In Vivo Hydroxylation of the Alkaloid Acronine, an Experimental Antitumor Agent

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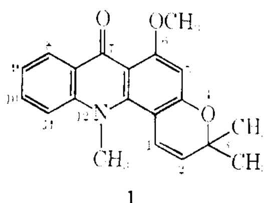
The nature of the metabolites of the alkaloid acronine has been studied in 5 species. All species were found to hydroxylate acronine to 9-hydroxyacronine and 11-hydroxyacronine. Four of these species, man, dog, rat and mouse, were also found to hydroxylate on the *gem*-dimethyl groups. The guinea pig and the mouse metabolized acronine in part by the O-demethylation route. A number of dihydroxy metabolites were also found among the terminal metabolic products. The metabolites are excreted predominately in the form of conjugates.

Acronine (**1**)[1-3] is an acridone alkaloid isolated from the bark of *Acronychia bauri* Schott, a scrub ash indigenous to Australia. It possesses broad-spectrum antitumor activity against experimental neoplasms in laboratory animals.[3,4] The structure of acronine has been deduced by chemical studies[5-9] and has been recently confirmed by synthesis.[10]



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The metabolism of this compound by mammalian systems was of particular interest since it is an alkaloid. Since alkaloids are metabolic end products of plants, it was of theoretical interest to study their further metabolism by animals. In this investigation the identities of the metabolites of acronine were determined by radiocarbon labeling, chromatographic procedures, mass spectroscopy, and nuclear magnetic resonance studies. No model compounds were available to facilitate the study.

Experimental Section

Acronine-*O*-methyl-^{14}C.—A mixture consisting of 153.5 mg (0.5 mmole) of *O*-desmethylacronine (*cf.* ref 10) and 25 mg of 50% NaH–mineral oil in 3 ml of DMF was heated at 50° in a H_2O bath until complete solution was attained. MeI-^{14}C (0.5 mmole, 1 mCi; Tracerlab, Inc.) was introduced *via* vacuum transfer and the resulting solution heated and stirred at 58° for 24 hr. Aqueous 2 *N* NaOH (8 ml) was added to the cooled reaction solution and the resulting mixture was extracted with 10 ml of PhH. The PhH solution was separated, washed with an equal volume of H_2O, and dried ($MgSO_4$). The PhH solution was evaporated to dryness *in vacuo*. The residual acronine-*O*-methyl-^{14}C was purified by tlc using preparative silica gel GF (Merck A.G.) plates and a PhH–EtOAc (1:1) solvent system for development. It weighed 124.0 mg (77%) and had a sp act. of 6.04 μCi/mg. The ^{14}C-labeled product cochromatographed identically with authentic material (R_f 0.4) and its radiochemical purity was demonstrated by autoradiography.

Acronine-*N*-methyl-^{14}C.—This compound was prepared in a 3-step synthesis as follows. **(A) *N*-Methyl-^{14}C-*O*-desmethyldihydroacronine.** MeI-^{14}C [249.6 mg, 1.76 mmoles, 5 mCi (Amersham-Searle)] was added by vacuum transfer to a mixture of 500 mg (1.7 mmoles) of *O*,*N*-didesmethyldihydroacronine[10] and 1.5 g of anhydrous K_2CO_3 in 3.5 ml of Me_2CO. The resulting mixture was stirred at reflux temperature for 24 hr. H_2O (5 ml) was added to the cooled reaction mixture and after vigorous shaking the phases were separated. The aqueous phase was extracted with 2 equal volumes of $CHCl_3$ and the combined $CHCl_3$ solution dried ($MgSO_4$). The $CHCl_3$ solution was evaporated to dryness *in vacuo* and the residual product was purified by development on preparative thin-layer silica gel (R_f 0.37) plates using a PhH–EtOAc (30:1) solvent system. The pure product (335 mg, 64%) was shown to be single spot material by tlc and autoradiography.

(B) *N*-Methyl-^{14}C-*O*-desmethylacronine.—2,3-Dichloro-5,6-dicyanoquinone (309 mg, 1.635 mmoles), was added portionwise over a period of 1 hr to a stirred solution of 335 mg (1.08 mmoles) of *N*-methyl-^{14}C-*O*-desmethyldihydroacronine in 15 ml of PhMe at reflux temperature. After complete addition the reaction mixture was stirred at reflux temperature for 2 hr. Upon cooling the reaction mixture was evaporated to dryness *in vacuo* and the residue dissolved in 30 ml of EtOAc. The EtOAc solution was washed successively with three 15-ml portions of 0.5 *N* NaOH and two 15-ml portions of saturated aqueous NaCl. The EtOAc solution, after drying ($MgSO_4$), was evaporated to dryness *in vacuo*. The product was purified by PhH–EtOAc solution (3:1) for development. The product, 73 mg (22%), was shown to be pure by tlc (R_f 0.5) and autoradiography.

