six or more interfaces above those of the minimum pathway.

Until now the time element has not been seriously considered in these deliberations. Since the main interest in work of this kind lies in the relative potencies of drugs as measured in a standard biological test, it is fair to limit our discussion to only those tests in which the observation period is the same in each instance. Thus, in the measurement of potency, time is considered to be held constant. In looking at the time variable one quickly realizes that the observation period is normally set by previous experience in dealing with drugs of the type being studied. So to begin with it is known that the test system allows sufficient time for at least some drug molecules to reach the receptors. Now if there are no membranes in the biological system, and a drug is administered as a concentrated dose in a small region at one end of the system, the time it takes for one of these added molecules to reach the other end is defusion controlled, and is a function of the molecules' average velocity. At a constant temperature, molecules of the same mass but of different chemical structures will have the same average velocities (there may be exceptions of course); but even if one molecule has twice the mass of another it will take it only 1.414 times as long to travel the same distance. Hence, other things being equal, the lighter molecules will have a slightly higher probability of reaching a particular distant point than the heavier ones in a given period.

However, these differences are negligible compared to those introduced when membranes and partition coefficients are considered, as is well known. The introduction of membranes to the system does not really alter a drug's rate of travel, what it does is to effectively increase the distance it must cover. It may be repelled at an interface; in order to penetrate it, a molecule must wander about randomly until, by chance, it strikes the interface again. The molecule may have to repeat this process many times. If it is not very successful, it will never reach the receptor to help effect the biological response; after all, there is a time limit.

At this point one may appreciate the above argument on equivalent most-probable routes. Those molecules which travel by the routes of greatest probability will be overwhelmingly represented at the receptor. Almost all others are wasted. What is important in determining the final outcome is the particular pathway the drug molecule travels, and the probability that a particular molecule penetrates an interface upon collision.

Suppose two molecules with different partition coefficients happen to follow exactly the same path, and are repelled at various interfaces exactly the same number of times: each molecule arrives at the receptor region at approximately the same time (we make a small correction for the different masses). An interesting event, but surely the molecule whose partition coefficient is least favorable has overcome greater odds to achieve its status. In a particular test system practically all molecules which reach the receptor travel by equivalent most-probable routes and arrive at approximately the same time. The problem therefore reduces to determining the probability that particular molecules will follow the most favorable routes, and this of course is done by the arguments leading to eq 15. Higher doses of drugs with unfavorable partition coefficients must be administered in order to offer more chances for enough molecules to get through to the receptor in time to effect a response. The foregoing argument may not apply to all possible types of drugs, but it is probably valid for many common cases.

Acknowledgments.—Thanks are due to Dr. L. H. Conover of these laboratories for his encouragement in preparing this work for publication.

Mass Spectral Analysis in the Identification of Human Metabolites of Warfarin[1]

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The mass spectra of warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin], a deuterium labeled analog, 5 hydroxylated analogs, and a reduced side chain analog are discussed and mechanistic rationalizations are given for the major fragmentation processes observed. These data together with uv, tlc, and synthetic data are used to identify and establish 6- and 7-hydroxywarfarin and the two diastereoisomers of 3-[α-(2-hydroxypropyl)benzyl]-4-hydroxycoumarin as metabolites of warfarin in normal man.

Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin, **1a**] is an oral anticoagulant commonly employed in this country. Among the problems encountered in its clinical use is the occasionally inordinate difficulty in the maintenance of a stable degree of anticoagulation; as a consequence hemorrhage can result from the same drug dose that previously produced acceptable hypoprothrombinemia. In addition the magnitude of the anticoagulant effect appears to be extremely sensitive to the influence of other drugs. These effects can usually be correlated with changes in the plasma clearance rate of the coumarin anticoagulants. For example[2] several

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(2) (a) H. M. Solomon and J. J. Schrogie, *J. Pharmacol. Exp. Ther.*, **154**, 660 (1966); (b) L. K. Garrettson, J. M. Perel, and P. G. Dayton, *J. Amer. Med. Ass.*, **207**, 2053 (1969).

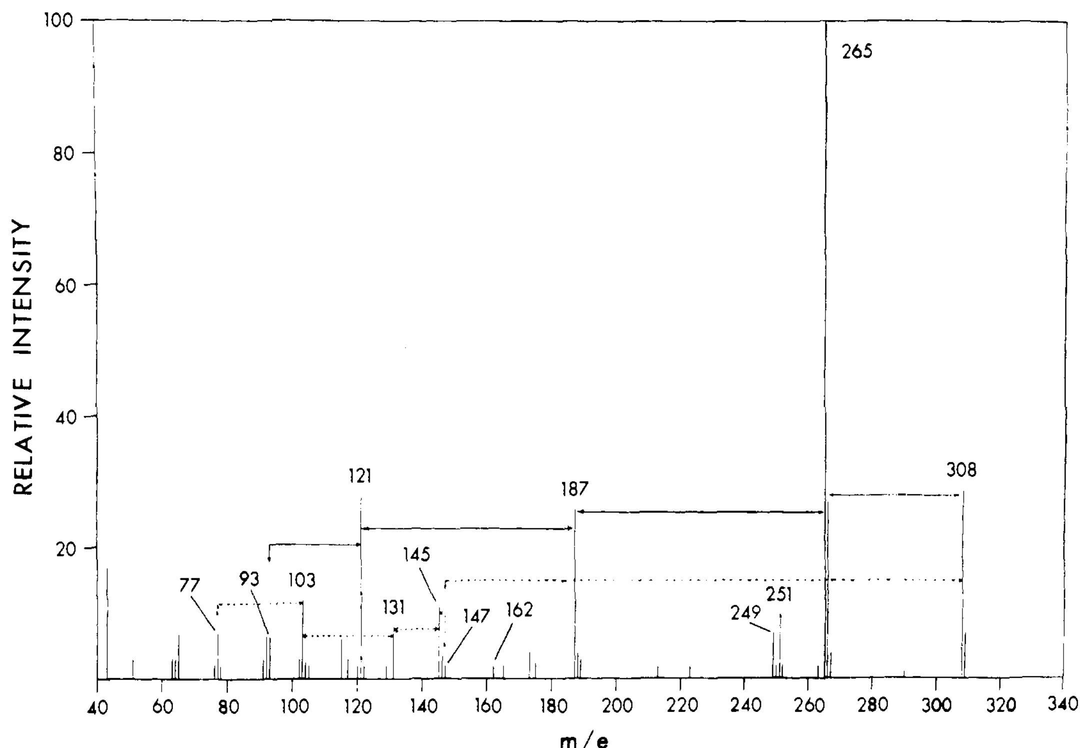
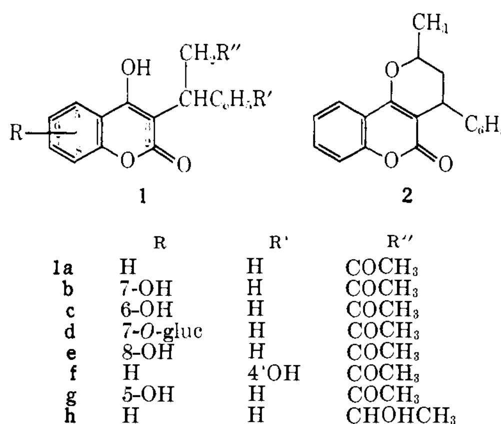


