Alkaloids of *Acronychia Baueri* Schott I

Isolation of the Alkaloids and a Study of the Antitumor and Other Biological Properties of Acronycine

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Utilizing a differential extraction technique for the examination of the bark of the Australian plant *Acronychia Baueri* Schott (*Bauerella australiana* Borzi), has resulted in the isolation of the triterpene lupeol and the alkaloids melicopine, acronycine, and normelicopidine. The experimental antitumor activity associated with the crude alkaloidal mixture obtained from the ether extract has been shown to be attributable to acronycine. Experimental evidence is herein given, showing acronycine to have the broadest antitumor spectrum of any alkaloid isolated to date in these laboratories. By virtue of its being chemically unrelated to any of the presently utilized antitumor agents it represents a new lead in the search for agents effective in the chemotherapeutic management of human neoplasms.

THE GENUS *Acronychia*, family *Rutaceae*, consists of approximately 20 species of trees and shrubs native to Australia and tropical Asia. Of the Australian species, one is also found in New Caledonia, six others being endemic. The species are characterized by opposite or alternate leaves, sepals, petals, and other flower parts being in fours (1).

Acronychia Baueri Schott, commonly called the scrub ash or scrub yellowwood, grows 50 to 60 ft. in New South Wales and Queensland. It resembles the evergreen *A. laevis* but has rather longer leaves and a hard fruit. The wood is said to be excellent for mallet and chisel handles (2). No references to its medicinal use were found.

While this species is usually classified as follows: family, *Rutaceae*; subfamily, *Toddalioideae*; tribe, Toddalieae; subtribe, Toddaliinae; Engler (3) considers it as being *Bauerella australiana* Borzi. "Whether or not *Bauerella* is a genus distinct from *Acronychia* is basically one of evaluation of separating characters. In 1931, Engler kept them distinct, but the material in the herbaria at Harvard cannot be distinguished at sight and the characters used by Engler to separate the two concepts are trivial indeed: for *Bauerella*, he cited sepals imbricate, stamen filaments treadlike, style long; for *Acronychia*, sepals valvate, filaments short and broad, style short. None of these is biologically critical. The genus must be based upon a significant biological difference. I, therefore, cannot accept *Bauerella*. The plant described in 1836 as *Acronychia Baueri* Schott was redescribed in 1897 as *Bauerella australiana* Borzi. I believe that the proper name of this plant should be *Acronychia Baueri* Schott." [1]

The choice of plants selected in the authors' phytochemical screening program is determined

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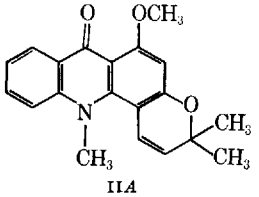
The drug used in this investigation was obtained from the Meer Corp., New York, N. Y.

Added in proof: Recently an alternative structure for acronycine has been proposed (IIA) (19):



IIA

Previous work was somewhat ambiguous in that either structure was possible, and the former structure had been selected on the basis of what could be considered as tenuous NMR data.

Oxidative degradation of acronycine has produced an acid which was identified and which could only have been derived from structure IIA.

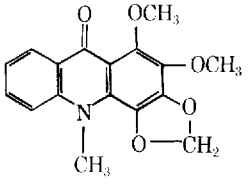
[1] Personal communication from Dr. Richard Evans Schultes, Curator, Botanical Museum, Harvard University, Cambridge, Mass.

by their reported folkloric usage and/or their reported alkaloidal content. Selection of *Acronychia Baueri* Schott falls into the second category, thereby being within the scope of the authors' original intent. Although the comprehensive work of Lahey *et al.* (4–10) had determined the alkaloidal content and the nature and structures of the various alkaloids, no biological activities were reported.

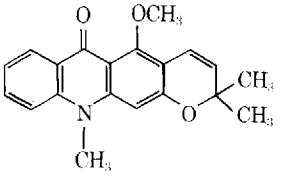
While the defatted ethanolic extract of the bark gave a negative response in the cancer screen *versus* the P-1534 leukemia, Mecca-lymphosarcoma, and adenocarcinoma 755, it elicited an interesting CNS depressant activity in the mouse behavior test (11). The observation of this latter activity was responsible for the ensuing phytochemical effort.

Inasmuch as the approach to the problem of isolation and purification involved a differential extraction technique, the results can be expected to be at variance with those of the total-extraction procedure with ethanol so frequently reported in the literature.

Defatting of the bark with *n*-hexane yielded an unexpectedly large amount of fat-soluble extractive, from which the triterpene lupeol was readily isolated. Extraction of the defatted drug with ether yielded an extract from which crystals readily deposited upon concentration. These crystals were eventually found to be a mixture of melicopine (I) and acronycine (II).



I



II

Although the original extract was inactive against the neoplasms used, it was found that the alkaloidal mixture was significantly effective *versus* the C-1498 leukemia, X-5563 myeloma, and AC-755. Fractional crystallization from methanol yielded the pure alkaloids. Melicopine was found to be ineffective against these experimental neoplasms, acronycine being responsible for the observed activity.

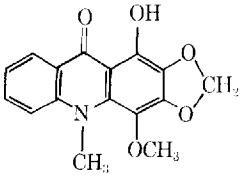
EXPERIMENTAL

A quantity of coarsely ground bark (15.0 Kg.) was extracted by stirring with two 35-L. portions of *n*-hexane. Concentration *in vacuo* yielded 253.0 Gm. of a nondrying oil. A copious quantity of fine needles deposited from the concentrate at room temperature. The oily mass was dissolved in 250 ml. of hot acetone, and the solution was allowed to cool spontaneously to room temperature, yielding 9.82 Gm. of fine colorless needles which exhibited parallel extinction and a high birefringence under polarized light, m.p. 210–212°. Its X-ray diffraction pattern was identical in all respects to that of the authentic triterpene lupeol.