(C) Acronine-*N*-methyl-^{14}C.—MeI (68 mg, 0.476 mmole) was allowed to react with 73 mg (0.238 mmole) of *N*-methyl-^{14}C-*O*-desmethylacronine in solution with 12 mg (0.250 mmole) of 50% NaH–mineral oil and 1.5 ml of PhMe in a manner identical with that described above for acronine-*O*-methyl-^{14}C. The product (57 mg, 75%), sp act. 9.0 μCi/mg, was shown to be pure by tlc (R_f 0.3) and autoradiography.

Methylation of Metabolites.—To prepare Me ethers of ring-hydroxylated acronine metabolites, the metabolite was dissolved in 0.5 ml of MeOH and 2.0 ml of ethereal CH_2N_2[11] was added. The reaction flask was stoppered lightly and allowed to stand at room temperature for 30 min. If the yellow color faded an additional 2.0 ml of CH_2N_2 solution was added. The reaction solution was evaporated to dryness *in vacuo* and the residue was dissolved in an appropriate solvent for tlc or glc purification.

Chromatographic Methods.—Tlc was carried out on silica GF (Merck A.G.) plates. Separation of original metabolic mixtures was accomplished using an EtOAc–MeOH–Et_2NH solvent system (85:10:5) for development. The R_f values for acronine and its metabolites are: acronine (0.89), **a** (0.55), **b** (0.41), **c** (0.71), **d** (0.30), and **e** (0). Purification of methylated derivatives, prepared by treatment of the individual metabolites with CH_2N_2,

(1) G. K. Hughes, F. N. Lahey, J. R. Price, and L. J. Webb, *Nature (London)*, **162**, 223 (1948).
(2) F. N. Lahey and W. C. Thomas, *Aust. J. Sci. Res. Ser. A*, **2**, 423 (1949).
(3) G. H. Svoboda, *Lloydia*, **29**, 206 (1966).
(4) G. H. Svoboda, G. A. Poore, P. J. Simpson, and G. B. Boder, *J. Pharm. Sci.*, **55**, 758 (1966).
(5) R. D. Brown, L. J. Drummond, F. N. Lahey, and W. C. Thomas, *Aust. J. Sci. Res. Ser. A*, **2**, 622 (1949).
(6) L. J. Drummond and F. N. Lahey, *ibid.*, **A2**, 630 (1949).
(7) R. D. Brown, and F. N. Lahey, *ibid.*, 593 (1950).
(8) P. L. MacDonald, and A. V. Robertson, *Aust. J. Chem.*, **19**, 275 (1966).
(9) T. R. Govindachari, B. R. Pai, and P. S. Subramaniam, *Tetrahedron*, **22**, 3245 (1966).
(10) J. R. Beck, R. Kwok, R. N. Booher, A. C. Brown, L. E. Patterson, P. Pranc, B. Rockey, and A. Pohland, *J. Amer. Chem. Soc.*, **90**, 4706 (1968).
(11) A. H. Blatt, "Organic Synthesis," Coll. Vol. II, Wiley, New York, N. Y., 1943, p 165.

was accomplished using an EtOAc–PhH solvent system (1:1) for development.

Glc was performed employing a 121.5-cm U-shaped glass column packed with 3.8% W-98 methylvinyl silicone gum on 80–100 mesh Diatoport S, contained in an F and M Model 402 biomedical gas chromatograph fitted with an H_2 flame detector. He at a flow rate of 60 ml/min was used as the carrier gas, the oven temperature was 275°. Purification and collection of individual metabolites and derivatives for mass spectrographic analysis was accomplished using the gas chromatograph modified with a stream splitter adjusted to a rate of 20:1 in favor of the collection side with He. Samples were collected in capillary tubes 10 cm in length. The retention times for the 11-methoxyacronine (**a**) and 9-methoxyacronine (**b**) were 4.0 and 7.0 min, respectively.

Mass Spectra.—The mass spectra of the purified metabolites were determined using a C.E.C. 21-110A double-focusing mass spectrograph operating at 230–300° and 70 eV. Samples were introduced directly into the ion source. Line spectra were drawn from the mass spectra and in each instance the most abundant ion was set at 100. Lines of relative abundance less than 5% were ignored.

Nmr studies were performed with a Varian Associates 60-Mc spectrometer.

Demethylation *in Vivo*.—The rates of *in vivo* demethylation were determined by following the rates of CO_2-^{14}C expiration after ip adminstration of the *O*-methyl-^{14}C- and *N*-methyl-^{14}C-acronines. For these experiments, the test animals were administered 25 mg/kg of the appropriately labeled acronine in polyethylene glycol (5 mg/ml). The rate of expiration of CO_2-^{14}C was determined using a radiorespirometer similar to that developed by Tolbert.[12] In our instrument the cage used for the rat and guinea pig studies had a volume of 500 ml and the volume of the ionization chamber was 500 ml. A male Purdue–Wistar rat (150 g) a 200-g male guinea pig or four 25-g C_3H mice were used with this cage. A flow rate of 500 cm^3/min of air was employed. *In vivo* demethylation studies in rabbits were effected using a 9.0-l. cage and a 1-l. ionization chamber. The flow rate of air in this sytem was 2.83 l./min.