Figure 1.—Mass spectrum of **1a**; (↔) indicates the fragmentation route shown in Scheme I, (↔) indicates the fragmentation route shown in Scheme III.

drugs which decrease the plasma clearance rate of the anticoagulant increase anticoagulant response. Conversely several drugs which increase the plasma clearance rate of the anticoagulant decrease the anticoagulant response.[3] However, several drugs exhibit paradoxical behavior in this regard. Phenylbutazone increases the plasma clearance rate of warfarin but at the same time increases the hypoprothrombinemic effect.[4] On the other hand, chlorobetaine[3] significantly increases the plasma clearance rate but has no effect on prothrombin response. It would seem possible that the metabolism of warfarin or changes in its metabolism could account for at least some of these effects. Unfortunately, nothing is known about the metabolism of warfarin in normal man.

In 1962 O'Reilly[5] proposed the 7-hydroxy derivative of warfarin **1b** as a metabolite. His suggestion was corroborated later by Hermodson and Link[6] who were able to isolate and identify **1b** and 6-hydroxywarfarin (**1c**) from the urine of an unusual patient[7] with hereditary resistance to warfarin. The metabolic fate of warfarin in the rat[6,8] has been well studied and 6-, 7-, the glucuronide of 7-, 8-, and 4′-hydroxywarfarin, **1c**, **b**, **d**, **e**, and **f**, respectively, as well as 2,3-dihydro-2-methyl-4-phenyl-5-oxo-γ-pyrano[3,2-c][1]benzopyran (**2**) have been identified. Cross species extrapolation of such data however is hazardous. An investigation of the metabolic fate of warfarin in normal man was thus initiated.



	R	R′	R″
1a	H	H	$COCH_3$
b	7-OH	H	$COCH_3$
c	6-OH	H	$COCH_3$
d	7-*O*-gluc	H	$COCH_3$
e	8-OH	H	$COCH_3$
f	H	4′OH	$COCH_3$
g	5-OH	H	$COCH_3$
h	H	H	$CHOHCH_3$

Mass Spectroscopy.—To establish a capability for elucidating the structures of unknown metabolites it was essential that a detailed study of the behavior of warfarin upon electron impact be carried out. The mass spectrum of **1a** is presented in Figure 1. The major fragmentation routes are supported by exact mass measurements, metastable scanning, and specific deuterium labeling (Schemes I, III, and IV). Specific structures and mechanisms have been invoked for the formation of the fragment ions primarily to produce a self-consistent rationale for the interpretation of the spectrum and are to be regarded as reasonable possibilities only. The base peak of the spectrum ($M^+ - 43$) (Figure 1) arises from initial ionization of the side chain C=O followed by heterolytic cleavage and loss of an acyl radical to form the dihydrofuranooxonium ion

(3) M. G. MacDonald, D. S. Robinson, D. Sylwester, and J. J. Jasse, *Clin. Pharmacol. Ther.*, **10**, 80 (1969).
(4) P. M. Aggeler, R. A. O'Reilly, L. S. Leong, and P. E. Kawitz, *N. Engl. J. Med.*, **276**, 496 (1967).
(5) R. A. O'Reilly, P. M. Aggeler, M. S. Hoag, and L. S. Long, *Thromb. Diath. Haemorrh.*, **8**, 82 (1962).
(6) M. A. Hermodson, "Biochemical Studies on Warfarin," Ph.D. Thesis, University of Wisconsin, Madison, Wis., 1968.
(7) R. A. O'Reilly, P. M. Aggeler, M. S. Hoag, L. S. Leong, and M. L. Kropatkin, *N. Engl. J. Med.*, **271**, 809 (1964).
(8) (a) W. M. Barker, "The Metabolism of 4-^{14}C-Warfarin Sodium in the Rat," Ph.D. Thesis, University of Wisconsin, Madison, Wis., 1965; (b) K. P. Link, D. Berg, and W. M. Barker, *Science*, **150**, 378 (1965); (c) W. M. Barker, M. A. Hermodson, and K. P. Link, *J. Pharmacol. Exp. Ther.*, **171**, 307 (1970).

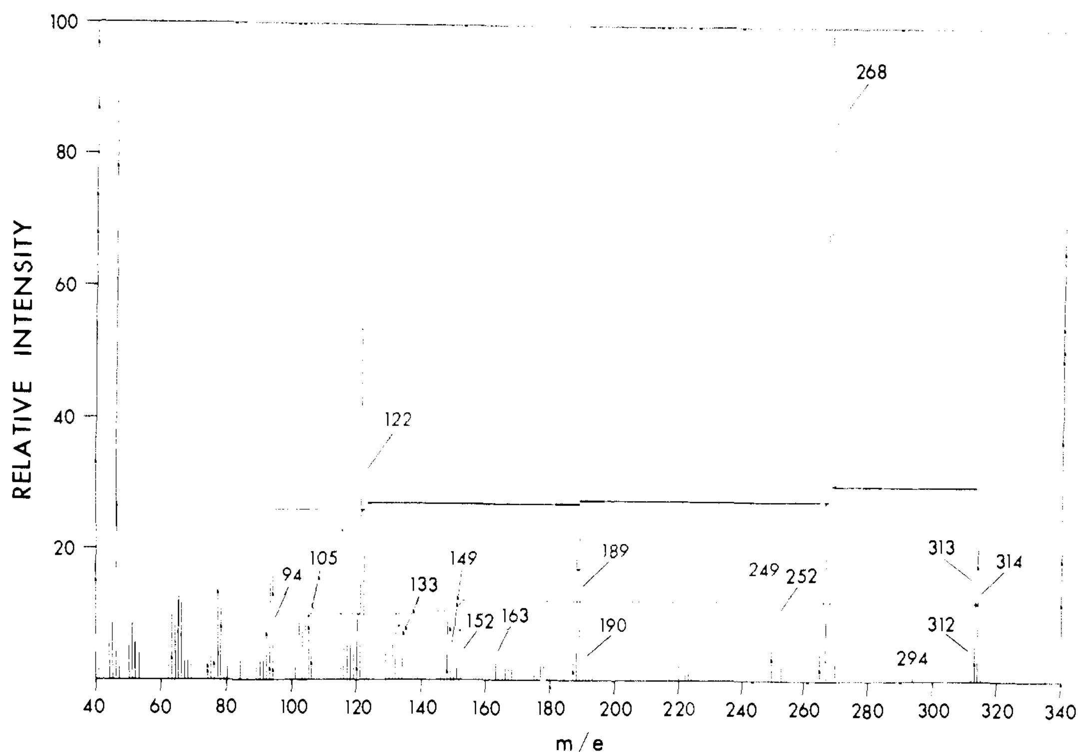
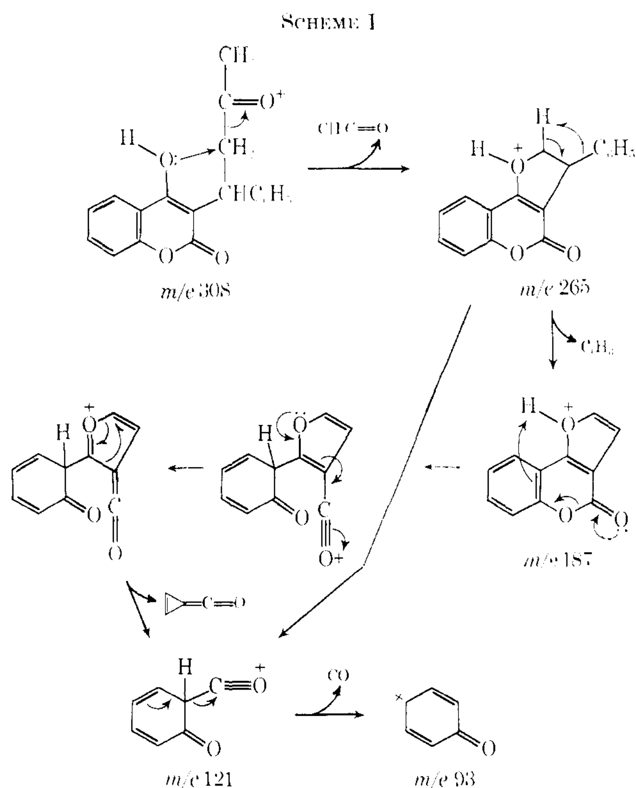
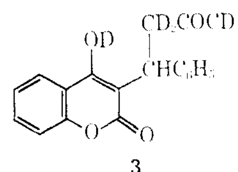