The crude mother liquor (CML) elicited some activity against the C-1498 leukemia, and it was assumed that this activity was due to the acronycine content, as this alkaloid was shown to be present when examined by thin-layer chromatography (1:1 ethyl acetate–benzene on Silica Gel G).

Dissolving 128.5 Gm. of the *n*-hexane extract·CML in 2 L. of ether, with the subsequent addition of ethereal HCl to the chilled solution, produced 13.444 Gm. of a red-orange amorphous hydrochloride. Suspending the salt in H_2O and extracting with ethylene dichloride yielded 9.37 Gm. of amorphous free bases. Crystallization from acetone yielded three crops of crystals: first crop, 0.348 Gm. of what appeared to be essentially normelicopidine (III); second crop, 1.248 Gm. of acronycine contaminated with some normelicopidine; third crop, 0.644 Gm. of a mixture of apparently equal parts of acronycine and normelicopidine. A small amount of normelicopidine (0.012 Gm.) was obtained by crystallizing the mother liquor from methanol.

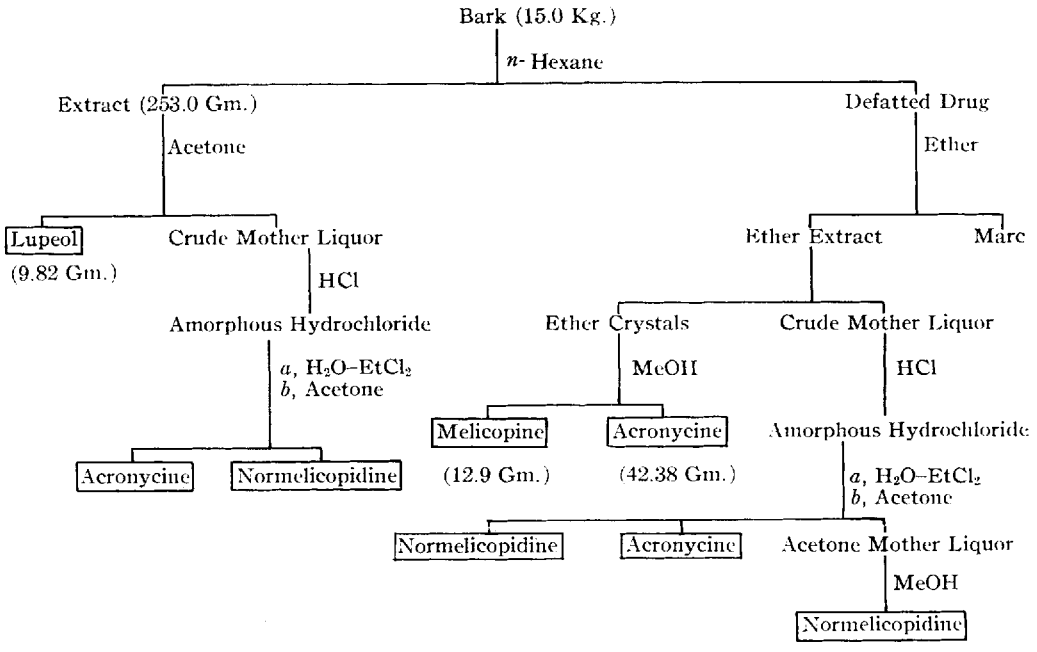
Recrystallization of the first crop from hot acetone yielded 0.298 Gm. of pure normelicopidine; recrystallization of the second crop from hot methanol yielded 0.028 Gm. of normelicopidine and 0.836 Gm. of acronycine; recrystallization of the third crop from hot methanol yielded 0.244 Gm. of normelicopidine and 0.236 Gm. of acronycine. The separation of normelicopidine from acronycine could be easily effected inasmuch as the former is quite insoluble in methanol.



III

The defatted bark was then extracted by stirring with three 40-L. portions of ether. Concentration of this extract to 10 L. produced 115.0 Gm. of yellow-gold crystals. Interpretation of physical data (X-ray, ultraviolet and infrared spectra, and TLC) indicated that this mixture was comprised of acronycine and melicopine, the former predominating.

Recrystallization of this mixture from methanol (10 ml./Gm.) afforded four crops of crystals: 12.9 Gm. of melicopine, 26.0 Gm. of slightly impure acronycine, 13.9 Gm. of a mixture of acronycine and melicopine, and 16.9 Gm. of slightly impure



Scheme I

acronycine, respectively. The second and fourth crops were combined (42.9 Gm.) and were recrystallized from methanol (8 ml./Gm.), yielding 41.4 Gm. of chromatographically pure acronycine. An additional 0.98 Gm. of pure alkaloid was obtained by crystallization of the finished mother liquor from acetone.

Treating an ether solution containing a 64.5-Gm. aliquot of the ether extract crude mother liquor[2] with ethereal HCl afforded 31.0 Gm. of a red-orange amorphous hydrochloride. The free bases were obtained (27.2 Gm.) by suspending the salt in H_2O and extracting with ethylene dichloride. Crystallization of normelicopidine (1.9 Gm.) was achieved with hot acetone (10 ml./Gm.). Concentration of the mother liquor to a low volume yielded 11.8 Gm. of essentially pure acronycine. (Chromatographic purity could be achieved by recrystallization from methanol as above.) An additional amount of normelicopidine (0.38 Gm.) was obtained by crystallization of the acetone mother liquor from methanol.

A flow diagram for the extraction scheme is presented in Scheme I.

Authentic samples of the alkaloids were not available for direct comparison, and it was necessary to resort to data published in the literature cited. All physical data[3] were in good agreement with those in the original references. As an additional aid to their identification, the infrared spectra of the individual alkaloids are presented in Fig. 1. NMR examination indicated that the integrated intensities, chemical shifts, and splitting patterns were consistent with the structures shown.

[2] Total weight = 169.0 Gm., but 100 Gm. reserved for chromatographic examination.

[3] Melting points were determined on a Kofler microstage. Ultraviolet absorption spectra were obtained using a Cary model 14 spectrophotometer; infrared spectra with a Perkin-Elmer model 21 double beam recording infrared spectrophotometer; NMR spectra with a Varian Associates 60-Mc. spectrometer. A standard Norelco powder camera, 114.6 mm. in diameter, was used in the X-ray examination.