***In Vivo* Elimination Studies.**—The *in vivo* fate of *O*-methyl-^{14}C- and *N*-methyl-^{14}C-acronine was studied in the mouse, rat, and guinea pig. After administration of 25 mg of ^{14}C-labeled acronine/kg ip (5 mg/ml of PEG_{200}) or orally (suspension of 5 mg/ml of 1% aqueous methocel), the animals were kept in stainless steel metabolism cages. Urine and feces samples were collected for 24 hr and the ^{14}C content of each determined by liquid scintillation counting. For the biliary elimination studies, 200-g male rats were anesthetized with Et_2O and a cannula was placed into the common bile duct. When guinea pigs were employed for biliary excretion studies, the animals were anesthetized with Et_2O and a cannula was placed into the bile duct posterior to the gall bladder. Immediately following the placement of the cannula, the ^{14}C-labeled acronine (25 mg/kg) in PEG_{200} was administered ip. The bile fluid was collected for a period of 24 hr and the ^{14}C content determined subsequently by liquid scintillation counting.

Isolation of Metabolites.—In order to investigate the nature of the metabolites present in the urine, bile, and blood, samples of these fluids were acidified to pH 5.5 with AcOH and were incubated at 37° for 24 hr with 1.0 ml of Glusulase solution (mixture of β-glucuronidase and sulfatase, Endo Products, Inc.)/100 ml of sample. The liberated metabolites were extracted into CH_2Cl_2. Separation and purification of individual metabolites was accomplished by means of tlc and glc.

In mice it was impractical to collect sufficient volumes of bile and instead feces were used as a source of metabolites. Dried, ground feces were extracted with several volumes of MeOH. The MeOH extracts were then taken to dryness and the residue taken up in pH 5.5 acetate buffer. Metabolites were then isolated as described above.

Results

Demethylation Studies.—The major routes by which acronine might be reasonably expected to be metabolized include N-demethylation, O-demethylation, or hydroxylation. Possible dealkylation was first investigated. By following the rate of CO_2-^{14}C expiration after administration of either *O*-methyl-^{14}C-labeled or *N*-methyl-^{14}C-labeled acronine it was possible to estimate the extent of *in vivo* O- or N-demethylation. Four species of laboratory animals; rat, mouse, rabbit, and guinea pig, were used in these experiments. The results obtained are summarized in Table I.

(12) B. M. Tolbert, M. Kirk, and E. Baker, *Amer. J. Physiol.*, **185**, 269 (1956).

TABLE I
In Vivo DEMETHYLATION OF RADIOACRONINE

Species[a]	% demethylation[d] O-Demethylation	N-Demethylation
Rat[b]	0	3
Mouse[b]	23	7
Guinea pig[b]	50	2
Rabbit[c]	8	0

[a] Dose in all cases was 25 mg/kg ip. [b] Results are the average obtained from 3 animals. [c] Results obtained from 1 animal. [d] Results are expressed as per cent of radiocarbon dose appearing as CO_2-^{14}C in 24 hr.

In vivo O-demethylation varies markedly with species tested. Thus, O-demethylation was of little importance in the metabolism of acronine by the rat or rabbit. This metabolic pathway was, however, of major importance in the guinea pig and to a lesser degree in the mouse. N-Demethylation was a minor pathway of metabolism of acronine in all species studied.

Routes of Metabolite Elimination.—The routes and extent of elimination of radioactive metabolites by rats receiving acronine-O-methyl-^{14}C was next studied. Since O-demethylation of acronine does not occur in rats, the *O*-methyl-^{14}C label was an ideal label for this study. When acronine-*O*-methyl-^{14}C was administered ip to rats 12.3% of ^{14}C was recovered in urine in 24 hr. On the other hand, recovery in feces in 48 hr amounted to 89.6% of the dose. In a separate experiment, 86% of the administered ^{14}C was excreted in the bile in 24 hr, thus confirming the results found in the intact animals. Excretion following oral administartion was also studied. As in the case of parenteral administration, excretion in the feces was the preponderant route of elimination. However, when acronine-*O*-methyl-^{14}C was given orally to rats excretion in the bile in 24 hr amounted to only 12% of the dose. These results suggest that acronine is poorly absorbed from the gastrointestinal tract in the rat.

Elimination studies in the guinea pig were conducted with acronine-*N*-methyl-^{14}C and acronine-*O*-methyl-^{14}C. Following the intraperitoneal administration of acronine-*N*-methyl-^{14}C, 85% of ^{14}C appeared in bile while only 10% was found in urine. Unlike the rat, the guinea pig metabolizes acronine extensively by O-demethylation (see above). Consequently, following ip administration of acronine-*O*-methyl-^{14}C to guinea pigs about 45% of the radioactivity appeared in bile, while urinary excretion was negligible.