Figure 2.—Mass spectrum of **3**; (↔) indicates the fragmentation route for Scheme I, (↔) indicates the fragmentation route for Scheme III.

SCHEME I



at *m/e* 265 (Scheme I). This ion loses benzene *via* homolytic cleavage with H abstraction to yield the resonance stabilized furanooxonium ion at *m/e* 187. The direct formation of the ion at *m/e* 121 from the ion at *m/e* 187 by the loss of C_4H_2O can be rationalized mechanistically as shown in Scheme I. The first part of the mechanism, *i.e.*, heterolytic cleavage of the 1–2 bond followed by an electron shift and abstraction of the proton from the furano oxygen has been invoked previously[9] for somewhat similar compounds and is supported by the occurrence of the corresponding ion at *m/e* 122 for the deuterated warfarin analog **3**. The second part of the postulated mechanism *i.e.*, rearrangement to form the ion at *m/e* 121 and a neutral cyclopropenyl ketone is analogous to the abundant formation of cyclopropenyl ions in the fragmentation of a number of substituted furans.[10] Moreover, the decomposition of an even electron ion into a neutral molecule and another even electron ion is known to be an energetically favorable process.[11] Alternately this ion can arise directly from the ion at *m/e* 265, presumably by a similar mechanism being operative prior to the loss of C_6H_6. The last established fragmentation in this sequence is simply loss of CO to generate the cyclohexadienone cation at *m/e* 93.



In order to gain evidence for the postulated mechanisms and to aid the elucidation of the major fragmentation routes of **1a**, **3** was prepared and its mass spectrum studied (Figure 2). Examination of the region of the molecular ion and accounting for ^{13}C isotopic abundance show that 34% is present as the hexadeuterio, 53% as a pentadeuterio, and 13% as a tetradeuterio compound.

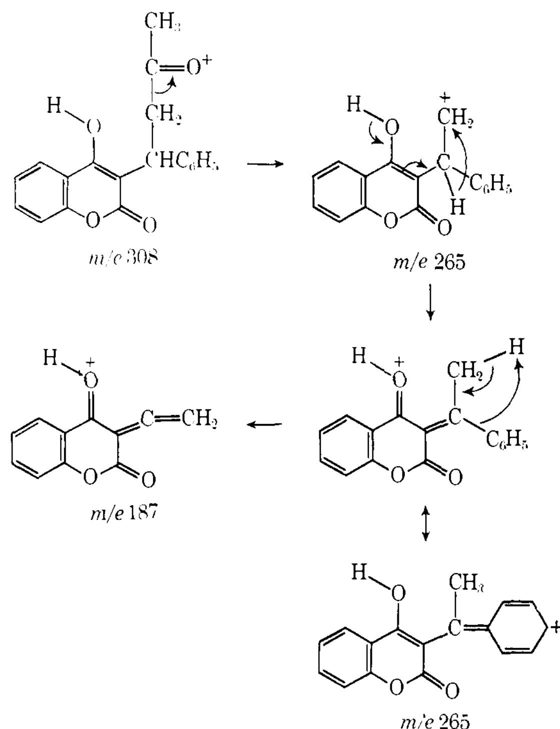
(9) R. A. W. Johnstone, B. J. Millard, F. M. Dean, and A. W. Hill, *J. Chem. Soc. C*, 1712 (1966).

(10) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Mass Spectrometry of Organic Compounds," Holden-Day, Inc., San Francisco, Calif., 1967, p 615.

(11) F. W. McLafferty, *Anal. Chem.*, **31**, 82 (1959).

However, for the sake of clarity, only the fragmentation of the hexadeuterio compound will be discussed. The base peak ion of warfarin *m/e* 265 is now shifted to *m/e* 268, and the furanooxonium ion *m/e* 187 appears at *m/e* 189. In addition, the ions at *m/e* 121 and *m/e* 93 are now shifted to *m/e* 122 and *m/e* 94, respectively. These data are consistent with the proposed fragmentation pathway. A reasonable alternative pathway, involving resonance-stabilized carbonium ions, is shown in Scheme II. However, if this pathway were mechanistically important then **3** should give rise to a significant amount of an ion at *m/e* 190 due to abstraction of the rearranged H by Ph in a 268 → 190 transformation. When ^{13}C isotopic abundance is taken into account, the ion at *m/e* 190 has an intensity of less than 4% of the ion at *m/e* 268. Thus this pathway would appear to be of minor importance.