Melicopine.—The base crystallizes from methanol as yellow orthorhombic blades elongated on the *c* crystallographic axis. Crystals show 100 and 010 faces and occasionally the 110 face crystals show pale yellow-dark yellow pleochroism, m.p. 179–181°.

Anal.—Calcd. for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.82; N, 4.47; O, 25.53. Found: C, 64.62; H, 4.96; N, 4.67; O, 25.66.

$\lambda_{max.}^{EtOH}$ 270 mμ (log ε 4.74), 301 mμ (log ε 4.13).

Acronycine.—The base crystallizes from methanol as opaque, yellow, porous particles of which neither the extinction positions nor crystal shape

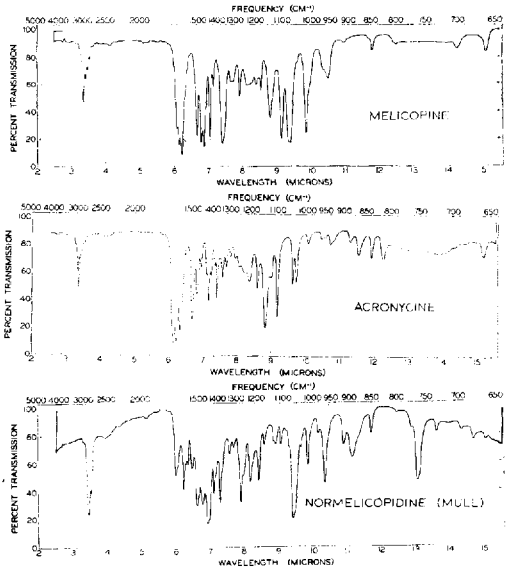


Fig. 1.—Infrared spectra of the alkaloids.

TABLE I.—EXPERIMENTAL TUMOR SPECTRUM OF ACRONYCINE

Tumor	Host, 10 Animals	i.p. Dosage, mg./Kg./Day	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm. T/C	Av. Life, T/C	% No Takes, T/C[a]	% Activity
		75 × 1 × 3					
B-82 leukemia	C58B1/6	37.5 × 1 × 7	−1.4/+0.4	...	23.7/14.6	...	61
C-1498 leukemia	C57B1/6	28 × 1 × 10	−1.5/+0.8	...	31.5/17.6	...	79 (7)
P-1534 leukemia	DBA/2	30 × 1 × 10	−0.2/−0.7	...	16.5/18.2	...	0
L-5178Y leukemia	DBA/2	28 × 1 × 10	+2.2/+3.2	...	24.2/15.0	...	62
AKR leukemia	AKR	28 × 1 × 10	+0.1/+0.8	...	38.3/21.5	...	78 (5)
Ehrlich ascites	Cox std.	30 × 1 × 10	+5.6/+7.8	...	21.8/18.4	...	0 (1)
Freund ascites	CAF[1]	48 × 1 × 10	+2.6/+6.5	...	9.8/14.5	...	0
S-180 ascites	CAF[1]	30 × 1 × 10	+3.0/+6.2	...	20.0/22.1	...	0
Taper hepatoma, ascites	SPF·ND4	48 × 1 × 10	+3.4/+8.6	...	17.7/14.8	...	20
Sarcoma 180	CAF[1]	30 × 1 × 10	+3.2/+6.0	7.1/11.9	...	0/0	40 (9)
Mecca-lymphosarcoma	AKR[c]	30 × 1 × 7	−0.4/+2.5	6.2/16.9	...	42/14	63 (7)
Ridgeway osteogenic sarcoma	AKR	48 × 1 × 9	−0.6/+3.4	0/9.6	...	100/0	100 (10)
X-5563 myeloma	C3H	30 × 1 × 8	+0.1/+0.3	0/9.1	...	100/13	100 (8)
Adenocarcinoma 755	C57B1/6	30 × 1 × 10	−0.5/+1.9	11.9/19.7	...	0/0	40 (10)
Shionogi carcinoma 115	dd/s (male)	36 × 1 × 9	+1.4/+1.4	0/15.3	...	100/5	100 (7)
High malignancy clone	C3H[d]	37.5 × 1 × 9	+1.6/+6.5	0/22.6	.	100/0	100 (5)
S-91 melanoma	DBA/1	36 × 1 × 9	−1.4/+0.1	0/14.1	...	100/0	100 (4)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors on solid tumors, indefinite survivors at 45 days on leukemia or ascitic tests. [c] Seven animals. [d] Five animals.

could be determined, from ethanol as yellow clear needles in fan-shaped clumps, the long crystals of which exhibited extinction values of approximately 18° from the elongation axis. Also in evidence were some short, thick crystals (almost cubic) that had parallel extinction to one side of the crystal, from acetone as monoclinic rods, having an extinction angle of approximately 22°. There were also a number of crystals twinned on the *a* and *c* axes, m.p. 174–176°.

Anal.—Calcd. for $C_{20}H_{19}NO_3$: C, 74.74; H, 5.96; N, 4.36; O, 14.94. Found: C, 74.76; H, 6.19; N, 4.29; O, 15.01.

$\lambda_{max.}^{EtOH}$ 280 mμ (log ε 4.60), 291 mμ (log ε 4.54), 304 mμ (log ε 4.28), 392 mμ (log ε 3.84).

Normelicopidine.—The base crystallizes from either methanol or acetone as red, equant monoclinic prisms in which occasional elongated forms are seen. The crystals show pronounced yellow-red to dark red pleochroism, m.p. 210–211°.

Anal.—Calcd. for $C_{16}H_{13}NO_5$: C, 64.21; H, 4.37; N, 4.68; O, 26.73. Found: C, 64.29; H, 4.55; N, 4.74; O, 26.46.