Identification of Acronine Metabolites.—The primary source of metabolites for these studies was from the bile of rats receiving acronine-*O*-methyl-^{14}C. Attempts to extract ^{14}C from bile at pH 7.0 with organic solvents were unsuccessful. Incubation of the bile with a mixture of β-glucuronidase and sulfatase, however, allowed over 85% of the biliary ^{14}C to be extracted

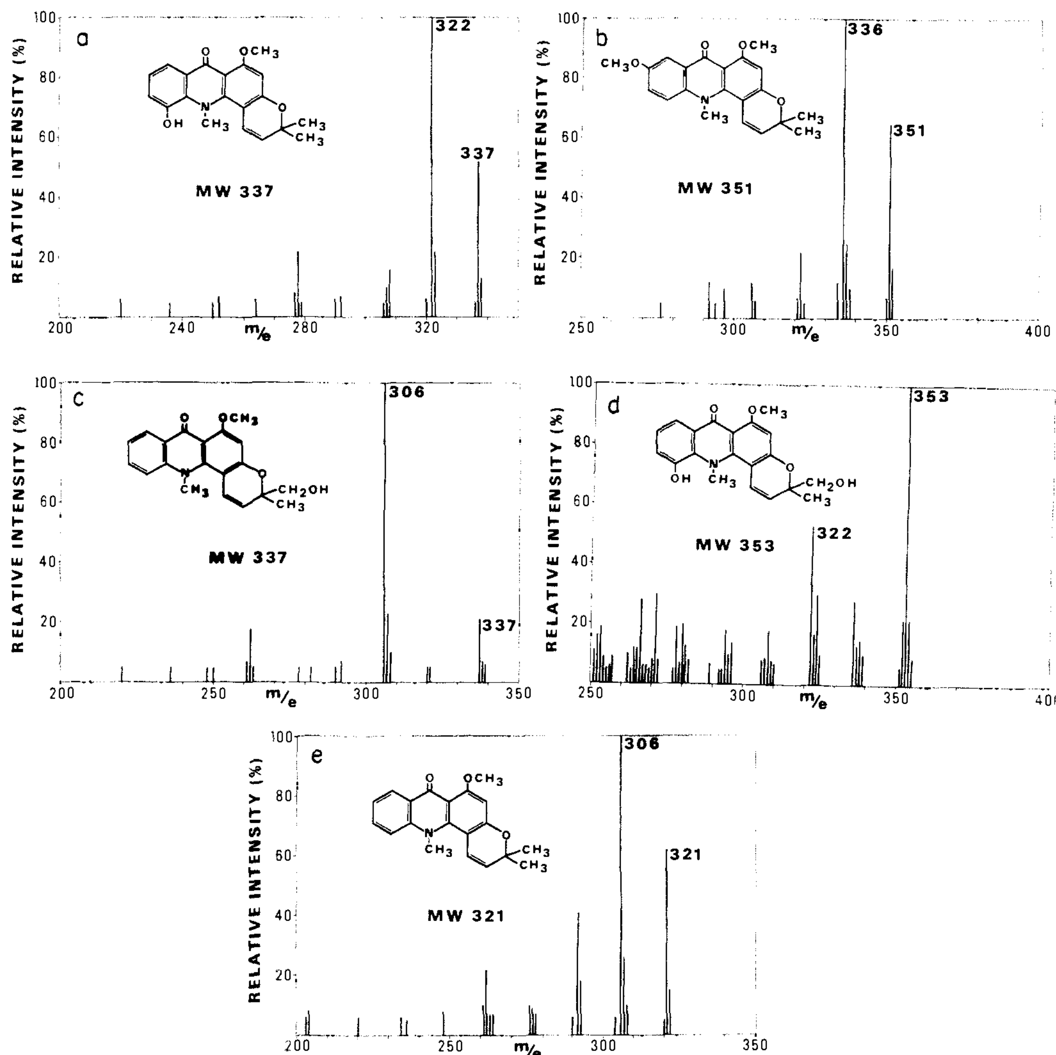


Figure 1.—Representative mass spectra: (a) 11-hydroxyacronine (metabolite **a**); (b) 9-methoxyacronine (methoxy derivative of metabolite **b**); (c) 3-hydroxymethylacronine (metabolite **c**); (d) 11-hydroxy-3-hydroxymethylacronine (metabolite **d**); (e) acronine.

into CH_2Cl_2. This result suggested that the metabolites were hydroxylated acronine derivatives.

Comparison tlc of the extracted radioactive metabolites with acronine identified acronine-^{14}C (3% of the biliary radiocarbon) as a minor metabolite. Five unknown radioactive metabolites were separated by tlc. Since each of these unknown metabolites was bright yellow, further work utilized bile from rats receiving unlabeled acronine. In order to facilitate the structure identification of these metabolites, 6 bile-cannulated rats were administered unlabeled acronine ip and the bile was collected. The combined bile was hydrolyzed and extracted as previously described. Five unknown metabolites were separated and isolated by tlc. After additional purification by tlc, the mass spectra of the 5 unknown metabolites were recorded. Some representative mass spectra are shown in Figure 1. From a consideration of the mass spectra of the metabolites, it was possible to subdivide the metabolites into 2 groups.