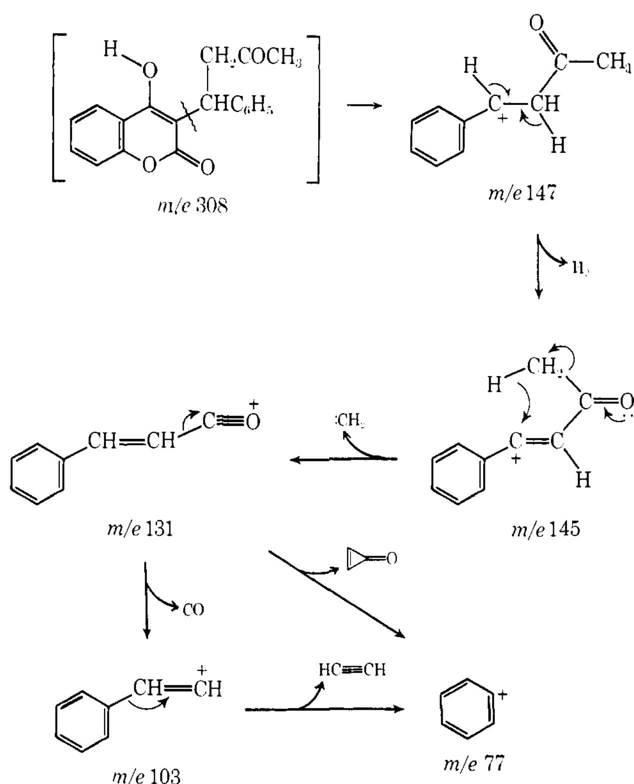
Scheme II



A second major pathway is initiated by heterolytic cleavage of the C_3-benzyl C–C bond (Scheme III). The benzyl carbonium ion, *m/e* 147, loses H to yield the resonance-stabilized vinyl carbonium ion at *m/e* 145. This ion in turn loses CH_2 presumably through the five-membered transition state shown with transfer of H^- to the benzyl C to give rise to an oxonium ion at *m/e* 131. This ion fragments further, first by loss of CO to give a styrene cation at *m/e* 103 followed by loss of acetylene to give the Ph cation at *m/e* 77. Alternately the oxonium ion at *m/e* 131 can fragment directly to the Ph cation at *m/e* 77. In addition to the detection of metastable ions for each of these transformations, support for the proposed mechanisms can be found in the spectrum of **3**. The benzyl carbonium ion *m/e* 147 is now shifted to 152, the vinyl carbonium ion 145 is shifted to *m/e* 149, the oxonium ion *m/e* 131 to *m/e* 133, and the ion at *m/e* 103 to *m/e* 105.

A third minor pathway centers around the formation of an ion at *m/e* 251, $C_{16}H_{11}O_3$, which arises from a num-

Scheme III



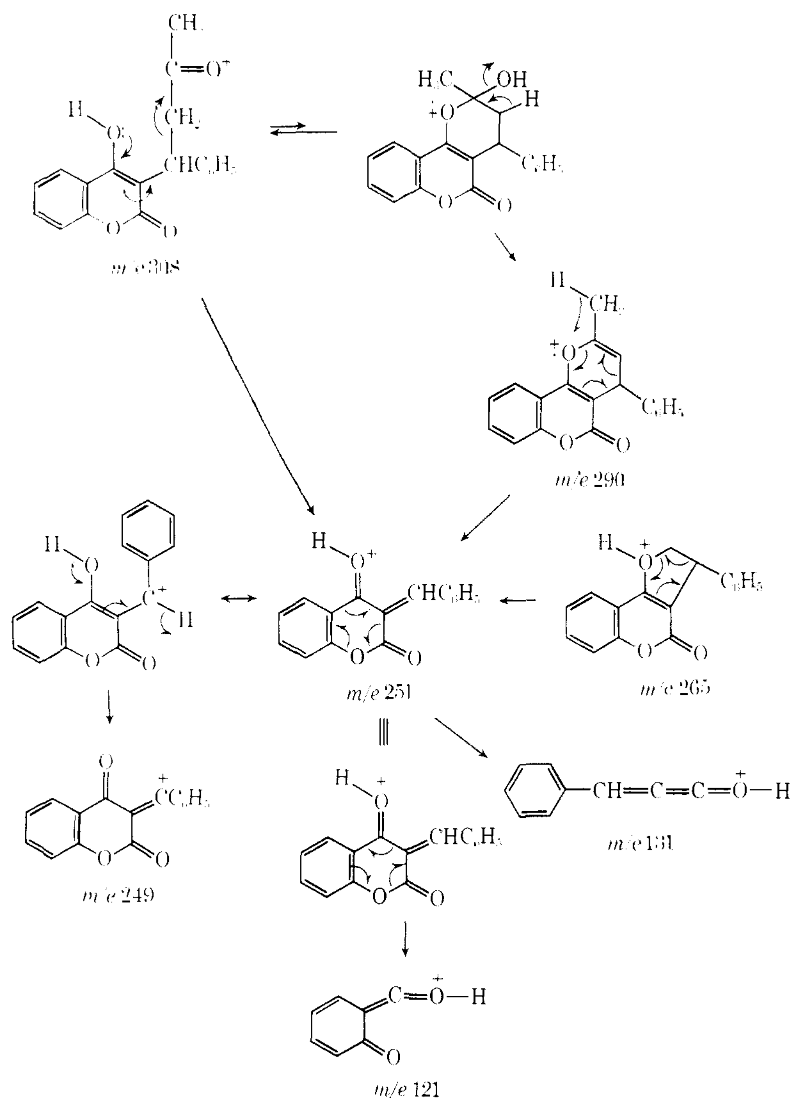
ber of ions and which in turn decomposes directly into a number of different ions (Scheme IV). The ion at *m/e* 290 presumably arises from thermal dehydration of a small amount of the hemiketal. Indeed it has been claimed that warfarin exists almost exclusively as the tautomeric hemiketal.[12] Our data, however, suggest that under the conditions of the determination of the mass spectrum it is of only minor importance. As indicated in Scheme III, metastable scanning shows that the ion at *m/e* 251 arises directly from the molecular ion, 308, the ion at *m/e* 290, and the base peak ion at 265. It then decomposes directly to yield ions at *m/e* 249, 131, or 121. As expected, the ions at *m/e* 251 and 290 are shifted to *m/e* 252 and 294, respectively, in the spectrum of **3**.

Since ionization of the acetonyl side chain of warfarin dominates its mass spectrum and since the fragmentation routes shown in Schemes I and III reflect the breakdown of the coumarin and Ph side chain portions of the molecule, respectively, it would seem reasonable to anticipate that metabolic changes in any or all of these portions of the molecule would be reflected in changes in the corresponding fragmentation route.

To examine this hypothesis, the mass spectra of 5-, 6-, 7-, 8-, and 4′-hydroxywarfarin, **1g**, **c**, **b**, **e**, and **f**, were determined and are shown in Figures 3–7, respectively. Exact mass measurement indicates that the fragmentation routes for warfarin (Schemes I, III, and IV) also occur in the spectra of the secompounds. In the case of **1g**, **c**, **b**, and **e**, Figures 3–6, the ions shown in Scheme III are "normal" whereas those shown in Schemes I and IV occur 16 mass units higher. These observations identify hydroxylation of the coumarin nucleus. As might be expected the mass spectrum of **1f**, Figure 7, shows just the opposite phenomenon, *i.e.*,

(12) E. Renk and W. G. Stoll, *Fortschr. Arzneimittelforsch.*, **11**, 226 (1968).