$\lambda_{max.}^{EtOH}$ 218 mμ (log ε 4.175), 282 mμ (log ε 4.53); shoulders at 251 mμ (log ε 4.35), 304 mμ (log ε 4.088); in alkaline solution a typical phenolic shift was seen: $\lambda_{max.}^{EtOH}$ 250 mμ (log ε 4.358), 284 mμ (log ε 4.448), 312 mμ (log ε 3.968).

BIOLOGICAL PROPERTIES

Acronycine possesses the broadest antitumor spectrum (Table I) of any alkaloid isolated to date in these laboratories. The two systems of choice for assaying its activity are the C-1498 myelogenous leukemia and the X-5563 plasma cell myeloma.

Both of these experimental neoplasms are unique in some characteristics: the C-1498 leukemia does not respond to any of the known clinically active chemotherapeutic agents presently available, while the X-5563 myeloma presents most of the same features that are associated with multiple myeloma in man. Also of special interest is the activity against the Shionogi carcinoma 115, an androgen-dependent tumor which might be of value in predicting clinical activity against prostatic cancer.

Methodology.—Procedures for animal tumor testing in these laboratories have previously been described (12) but consist essentially of subcutaneous trocar implantation of solid tumors in the axilliary region and intraperitoneal inoculation of ascitic and leukemic cells with standard cell inocula. Treatment is usually initiated 24 hr. later, exceptions being made with the X-5563 myeloma, Shionogi carcinoma 115, and the S-91 melanoma. In the case of the X-5563, treatment was initiated 3 days after transplantation, while with the other two it was begun 5 days after transplantation. Activity of a compound is defined in terms of per cent inhibition, using treated *versus* controls (T/C) when testing against the solid tumors and in terms of per cent prolongation of life, using treated *versus* controls (T/C) when testing against the leukemias and ascites systems.

Preparation of Acronycine for Testing.—The relative insolubility of this alkaloid has obviously not precluded its being tested. A suitable suspension was prepared by grinding the compound with small volumes of a nonionic dispersant[4] to obtain a uniform suspension and diluting with this diluent to the desired volume. (The dispersant is a polyoxyethylated fatty acid that is H_2O-miscible and nontoxic when diluted to the proper concentration of 1:10 with either sterile distilled H_2O or sterile physiological saline solution.) Acronycine was neither soluble in, nor were satisfactory suspensions obtained with carboxymethylcellulose, saline, sesame oil, olive oil, or acacia solution.

ANTITUMOR ACTIVITY OF ACRONYCINE

In this laboratory acronycine has been shown to be a potent antitumor agent against a multiplicity of mouse neoplasms, significant activity having been demonstrated against 12 of 17 tumors tested with a wide range of dose levels. Not only is it broad

[4] Marketed as Emulphor by General Aniline and Film Corp., Melrose Park, Ill.

Table II.—Activity of Acronycine *Versus* C-1498 Leukemia *Via* Various Routes of Administration

Route	Dosage, mg./Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Life, T/C	% Prolongation	Indefinite Survivors
Intraperitoneal	15	−0.8/+0.8	25.9/17.6	47	..
	20	−1.1/+0.8	29.0/17.6	65	6
	24	−1.9/+0.8	31.6/17.6	80	3
	28	−1.5/+0.8	31.5/17.6	79	7
	30	−1.2/+0.8	31.0/17.6	76	3
	75	−2.8/−0.2	23.0/13.3	72	..
	80	−3.1/+0.1	24.0/16.1	49	5
	90	−2.7/+0.1	29.2/16.1	81	2
	100	−2.0/+0.1	19.6/16.1	21	7
	125	−2.4/−0.2	14.6/13.3	0	..
	150	−2.5/−0.2	13.0/13.3	0	..
	175	−1.0/−0.2	12.4/13.3	0	..
Oral	30	−1.1/+0.4	23.6/17.9	31	..
	45	−1.4/+0.4	31.4/17.9	76	5
	60	−1.8/+0.4	31.3/17.9	75	7
	75	−1.4/+0.4	24.4/17.9	37	5
	80	−2.1/+0.4	18.7/17.9	0	6
Subcutaneous	15	−0.8/+0.4	19.9/18.5	0	..
	20	−1.5/+0.4	20.4/18.5	0	..
	24	−1.1/+0.4	22.8/18.5	23	..
	28	−1.8/+0.4	27.8/18.5	57	..
	30	−1.8/+0.4	27.3/18.5	48	1

Table III.—Activity of Acronycine *Versus* C-1498 Leukemia with 3-Day Delayed Treatment

Route	Dosage, mg./Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Life, T/C	% Prolongation
Intraperitoneal	15	−0.1/+1.6	23.3/16.2	43
	20	−0.3/+1.6	25.1/16.2	55
	24	−1.2/+1.6	26.0/16.2	61
	28	−1.0/+1.6	26.1/16.2	61
	30	−0.8/+1.6	25.6/16.2	58
Oral	30	−1.4/+0.4	21.4/16.6	29
	45	−1.6/+0.4	27.1/16.6	64
	60	−2.3/+0.4	26.8/16.6	61
	75	−0.8/+0.4	30.6/16.6	85
	80	−2.0/+0.4	28.8/16.6	73
Subcutaneous	15	+0.8/+1.1	18.7/16.8	0
	20	+0.5/+1.1	19.1/16.8	0
	24	−0.5/+1.1	20.3/16.8	21
	28	−0.2/+1.1	19.7/16.8	0
	30	+0.9/+1.1	19.7/16.8	0
Intravenous	3.75	+0.6/+0.7	17.8/15.6	0
	7.5	−0.4/+0.7	18.4/15.6	0
	10	−1.6/+0.7	20.2/15.6	29

Table IV.—Activity of Acronycine *Versus* C-1498 Leukemia with 6-Day Delayed Treatment