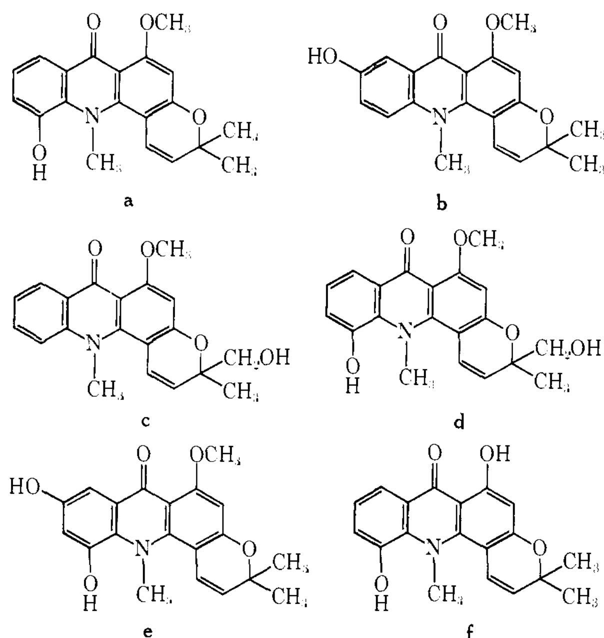
The first group consisted of 3 metabolites, M^+ peak *m/e* 337 (*cf.* Figure 1a as an example). These metabolites thus were isomeric and possessed a mol wt 16 units greater than that of acronine (321). This suggested that these metabolites were monohydroxylated derivatives of acronine. The mass spectrum of acronine itself (Figure 1e) showed an initial fragmentation of *m/e* 321 → *m/e* 306, which has been attributed to the loss of one of the 3-Me groups, *m/e* 15. Compounds structurally similar to acronine and containing the *gem*-Me_2 group, 2,2-dimethylchromenes, are known to fragment in this manner.[13] The mass spectra of 2 of these metabolites (**a** and **b**) showed an initial *m/e* 337 → *m/e* 322 fragmentation attributed to the loss of one of the 3-Me groups. This result indicated that the 2 metabolites were acronine derivatives containing unaltered *gem*-Me_2 groups. Treatment of these monohydroxylated acronine derivatives with ethereal CH_2N_2 resulted in an increase of 14 *m/e* units of the M peak to 351 in their mass spectra (see Figure 1b). This increase reflected the conversion of a phenolic OH to an aromatic OMe.

The mass spectrum of the third 337 metabolite (**c**) however, showed an initial *m/e* 337 → *m/e* 306 fragmentation (Figure 1c). The presence of a strong M-31 peak was attributed to the loss of a CH_2OH group. The treatment of this metabolite with D_2O and subsequent mass spectroscopic analysis of the deuterated product showed a strong M peak of *m/e* 338 units and a strong M-32 peak resulting from the loss of the CH_2OD ion. These mass spectroscopic data showed, therefore, that this acronine metabolite contained an ali-

(13) C. S. Barnes and J. L. Occolowitz, *Aust. J. Chem.*, **17**, 975 (1964).

phatic OH and, because this OH was lost in the first fragmentation, that one of the 3-Me groups of the acronine molecule had been hydroxylated. The fact that this metabolite did not react with CH_2N_2 provided additional evidence for the aliphatic nature of the OH of this metabolite. The structure of metabolite **c** is shown in Chart I, **c**.

CHART I
STRUCTURES OF THE METABOLITES OF ACRONINE WHICH HAVE BEEN ISOLATED AND IDENTIFIED



The second group of acronine metabolites consisted of 2 compounds having an M peak of *m/e* 353 in their mass spectra (*cf.* Figure 1d, as an example). This mol wt was 32 *m/e* greater than that of acronine and suggested that these isomers were dihydroxylated derivatives of acronine. While these compounds were found to be isomeric their mass spectra were found to possess different fragmentation patterns. The mass spectrum of one dihydroxylated metabolite (**d**) possessed a moderate peak at M-17, attributed to the loss of an OH^-, and a major fragment at M-31 (Figure 1d). The latter fragment, *m/e* 322, resulted from the loss of a CH_2OH ion from the mass ion, *m/e* 353. This initial fragmentation, loss of *m/e* 31, was identical with that observed for the monohydroxylated metabolite **c** containing the aliphatic 3-CH_2OH group and was therefore indicative of the hydroxylation on a 3-Me group of acronine. When metabolite **d** was treated with CH_2N_2 it was found to react with 1 mole of the reagent. This result was direct evidence for the presence of one aromatic OH and also indirectly confirmed the presence of the aliphatic OH group.