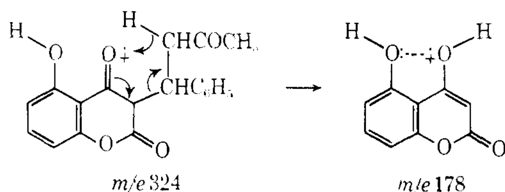
SCHEME IV



those ions after loss of Ph shown in Schemes I and IV are "normal" whereas those shown in Scheme III occur 16 mass units higher, indicating that Ph must be the portion of the molecule that is hydroxylated.

Among all the compounds discussed so far, only the mass spectrum of **1g** shows an extremely intense ion, 85% base peak, at *m/e* 178, $C_9H_6O_4$. Metastable scanning shows that the ion arises directly from the parent ion. If one assumes some small amount of the keto form, its formation can be rationalized mechanistically according to Scheme V. Support for this mechanism is suggested by the fact that the corresponding ion *m/e* 162 in the mass spectrum of warfarin shifts to *m/e* 163 in the mass spectrum of **3**, Figure 2. The stability of this ion in the mass spectrum of **1g** could be accounted for by a sharing of a nonbonded electron between the 4- and 5-OH groups. Having now gained a knowledge of how warfarin and similar compounds behave upon electron impact the problem of the isolation and identification of potential metabolites was undertaken.

SCHEME V



Isolation and Identification of Metabolites.—1,2-Dichloroethane–BuOH extracts of acidified urine obtained from individuals both before and after receiving warfarin were examined by tlc on silica gel. Coumarin and coumarin derivatives are known to fluoresce[13] upon irradiation with short-wave uv light and it was upon this basis that suspected metabolites were detected and followed through the isolation and purification procedures employed. Fluorescent loci, not present in control samples, were enumerated according to their chromatographic behavior on silica gel after an initial development in a 1,2-dichloroethane–Me_2CO system, 9:1. With this system blue fluorescent spots were noted at R_f's of 0.75, 0.50, 0.23, 0.10, and 0 and were designated compounds A through E, respectively. A compound with yellow fluoresence migrated with an R_f of 0.19 and was designated Cy. Determination of the mass spectra of compounds C and Cy and exact

(13) Y. Ichimura, *Yakugaku Zasshi*, **79**, 1079 (1959).

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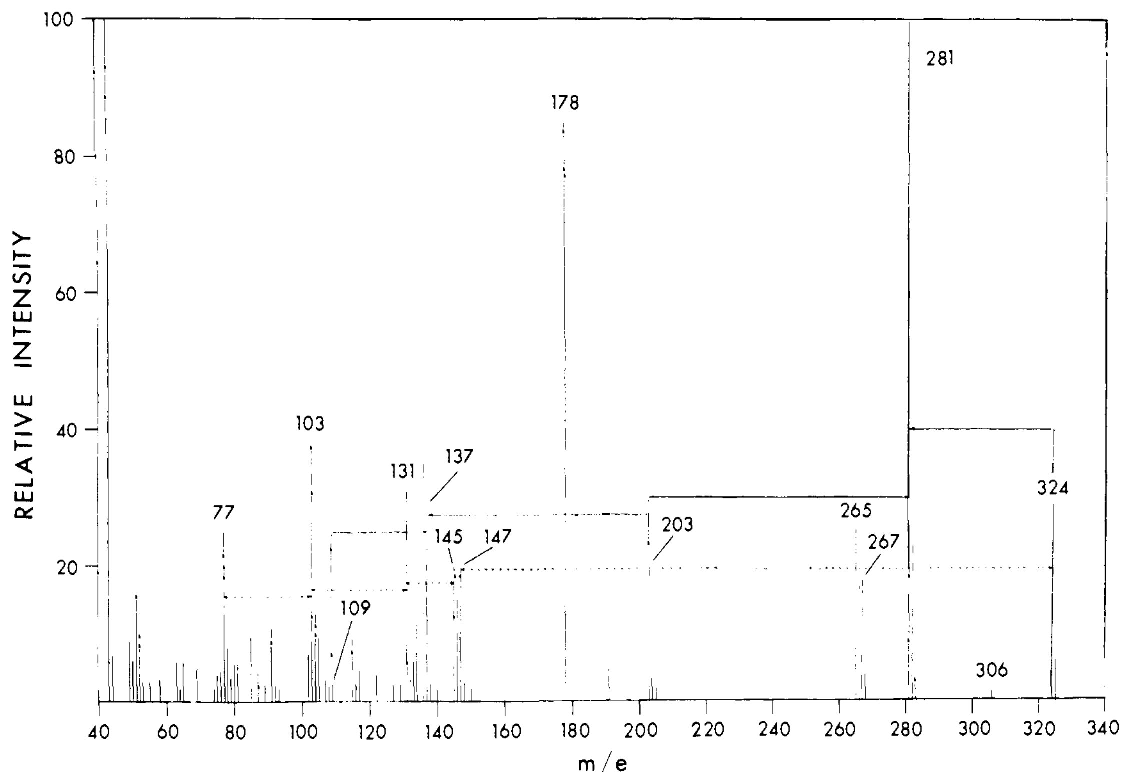


Figure 3.—Mass spectrum of **1g**; (↔) indicates the fragmentation route analogous to that shown in Scheme I, (↔) indicates the fragmentation route shown in Scheme III.