Route	Dosage, mg./Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Life, T/C	% Prolongation
Intraperitoneal	15	−1.7/−2.1	19.0/15.8	20
	20	−3.0/−2.1	21.8/15.8	34
	24	−3.0/−2.1	20.6/15.8	27
	28	−2.3/−2.1	24.0/15.8	51
	30	−2.8/−2.1	23.0/15.8	45
Oral	30	−2.0/0	18.7/16.2	0
	45	−2.6/0	19.6/16.2	21
	60	−3.4/0	19.9/16.2	23
	75	−3.3/0	26.7/16.2	65
	80	−3.0/0	22.7/16.2	33
Subcutaneous	15	−1.0/+0.6	17.4/16.2	0
	20	−1.1/+0.6	18.2/16.2	0
	24	−0.9/+0.6	17.9/16.2	0
	28	−1.8/+0.6	18.5/16.2	0
	30	−1.4/+0.6	18.7/16.2	0
Intravenous	3.75	−0.4/0	17.8/16.4	0
	7.5	−0.4/0	18.2/16.4	0
	10	−1.0/0	19.0/16.4	0

TABLE V.—ACTIVITY OF ACRONYCINE *Versus* X-5563 MYELOMA

Route	Dosage, mg./ Kg. × 1 × 8	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	24	−0.6/+0.3	0/9.1	100/13	100 (8)
	28	+1.3/+0.3	0/9.1	100/13	100 (6)
	30	+0.1/+0.3	0/9.1	100/13	100 (8)
	36	+2.5/+0.3	0/9.1	100/13	100 (3)
	45	−1.4/+0.3	0/9.1	100/13	100 (2)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors.

TABLE VI.—ACTIVITY OF ACRONYCINE *Versus* X-5563 MYELOMA WITH 3-DAY DELAYED TREATMENT

Route	Dosage, mg./ Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Tumor Size mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	15	−3.8/−0.6	1.9/10.8	55/6	82 (8)
	20	−1.4/−0.6	0.7/10.8	55/6	94 (6)
	24	+0.1/−0.6	0/10.8	100/6	100 (4)
	28	−1.2/−0.6	0/10.8	100/6	100 (6)
	30	−2.5/−0.6	0/10.8	100/6	100 (2)
Oral	30	−3.5/+0.9	1.3/13.3	67/0	90 (8)
	45	−/+0.9	−/13.3	−/0	N.S.[c]
	60	−/+0.9	−/13.3	−/0	N.S.
	75	−/+0.9	−/13.3	−/0	N.S.
	80	−1.1/+0.9	0/13.3	100/0	100 (1)
Subcutaneous	15	−2.9/−0.5	7.9/14.6	11/0	46 (8)
	20	−1.6/−0.5	4.9/14.6	22/0	67 (9)
	24	−3.5/−0.5	5.5/14.6	0/0	62 (8)
	28	−2.4/−0.5	6.5/14.6	11/0	56 (9)
	30	−3.0/−0.5	7.0/14.6	11/0	52 (9)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

TABLE VII.—ACTIVITY OF ACRONYCINE *Versus* X-5563 MYELOMA WITH 6-DAY DELAYED TREATMENT

Route	Dosage, mg./ Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Tumor Size mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	15	+0.6/+3.9	13.4/18.3	0/7	28 (7)
	20	−0.8/+3.9	10.3/18.3	14/7	43 (3)
	24	+0.9/+3.9	13.7/18.3	0/7	25 (4)
	28	+2.6/+3.9	16.5/18.3	0/7	0
	30	−1.8/+3.9	8.2/18.3	0/7	55 (2)
Oral	30	−2.1/+3.5	10.2/15.7	14/7	35 (5)
	45	−1.2/+3.5	16.0/15.7	0/7	0
	60	−2.7/+3.5	7.0/15.7	0/7	56 (1)
	75	−/+3.5	−/15.7	−/7	N.S.[c]
	80	−3.0/+3.5	9.0/15.7	0/7	43 (1)
Subcutaneous	15	+0.7/+5.4	14.0/18.4	14/7	0
	20	+0.7/+5.4	16.9/18.4	0/7	0
	24	+0.2/+5.4	15.0/18.4	0/7	0
	28	−3.5/+5.4	13.2/18.4	14/7	28 (6)
	30	−1.8/+5.4	14.1/18.4	0/7	0
Intravenous	3.75	+2.8/+5.2	16.9/24.4	0/0	30 (7)
	5	+1.0/+5.2	15.1/24.4	14/0	38 (7)
	7.5	+1.9/+5.2	19.3/24.4	0/0	0

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

spectrum in character, but it is also effective by various routes of administration. The oral and subcutaneous activities are of special interest since most of the clinically proved oncolytic agents presently used are ineffective orally and elicit intolerable side effects when administered subcutaneously.

When tested against the adenocarcinoma 755, C-1498 leukemia, and the X-5563 myeloma, acronycine also displayed significant activity by both the oral and subcutaneous routes. Furthermore, there was no evidence of skin irritation or alopecia when administered subcutaneously.

Only minimum activity was observed when the alkaloid was administered intravenously. In all probability this is attributable to its insolubility, sufficiently high blood levels not being attained. In several instances blockage of the circulatory system was evidenced, autopsy revealing an actual aortic block by the compound itself.

In determining the efficacy of a potential clinically useful compound it is always of interest to determine its effect under conditions which simulate, to the best of one's ability, the human situation, *i.e.*, its effectiveness against a well-established neoplasm. This condition can be achieved experimentally by implanting the mice with the tumors and withhold-

TABLE VIII.—ELECTROPHORETIC STUDIES ON X-5563 TUMOR-BEARING, ACRONYCINE-TREATED MICE

Dosage, 4 Animals, mg./Kg. × 1 × 10	Serum Fraction	% on Day of Sampling 0	7	12	19
20	γ-like	18	24	17	21
	β	23	24	19	19
	α	11	13	13	7
	Albumin	50	39	50	53
	Protein[a]	130	98	87	64
28	γ-like	14	23	18	18
	β	21	21	21	22
	α	12	13	14	7
	Albumin	53	43	47	53
	Protein[a]	130	96	84	58
36	γ-like	18	24	20	15[b]
	β	21	21	15	17[b]
	α	11	15	14	0[b]
	Albumin	50	60	51	68[b]
	Protein[a]	119	100	70	53[b]
Saline controls	γ-like	18	22	21	32
	β	23	20	18	17
	α	11	13	11	9
	Albumin	48	45	50	42
	Protein[a]	148	89	85	84

[a] Total count of Analytrol integrator teeth under each protein fraction peak. The number of teeth would be directly proportional to the density of the dyed protein band on the actual electrophoretic strip. [b] One animal only.

ing treatment until the neoplasm is ravaging its host. It should be noted that animals inoculated with the C-1498 leukemia at a known cell concentration normally live for a period of 14–18 days. Therefore, a delay of 6 days after inoculation before initiating therapy has without question allowed the tumor to become well established.

