cloxacillin (6)], only a few studies have dealt with the mechanism of the hydrolytic reactions. As a part of a study concerning chemical reactions possibly involved in penicillin allergy, this paper reports preliminary results about the mechanism by which benzylpenicillin methyl ester in aqueous solution isomerizes to methyl benzylpenicillenate.

On the basis of experimental data by Krejci (7), Schwartz (8) showed that the degradation of benzylpenicillin in acidic aqueous solution is characterized by two parallel reactions. The formation of penicillenic acid is thought to be a result of the hydrogen-ion-catalyzed hydrolysis of the penicillinate ion or the kinetically equivalent uncatalyzed rearrangement of undissociated penicillinic acid.

It has now been found that the formation of penicillenic acid from the penicillin molecule goes through a metastable intermediate and that both undissociated and dissociated penicillinic acid are isomerized but to a different extent. In this brief report, only the results obtained with benzylpenicillin methyl ester are presented;