The second dihydroxylated metabolite **e** had in its mass spectrum an intense M-15 peak (*m/e* 353 → *m/e* 338). This fragmentation, identical with that found in the spectrum of acronine and shown to be due to the loss of one of the 3-Me groups, showed the *gem*-Me_2 group to be intact in this metabolite. When treated with CH_2N_2, this metabolite was found to react with 2 moles of reagent and consequently contained two phenolic OH groups. High resolution mass spectroscopic analysis of the dimethoxy derivative showed an M ion at *m/e* 381 and an intial fragmentation of *m/e* 381 → *m/e* 352. This strong M-29 peak was attributed to the loss of the CHO+ ion. Similar studies on dimethoxybenzenes[14] revealed that *m*-dimethoxybenzene fragmented in an identical manner while *o*- and *p*-dimethoxybenzene fragment by the successive loss of 2 Me groups, 30 *m/e*. This evidence therefore indicated the positions of the two phenolic OH groups in this acronine metabolite to be *meta* in respect to one another. The suggested structure of metabolite **e** is thus the structure shown in Chart I, **e**.

The availability of clinical urine samples from patients receiving orally administered acronine (100-mg capsule daily) offered a possible source of sufficient quantities of metabolites to complete the structural studies. The acronine metabolites present in the human urine were isolated by the procedures used for rat bile, and were separated by tlc. The results obtained showed that 4 metabolites (**a**, **b**, **d**, **e**) were present in human urine. Metabolite **c**, 3-hydroxymethylacronine (Chart I, **c**) was not present.

The human urinary metabolites were derivatized by reaction with CH_2N_2. The resulting Me ethers were found by comparison glc and tlc to be identical with the Me ethers obtained in a similar manner from the corresponding rat biliary metabolites. Sufficient quantities of these metabolites and their Me ethers were obtained in pure form to allow the completion of the structure elucidation studies using nmr.

One phenolic monohydroxylated metabolite (**a**) was identified directly from its nmr spectrum at 100 MHz in 1% $CDCl_3$ and in 1% CD_3OD solution as 11-hydroxyacronine (Chart I, **a**). The most convincing evidence for this assignment was the upfield shift of 0.52 ppm of the broad signal for the 8 proton (Table II). If the OH group had been at position 10, the signal for the 8 proton would have appeared at about 8.3 ppm as a broad doublet with a coupling constant, $J_{8,9}$, of 8 cps. If the position of hydroxylation had been at 9, the signal for the 8 proton would have been near 7.9 ppm

TABLE II
NMR SPECTRA OF ACRONINE AND METABOLITES[a] CHEMICAL SHIFTS (δ)

Position of proton	Acronine	a		b	d
	$CDCl_3$	$CDCl_3$	CD_3OD	CD_3OD	CD_3OD
C–CH_3 (3)	1.52(s)	1.52	1.51	1.53	1.45
N–CH_3 (12)	3.76(s)	3.64	3.70	3.88	3.63
O–CH_3 (6)	3.95(s)	3.92	3.90	3.88	3.88
1	6.50(d)	6.70	6.75	6.68	6.81
2	5.47(d)	5.57	5.68	5.60	5.66
5	6.30(s)	6.31	6.39	6.39	6.45
8	8.38	7.86[b]	7.72[b]	7.72(d)	7.79[b]
9	7.21	7.10	7.12		7.28
10	7.55	7.13	7.14	7.35(q)	7.28
11	7.31			7.54(d)	
O–CH_3 (9 or 11)			3.92	3.99	3.92
					3.62
–CH_2OH (3)					3.72

[a] The following coupling constants were observed for acronine: $J_{1,2}$ = 9.7 cps, $J_{8,9}$ = 8.0 cps, $J_{9,10}$ = 8.6 cps, $J_{9,11}$ = 1.3 cps. For metabolite a and the Me ether of b and d, $J_{1,2}$ = 10 cps. $J_{10,11}$ = 9.0 cps and $J_{8,10}$ = 2.7 cps for the Me ether of b. [b] Broad complex signal; (s) singlet, (d) doublet, (q) quartet.

(14) C. S. Barnes, and J. L. Occolowitz, *Aust. J. Chem.*, **16**, 219 (1963).

in $CDCl_3$ (7.7 in CD_3OD) but would have appeared as a narrow doublet with a $J_{8,10}$ of 2.5 cps.

The second phenolic monohydroxylated metabolite **b** was converted into its Me ether in order to render it soluble enough in CD_3OD to obtain its nmr spectrum. It was also necessary to employ a computer summation of 36 scans of the range of 6.0 to 8.0 ppm in order to obtain a significant nmr spectrum. From this spectrum it was possible to assign the structure of metabolite **b** as 9-hydroxyacronine (Chart I, **b**). The confirming feature of this spectrum was the signal for the proton at position 8. It appeared as a narrow doublet with a $J_{8,10}$ of 2.5 cps at 7.7 ppm in CD_3OD.