Acronycine also has the demonstrable ability to produce "cures" or indefinite survivors of animals inoculated with the C-1498 leukemia. An indefinite survivor is defined as one which survives 45 days or longer after inoculation, having undergone the usual 9- or 10-day regimen of daily treatment. Furthermore, it has demonstrated the ability to inhibit the growth of several neoplasms. These phenomena are detailed in the tables and summaries which follow.

Activity Against the C-1498 Leukemia.—As stated earlier, this neoplasm is singularly nonresponsive to chemotherapeutic agents. Table II summarizes the dose-response relationships for the various routes of administration, while Tables III and IV give the results of 3- and 6-day delayed therapy *via* various routes. It is readily seen that acronycine is effective when administered by any of several routes and even when treatment is delayed, the neoplasm having become well established.

Activity Against the X-5563 Plasma Cell Myeloma (Tables V, VI, and VII).—This neoplasm is intended to serve as a model for human multiple myeloma. In both the animal and human situations an abnormal protein is evidenced in the γ-globulin area of the blood serum.[5] The presence of this abnormal protein seems to increase with the progressive growth of the tumor in both hosts. The depression of this protein has been observed in patients responding to active chemotherapeutic agents, and this has also been seen in the serum of these experimental animals (Table VIII, Figs. 2 and 3).

[5] Beckman model R paper electrophoresis system, using Spinco procedure A, a method for serum proteins utilizing bromphenol blue dye in aqueous solution. The procedure is described in "Technical Bulletin 6095A," Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, Calif.

It should be noted, however, that in man it has been possible to witness a regression of the tumor mass without a depression of this protein. In mice bearing the X-5563 neoplasm and responding to treatment with acronycine, regression of the tumor has always been accompanied by a drop in this abnormal protein. This technique, therefore, provides one with an interesting correlative tool in working with a compound active against this particular tumor.

Activity Against the Shionogi Carcinoma 115.—Of special interest in the tumor spectrum is the activity against this neoplasm. This is an androgen-dependent system which may be of value in serving as a model for activity against prostatic cancer in man. While this tumor originally arose in females, it will not grow in female mice, castrated males, or males treated with progesterone, estradiol, or other female sex hormones. Dose-response relationships are presented in Table IX.

Activity Against the Adenocarcinoma 755.—The activity of acronycine against this neoplasm by various routes of administration (Table X) is another feature which distinguishes it from the alkaloids of *Catharanthus roseus* G. Don (*Vinca rosea* Linn.).

OTHER BIOLOGICAL PROPERTIES

Electrophoretic Patterns and Leukopenia.—The methodology involved in these determinations is herein described. Sixteen C3H mice[6] were randomly set up in small hanging cages with four animals in each cage and one group serving as saline controls. The first bleeding was done on day zero, by the orbital technique, to establish both the normal white blood cell counts and the normal electrophoretic patterns for each group. After the blood samples were taken, all of the animals were implanted subcutaneously with the X-5563 tumor. Food and water were supplied *ad libitum* for the

[6] Supplied by Microbiological Associates Laboratory, Bethesda, Md.

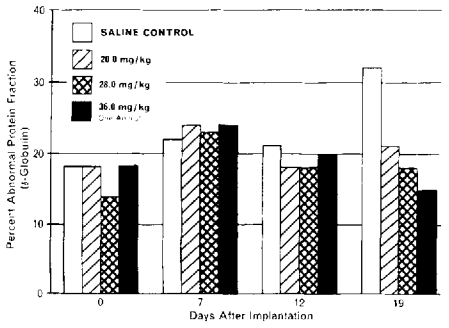


Fig. 2.—Electrophoretic patterns of X-5563 tumor-bearing, acronycine-treated mice.

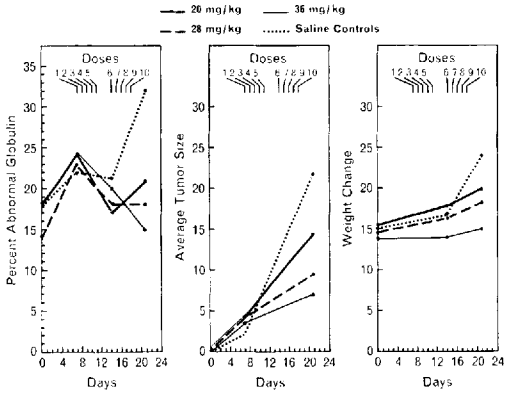


Fig. 3.—Additional parameters of electrophoresis WBC experiment.

next 6 days. On day 7 another bleeding was done, tumor measurements were made, and all animals were weighed. Intraperitoneal treatment was then initiated, three groups of mice receiving acronycine, one group receiving only saline for a total of 10 injections. Similar bleedings were done on day 12, after five injections, and on day 19, the day after the tenth and final injection. On day 19 all animals were weighed, tumor measurements were made, and the experiment was terminated (Table XI).

The electrophoresis was carried out in the following manner: 10 μl. of mouse plasma was used on each strip with Beckman B2 barbital[7] buffer, pH 8.6, having a 0.075 *M* strength. A 16-hr. run at room temperature was made, employing a constant current of 5 ma./cell. At the end of this period, the strips were stained by the Beckman method, scanned, and evaluated with a Beckman Analytrol.