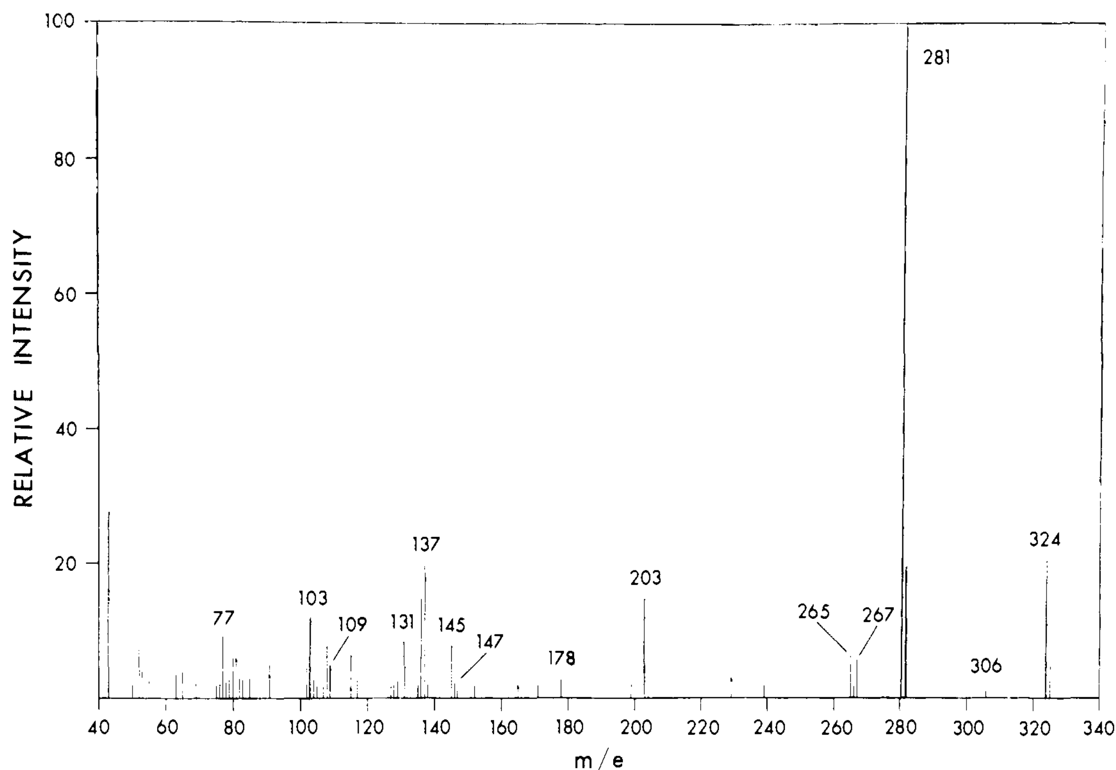


Figure 4.—Mass spectrum of **1c**.

mass measurement of structurally significant ions (see preceding section) indicated that these compounds were coumarin ring hydroxylated warfarin metabolites. The uv spectrum of compound C (λ_{max} 329 mμ, 0.25 *N* NaOH) was found to be identical with that obtained from a synthetic sample of **1b** and different from those obtained for **1g**, **c**, and **e**. In addition, the behavior of compounds C and **1b** was found to be identical in several different tlc systems.

The uv spectrum (λ_{max} 302 mμ, 0.25 *N* NaOH) for Cy was found to be identical with that obtained from both **1c** and **1e**. Together with the above data comparative tlc in cyclohexane–ethyl formate–formic acid, 100:200:1 established this compound as **1c**.

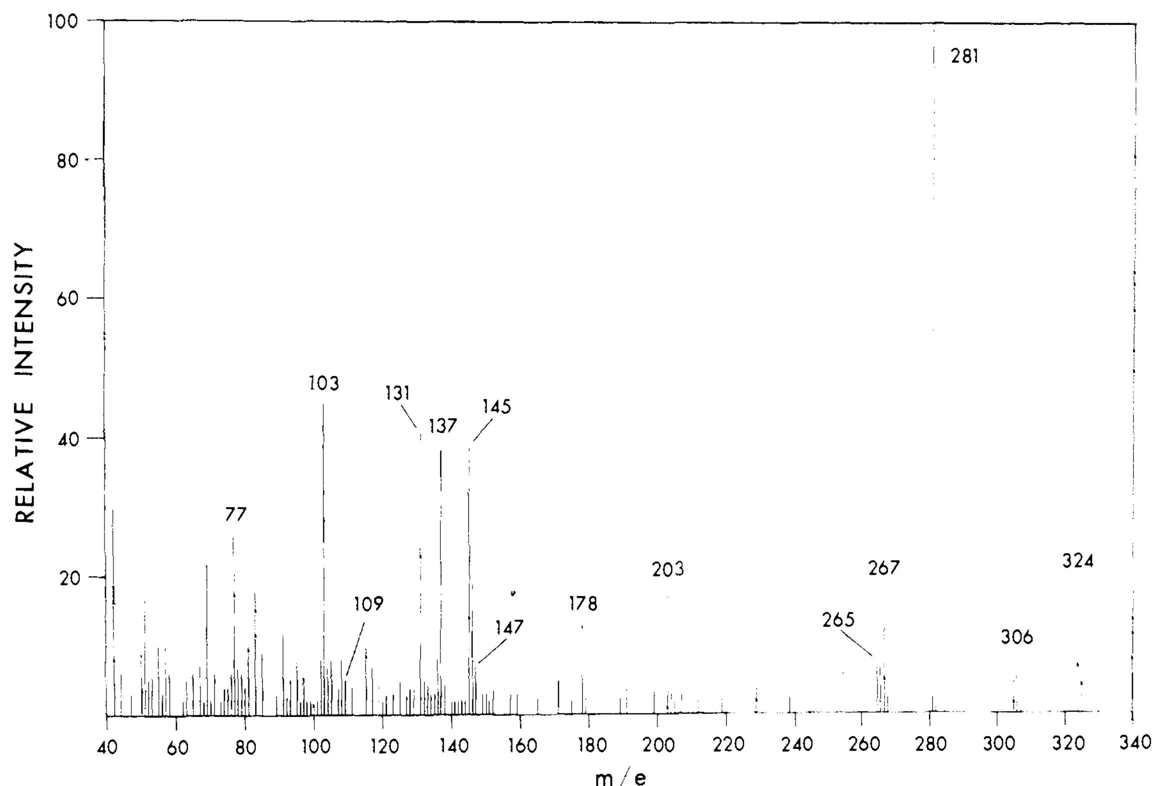


Figure 5.—Mass spectrum of **1b**.

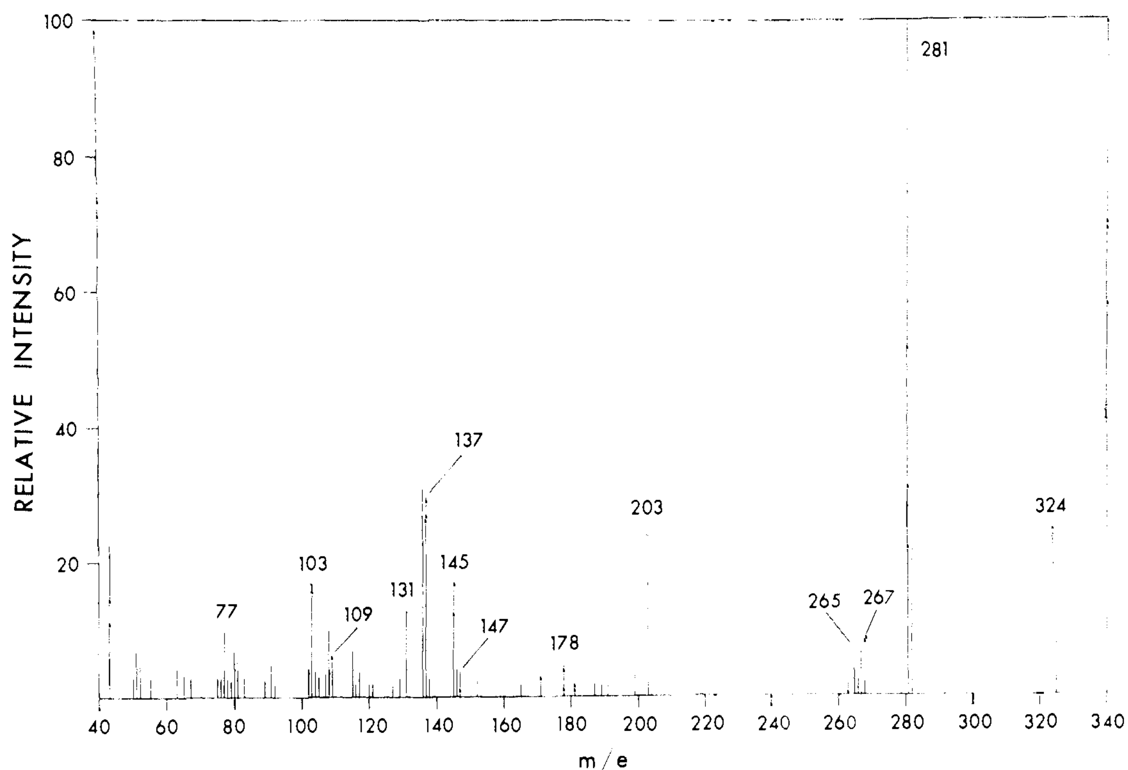


Figure 6.—Mass spectrum of **1e**.