The structure of metabolite **d** was unequivocally assigned as shown in Chart I, **d** based on nmr data. Thus its spectrum showed the upfield shift of the broad signal for the 8 proton which established the position of the phenolic OH group at position 11. Also the aliphatic OH was assigned to a Me group at position 3 because of the observed intensity for only one Me group at position 3 and of a two proton AB multiplet for CH_2OH which could be nonequivalent only at positions 3. Table II summarizes the chemical shifts (δ) for acronine and its metabolites.

The determination of positions of hydroxylation as 11 and 9, respectively, for metabolties **a** and **b** was confirming evidence for the structure assignment for the dihydroxylated metabolite **e** containing the two phenolic OH groups (see Chart I, **c**). Likewise the elucidation of the structure of metabolite **d** was confirmatory evidence for the structure of metabolite **c** found in rat bile but absent in human urine.

In vivo demethylation studies showed that in the guinea pig, O-demethylation played an important role in the metabolism of acronine. The structure of the biliary metabolites from the guinea pig was therefore of particular interest. Bile fluid, collected from guinea pigs administered acronine-*N*-methyl-^{14}C ip, was processed in a manner identical with that described for the rat bile. Tlc revealed the presence of 3 radioactive metabolites in the extraction mixture. Two of these metabolites were identified as 11- and 9-hydroxyacronines (metabilites **a** and **b**) by comparison tlc of the metabolites with known samples and by comparison glc of their Me ethers with those used for structure determination. The third radioactive metabolite **f** was shown by mass spectrographic analysis to have M^+ 323. Analysis of the spectrum showed the *gem*-Me_2 group at position 3 to be intact (M-15 peak). Exchange with D_2O followed by mass spectroscopic analysis showed the compound to contain two OH groups. Reaction with CH_2N_2, however, yielded a derivative that was identified as 11-methoxyacronine, M^+ 351. With the knowledge that the guinea pig does O-demethylate acronine and with the above data it was not unreasonable to assign the structure as 11-hydroxydesmethyl acronine (methabolite **f**, Chart I, **f**). Additional evidence for this assignment was obtained from the reaction of this metabolite with $C_2H_4N_2$. Mass spectroscopic analysis of the reaction product showed an M peak at 379. This was an increase of 58 *m/e* units over the molecular weight of the metabolite itself (323) and was indicative of the addition of 2 moles of diazoethane. Scheme I summarizes the results of these reactions.

The nature of the urinary metabolites obtained following oral administration of acronine to dogs was also investigated. The urine was processed in a manner already described and the metabolites were extracted into CH_2Cl_2. Initial chromatographic separation showed that 3 metabolites of acronine were present in this mixture. These metabolites and their Me ethers were identified by comparison chromatographic techniques as being 11-hydroxyacronine (metabolite **a**), 9-hydroxyacronine (metabilite **b**), and 11-hydroxy-3-hydroxymethylacronine (methabolite **d**). No unchanged drug was found in the urine of dogs.

Finally, the fate of acronine in mice was investigated. Like the rat, this species excretes the metabolites of acronine almost entirely *via* the bile duct. The conjugated metabolites were recovered from feces by MeOH extraction. After hydrolysis of the conjugates the liberated metabolites were isolated and identified as before. The mouse was found to excrete all 5 of the metabolites previously found in rats. Although metabolite **f**, a result of O-demethylation, found in guinea pig bile would have been expected, no detectable quantity was present.

The species differences in the *in vivo* metabolism of acronine are summarized in Table III.

SCHEME I

REACTION OF METABOLITE **f** WITH DIAZOMETHANE AND DIAZOETHANE

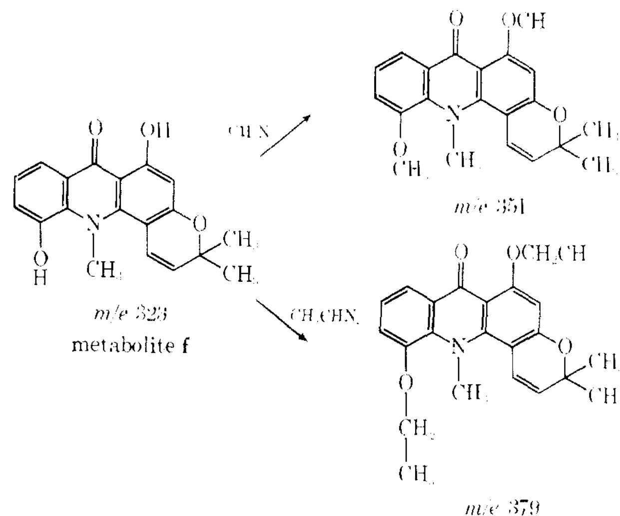


TABLE III

ACRONINE METABOLITES FOUND IN LABORATORY ANIMALS AND IN MAN

Metabolite	Name	Species: Man	Rat	Guinea Pig	Dog	Mouse
a	11-Hydroxyacronine	x	x	x	x	x
b	9-Hydroxyacronine	x	x	x	x	x
c	3-Hydroxymethylacronine		x			x
d	11-Hydroxy-3-hydroxy-methylacronine	x	x		x	x
e	9,11-dihydroxyacronine	x	x			x
f	11-Hydroxy-O-desmethyl-acronine			x		

The urine of cats adminstered acronine ip was found to contain no detectable quantities of the parent drug or its metabolites. The blood plasma obtained from these cats, however, was found to contain 2 metabo-

lites: 11-hydroxyacronine (**a**) and 9-hydroxyacronine (**b**).