Some leukopenia was evidenced at all dose levels. The method used to determine the white count was as follows: 20 μl. of whole blood was taken from each of the treated and control mice by the orbital technique. The fresh whole blood was then diluted with 10 ml. of particle-free physiological saline. Hemolysis of the red cells was achieved by adding a small amount of 2% saponin solution, allowing all of the intact white cells to be enumerated. Counts were made using a Coulter counter, the instrument being set to correlate with counts done by the hemocytometer method.

It should be noted that mice are somewhat limited in the amount of stress that can be tolerated, and that those on the electrophoresis-white blood cell count experiment were subjected to a great deal of stress because of a combination of factors—treatment with the alkaloid, several bleedings, the amount of blood withdrawn, and daily handling. The drug-treated animals were apparently unable to replenish the protein removed by the blood samplings under the conditions of daily therapy. This factor could be partly responsible for some deaths occurring during the experiments.

Mitotic Studies.—Acronycine was compared with vincaleukoblastine sulfate in several tissue culture cell lines for its ability to exert metaphase arrest. The procedures and methods have been described in detail elsewhere (13) but may be briefly summarized.

Two different cell lines were used, a diploid Chinese hamster lung and the polyploid HeLa cell. The alkaloid was dissolved in undenatured ethanol, sterile-filtered, and diluted in the appropriate tissue culture medium to a maximum concentration of 25 mcg./ml. in 0.25% ethanol. Log-phase cultures were exposed to the compounds for 4 and 24 hr. The cells were prepared according to two different methods—a hematoxylin-eosin stain (H and E) and a chromosome preparation according to the method of Puck (13).

No differences in effect were seen in the two cell lines and there was no apparent mitotic arrest associated with acronycine. At concentrations of 25 mcg./ml., acronycine exerted a very rapid cytotoxicity to cells which was produced in less than 3 hr.; yet at 10 mcg./ml. there was apparently no cytological effect. The TCD_{50} had previously been established at 13 mcg./ml. The results of a typical experiment are shown in Table XII.

Adrenal Apoplexy.—Acronycine exhibited significant activity in blocking the apoplexy effect of 7,12-dimethylbenz(a)anthracene (7,12-DMBA) when administered orally at 40 and 80 mg./rat in a single feeding 24 hr. prior to the oral administration of the 7,12-DMBA. Huggins *et al.* (14) orally administered 20 mg. of 7,12-DMBA to 50-day-old virgin female Sprague-Dawley rats and on necropsy found that the adrenals were completely engorged with blood and greatly hypertrophied. The same investigators further found that this effect could be blocked by certain other polycyclic hydrocarbons such as 3-methyl cholanthrene.

The strain, age, and sex of the animal are important factors in inducing this phenomenon. There is apparently some interrelationship between the female hormones being produced at this age, the role of the pituitary and that of the adrenal cortex, but it is not yet completely understood.

The same effects have been seen in these laboratories and the assay was performed in the following manner. Sprague-Dawley virgin female rats were received at 43 days of age. They were given food and water *ad libitum* until day 49. On this day they were weighed and dosed orally with the test compound, the controls receiving only saline. Twenty-four hours later they received the 7,12-DMBA, along with food and water *ad libitum*. On day 53 all of the animals were sacrificed by decapita-

[7] Marketed as Veronal by Winthrop Laboratories, Inc., New York, N. Y.

TABLE IX.—ACTIVITY OF ACRONYCINE *Versus* SHIONOGI CARCINOMA 115

Route	Dosage, mg./ Kg. × 1 × 9	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	36	+1.4/+1.4	0/15.3	100/5	100 (7)
	45	−0.8/+1.4	0/15.3	100/5	100 (4)
	50	−0.4/+1.4	0/15.3	100/5	100 (1)
	60	−/+1.4	−/15.3	−/5	N.S.[c]

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

TABLE X.—ACTIVITY OF ACRONYCINE *Versus* AC-755 *Via* VARIOUS ROUTES OF ADMINISTRATION

Route	Dosage, mg./ Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	15	+0.3/+1.9	15.7/19.7	0/0	0
	20	+0.2/+1.9	14.4/19.7	0/0	27 (10)
	24	−1.2/+1.9	12.6/19.7	0/0	36 (10)
	28	−0.3/+1.9	12.3/19.7	0/0	38 (10)
	30	−0.5/+1.9	11.9/19.7	0/0	40 (10)
	75	−0.1/+2.0	13.3/15.6	0/0	0
	80	−2.3/+2.0	13.8/24.1	10/0	42 (10)
	90	−2.2/+2.0	11.1/24.1	10/0	54 (10)
	100	+0.6/+2.0	14.0/24.1	10/0	42 (10)
	125	+0.3/+2.0	8.0/15.6	10/0	49 (3)
	150	−/+2.0	−/15.6	−/0	N.S.[c]
	175	−/+2.0	−/15.6	−/0	N.S.
Oral	30	−1.6/+0.9	11.1/20.4	0/0	46 (10)
	45	−2.5/+0.9	10.6/20.4	0/0	48 (10)
	60	−3.9/+0.9	9.7/20.4	0/0	52 (9)
	75	−5.1/+0.9	7.6/20.4	0/0	63 (10)
	80	−4.2/+0.9	6.5/20.4	0/0	68 (10)
Subcutaneous	15	+1.0/+2.7	13.9/20.9	0/0	34 (10)
	20	−0.1/+2.7	13.0/20.9	0/0	38 (10)
	24	−0.3/+2.7	11.7/20.9	0/0	44 (10)
	28	−0.8/+2.7	10.5/20.9	0/0	50 (7)
	30	−0.8/+2.7	13.0/20.9	0/0	38 (10)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

tion and exsanguinated. The adrenals were then removed and weighed.