The mass spectrum of the compound designated D gave an ion at *m/e* 310 which upon exact mass measurement was found to have the empirical formula $C_{19}H_{18}O_4$, suggesting a compound similar to warfarin but having additional H. The lack of any significant amount of an ion at *m/e* 267 and the appearance of an intense ion at 292 ($M^+ - H_2O$) strongly implied reduction of the side chain CO of warfarin to 3-[α-(2-hydroxypropyl)benzyl]-4-hydroxycoumarin, **1h**, as a metabolic transformation. In addition ions at *m/e* 265, 187, 121, and 93 were found to be present and were found to have an empirical formula consistent with the ions shown in

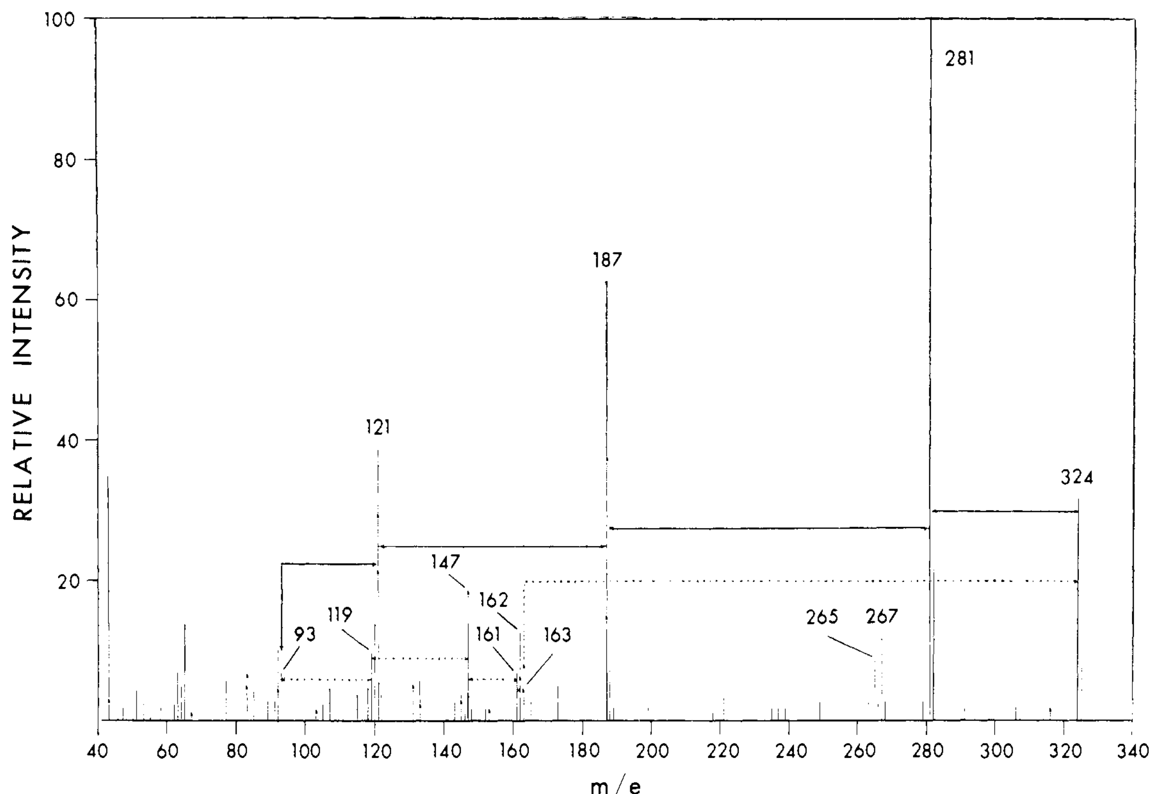


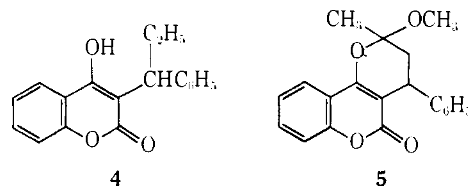
Figure 7.—Mass spectrum of **1f**; (↔) indicates the fragmentation route analogous to that shown in Scheme I, (↔) indicates the fragmentation route analogous to that shown in Scheme III.

Scheme I. Reduction of warfarin with $NaBH_4$ gave an essentially quantitative yield of the two possible diastereoisomers[14] of **1h**. The mass spectrum of this mixture is shown in Figure 8. Not unexpectedly the fragmentation pattern is considerably different from that given by **1a–g**; however, a fragmentation route corresponding to that shown in Scheme I is present. The origin of this route is presumably due to initial ionization of the side-chain OH followed by heterolytic cleavage and loss of the radical $CH_3\dot{C}HOH$ to form the dihydrofuranooxonium ion at m/e 265 in a fashion analogous to that shown (Scheme I). Compound D and synthetic mixture **1h** gave identical uv spectra (λ_{max} 309 mμ, 0.25 N NaOH) and ran identically in the 1,2-dichloroethane–Me_2CO (9:1) tlc system. These data would seem to establish clearly the identity of D as one or a mixture of all four possible[14] isomers of **1h**. Subsequently it was found that the mixture of diastereoisomers of **1h** could be separated in a tlc system of cyclohexane–ethyl formate–formic acid, 100:200:1. When D was cochromatographed with **1h** in this system it separated into two spots D_1 and D_2, each of which corresponded to one of the synthetic diastereoisomers. Elution of D_1 and D_2 and comparison of the uv and mass spectra to those given by the corresponding diastereoisomers of **1h** established the identity of D_1 and D_2 as the two diastereoisomers of **1h**.

Discussion

The finding of 6- and 7-hydroxywarfarin now establishes these oxidative biotransformations in "normal man," a patient with hereditary warfarin resistance, and the rat. In addition it should be noted that these compounds have been shown to be without significant anticoagulant activity at least in the rat.[8a,c]

On the other hand there would seem to be reason to suspect that the two diastereoisomers of **1h** may have significant anticoagulant activity on the following grounds. On a molar basis phenprocoumon (**4**) is known to be a more potent anticoagulant than warfarin while cyclocumorol (**5**)[15] although not as potent as warfarin, still has significant activity. Thus it would appear that molecular modification of the side chain does not abolish or greatly decrease the response. This possibility is currently being investigated and preliminary data from the Rhesus monkey suggest that **1h** is indeed active.