Discussion

Although acronine contains both an *O*-Me and an *N*-Me group, dealkylation does not appear to be an important route of biotransformation. An exception to this observation occurs in the guinea pig. In this species and to a lesser extent in mice, O-demethylation does account for a portion of the metabolic conversion of acronine. In general, however, hydroxylation appears to be the major route of oxidation. Of the 5 species studied, all hydroxylate acronine at C-9 and C-11, indicating a similarity of the enzyme systems involved in each animal. The major species difference observed involved the ability to hydroxylate the methyl group at the quaternary C. This route was observed in rat, mouse, dog, and man, but not in guinea pig.

The observation that the alkaloid acronine is readily oxygenated by mammalian enzymes at a number of different positions in the molecule is of some interest since none of these metabolites have been reported as metabolites of acronine in *Acronychia bauri.* It would be of some interest to reinvestigate the alkaloids of this plant to see if any of the hydroxyacronines occur naturally.

The work described emphasizes the value of contemporary chromatographic methods used in conjunction with mass spectroscopy for the determination of the structure of natural products.

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Choline Acetyltransferase Inhibitors. Physicochemical Properties in Relation to Inhibitory Activity of Styrylpyridine Analogs

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Hückel molecular orbital and Hansch calculations were performed on various styrylpyridine derivatives and analogs, some of which are potent inhibitors of choline acetyltransferase (ChA). The results are consistent with the view that these compounds bind to ChA *via* hydrophobic and π donor contributions of the aryl moiety, and π acceptor interactions, presumably by the pyridinium-like portion.

Previous reports[1,2] have described some derivatives and analogs of styrylpyridine which include quite potent inhibitors of choline acetyltransferase (EC 2.3.1.6)(ChA). These papers delineated, in a qualitative manner, steric and electronic features contributing to optimal inhibitory activity. Some speculations were made concerning the nature of possible interactions of these inhibitors with the enzyme at a molecular level. The present report describes some efforts to quantitate certain structure–activity relationships (SAR) among these types of compounds. Such exercises could serve to more critically evaluate proposed[2] inhibitor–enzyme interactions and also provide additional guidance to directions of future molecular design.

The application of computer based techniques for quantitative assessment of some S variables in SAR for various classes of medicinals has met with moderate success.[3] Of these methods, Hückel molecular orbital calculations[4] and the Hansch technique[5,6] have now been applied to the interpretation of the structural variables among styrylpyridine-like compounds with ChA inhibitory activity.

Results and Discussion

Hückel molecular orbital (HMO) calculations were performed on 21 styrylpyridine derivatives and analogs. The method and parameters utilized are discussed in the Experimental Section. Compounds were divided into three structural groups (A, B, and C) for initial ease of comparison. These groups are presented in Table I together with the *in vitro* activity data[2] (expressed for convenience as log $1/I_{50}$) and various pertinent HMO derived quantities.

It has been suggested[2] that these compounds may bind to ChA *via* a charge transfer acceptor contribution by the pyridinium-like moiety and charge transfer donor interactions involving the aryl group. Using this as a working hypothesis, the data generated from the HMO calculations were examined for possible correlations. Disappointing correlations were obtained using either E_{HOMO} and/or E_{LEMO}, the energies of the highest occupied and lowest empty molecular orbitals, respectively. These quantities are related to the ability of the molecule as a *whole* to act as a charge transfer donor or acceptor. This is not incompatible with the working hypothesis since inhibitor activity appears to require a separation of donor and acceptor units as in styrylpyridine; ring fusion of components as in phenanthridinium[1] results in loss of activity.

(1) C. J. Cavallito, H. S. Yun, J. C. Smith, and F. F. Foldes, *J. Med. Chem.*, **12**, 134 (1969).
(2) C. J. Cavallito, H. S. Yun, T. Kaplan, J. C. Smith, and F. F. Foldes, *ibid.*, **13**, 221 (1970).
(3) See for example, (a) J. G. Beasley and W. P. Purcell, *Biochem. Biophys. Acta*, **178**, 175 (1969); (b) R. W. Fuller, M. M. Marsh, and J. Mills, *J. Med. Chem.*, **11**, 397 (1968).
(4) A. Streitweiser, "Molecular Orbital Theory for Organic Chemists," Wiley, New York, N. Y., 1961.
(5) C. Hansch, R. M. Muir, T. Fujita, P. P. Maloney, F. Geiger, and M. Streich, *J. Amer. Chem. Soc.*, **85**, 2817 (1963).
(6) T. Fujita, J. Iwasa, and C. Hansch, *ibid.*, **86**, 5175 (1964).