The question of the optical purity of D_1 and D_2 remains to be resolved. These compounds could of course be racemic mixtures but the fact that alcohol dehydrogenases are known[16] to be stereospecific reducing enzymes leads one to speculate that perhaps the enzyme(s) responsible for reduction of the warfarin side chain is also stereospecific. It would appear that at

(14) It is recognized that this statement is only true provided **1h** exists exclusively in the enolic 4-OH form. If the tautomeric 4-keto form is of importance which it well may be, then 8 isomers are possible. However, if the tautomerism is facile, separation and characterization of the isomeric pairs about C3 would be extremely difficult if not impossible.

(15) L. B. Jaques, "Anticoagulant Therapy," Charles C Thomas, Inc., Springfield, Ill., 1965, p 92.

(16) (a) F. A. Loewus, F. H. Westheimer, and B. Vennesland, *J. Amer. Chem. Soc.*, **75**, 5018 (1953); (b) G. J. Karabatsos, J. S. Fleming, N. Hsi, and R. H. Abeles, *ibid.*, **83**, 2676 (1961).

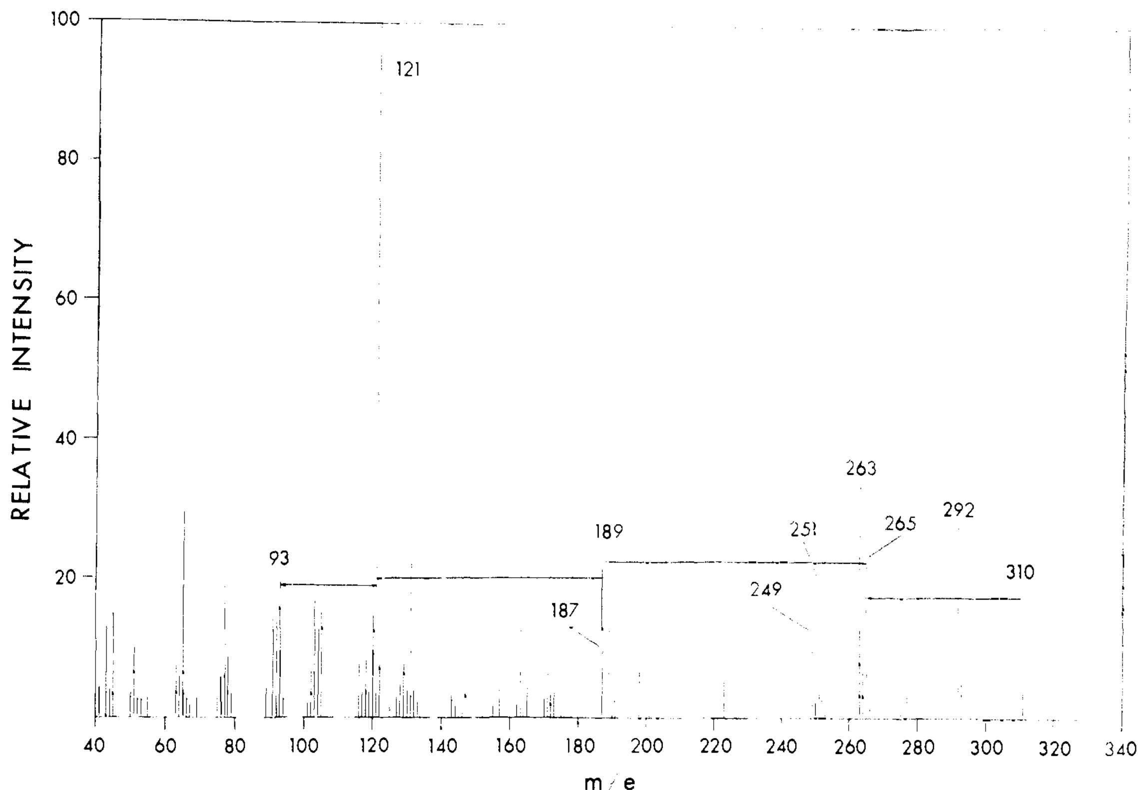


Figure 8.—Mass spectrum of **1h**; (↔) indicates the fragmentation route analogous to that shown in Scheme I.

least the oxidative enzymes are stereospecific[6] since the 7-hydroxywarfarin isolated from the patient with warfarin resistance was shown to be optically active. In addition it is known that in the rat the (S) enantiomer of both phenprocoumon[17] and warfarin[18] is about 5 times as active as the corresponding (R) enantiomer suggesting that each of the 4 isomers of **1h** may have significantly different anticoagulant activities. This possibility and the establishment of both the relative and absolute configurations of these 4 isomers is currently under investigation.

Experimental Section

All of the compounds studied were introduced into an AEI Model MS-902 mass spectrometer *via* the direct insertion probe and were vaporized at temps between 200 and 250°. Exact mass measurements were made by the electrical peak-matching technique using perfluorotributylamine as standard. The metastable scanning was done employing the techniques described by Jennings, *et al.*[19] Uv spectra were recorded on a Zeiss PMO II or a Carey Model 11 spectrophotometer. Details concerning the extraction and tlc technique employed have been described elsewhere.[20] Samples of 6-, 7-, 8-, and 4'-hydroxywarfarin were graciously supplied by M. Hermodson and K. Link while 5-hydroxywarfarin was synthesized in this laboratory according to the procedure described by Hermodson.[21]

3-[α-(2-Oxopropyl-1,1,3,3,3-d_5)benzyl]-4-deuterioxycoumarin (2).—A solution of 0.10 g of the Na salt of **1a** in 10 ml of 99.8% D_2O was stirred at room temp for 72 hr. The exchanged product was pptd by addition of a few drops of concd HCl and extracted with $CHCl_3$. The $CHCl_3$ was dried (Na_2SO_4) and then evapd and the residue subjected to mass spectral analysis.

3-[α-(2-Hydroxypropyl)benzyl]-4-hydroxycoumarin (1g).—A solution of 50 g (0.152 mole) of **1a** and 4.3 g (0.114 mole) of $NaBH_4$ in 250 ml of H_2O was stirred at 25° for 1 hr. Excess $NaBH_4$ was destroyed and the product pptd upon acidification with dil HCl. The amorphorous ppt was filtered, washed with H_2O, and vacuum dried. This material ran as a single fluorescent spot, different from starting material, on tlc, silica gel, MeOH–benzene, 33:77. The ir, uv, and mass spectra were consistent with the assigned structure, mp 84–110°.

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(21) Hermodson's synthetic sequence was utilized as reported except dioxane was substituted for PhH in the condensation of 2,6-dicarboethyloxyacetophenone to 5-carboethyloxy-4-hydroxycoumarin.