Determinations of hemoglobin were not done, the adrenals instead having been observed macroscopically and rated according to the degree of apoplexy present. With an active blocking agent the adrenals appeared yellowish in color as in normal untreated rats and weighed less than those of the controls. Per cent activity of a compound in preventing apoplexy was carried by the following arbitrary protocol:

4+ = total apoplexy (inactive compound)
3+ = 30% absence of apoplexy
2+ = 60% absence of apoplexy
1+ = 80% absence of apoplexy
0 = 100% absence of apoplexy

In assaying acronycine in the preceding manner 72 and 92% blocking effects were observed at 40 and 80-mg. doses per rat, respectively.

Effect of Topical Application.—Acronycine was suspended in the nonionic dispersant[4] as previously described and a suspension of 3 mg./ml. was applied daily to the skin of normal SPF-ND4 mice for a total of 22 applications. There was no evidence of skin irritation, epilation, or hyperplasia.

Algae, Protozoa, and Mammalian Cell Studies.—A dose response of acronycine was run against six algal and protozoan agar-diffusion type assays by the method of Johnson *et al.* (15). The systems involved were *Chlorella vulgaris*, *Scenedesmus basiliensis*, *Tetrahymena pyriformis*, *Ochromonas malhamensis*, *O. danica*, and *Euglena gracilis*. Trace activities were seen against the *S. basiliensis* and *T. pyriformis*, while significant measurable zones of inhibition were observed against both of the *Ochromonas* sp. from paper disks dipped into an ethanol solution containing 500 mcg./ml. of the alkaloid.

Acronycine was further found to be inactive against the bacterial agar diffusion systems of *Clostridium feseri* and *Lactobacillus casei*. However, significant activity was observed in the tissue culture bioautograph systems employing the HeLa human epidermoid cancer cells and the AV_2 human amniotic cells. While several dose levels were used, the lowest dose level employed to give significant zones of activity was 500 mcg./ml.

These data are summarized in Table XIII.

Tetrahymena Motility.—*Tetrahymena pyriformis* is an actively motile protozoan cell. The ciliate's powerful swimming ability allows it to remain in suspension for long periods despite its almost macroscopic size. Any compound exerting a gross cellular toxicity in general or a neurotoxic effect in particular would disrupt the ciliary apparatus causing sedimentation of the immobilized cell. The effect of acronycine upon *Tetrahymena* motility was studied by comparing the sedimentation rate of alkaloid-treated cell suspensions with untreated controls. Significant biological response in this system would suggest a rapid and economical method for comparison of structural modifications

TABLE XI.—W.B.C. AT VARIOUS TIME INTERVALS DURING TREATMENT OF X-5563 TUMOR-BEARING MICE WITH ACRONYCINE

Dosage, 4 Animals, mg./Kg. × 1 × 10	Count/mm.,[a] 0 (Normal)	Day of Sampling 7	12	19	Av. Wt. of Animals, Day of Sampling 0	7	12	19	Mean Tumor Diam., mm., Day of Measurement 7	19	% Inhibition
20	4900	6000	8200	2300	15.6	16.9	19.3	20.0	4.1	14.5	34
28	4700	6200	6000	2500	14.7	16.3	17.5	18.2	4.1	9.5	57
36	4800	6200	6300	2300	13.7	14.0	15.0	15.0	2.3	7.0	68
Saline controls	5500	6100	9400	14800	15.0	16.6	19.9	23.9	2.2	21.9	...

TABLE XII.—EFFECT OF ACRONYCINE ON HELA CELLS, 24-hr. EXPOSURE–CHROMOSOME PREPARATION

Compd.	Concn., mcg./ml.	No. Mitosis	Total No. Cells Counted	% Mitoses
Acronycine	25	Too cytotoxic	...	...
	10	28	712	3.9
	5	24	650	3.7
	1	14	496	2.8
	0.5	27	725	3.7
EtOH control	0.25%	16	422	3.8
VLB sulfate	0.5	120	725	16.6
Control	...	30	830	3.6

TABLE XIII.—AGAR-DIFFUSION PAPER-DISK TYPE ASSAYS OF ACRONYCINE ON ALGAE, PROTOZOA, AND MAMMALIAN CELLS

Concn., mcg./ml.[a]	System *C.v.*[b]	*S.b.*	*T.p.*	*O.m.*	*O.d.*	*E.g.*	*C.f.*	*L.c.*	HeLa	AV_2
2000	...	H, Tr	Tr	H, 20	H, 22	...	...	...	21	16
1000	...	H, Tr	Tr	H, 18	H, 21	...	...	...	18	15
500	...	...	...	H, 17	H, Tr	...	...	...	12	14
250	...	...	...	H, 15	H, Tr	...	...	...	10	Tr

[a] Figures represent mm. inhibitory zone diameter; ..., inactive; Tr, zone less than 10 mm.; H, hazy zone. [b] *C.v., Chlorella vulgaris; S.b., Scenedesmus basiliensis; T.p., Tetrahymena pyriformis; O.m., Ochromonas malhamensis; O.d., O. danica; E.g., Euglena gracilis; C.f., Clostridium feseri; L.c., Lactobacillus casei;* HeLa, human epidermoid cancer cells; AV_2, human amniotic cells.

of the compound and as an additional tool for future mechanism of action studies. Other workers have utilized *Tetrahymena* to study the effect of the phenothiazine tranquilizers (16) and the neuromuscular blocking action of some methonium compounds (17). This is not to say that these protozoa will detect all neurologically active agents—curare and leurocristine sulfate show no toxic effect upon motility of the organism. These motility studies represent a relatively simple method of observing the biological effect of a compound. The import of these observations depends upon possible correlation to the biological effects seen in drug-treated animals.

Dilute suspensions of viable *Tetrahymena* cells were prepared in particle-free saline. Acronycine was freshly dissolved in undenatured ethanol and added to the test suspension at zero time. The final drug concentration in the cell suspension was 50 mcg./ml., the final ethanol concentration being 1% (v/v). A similar volume of ethanol was added to the cell control. The sedimentation rate of the protozoa was measured by carefully inserting the counting stylus of the Coulter counter, an electronic particle counter, into the upper third of the suspension and counting the swimming cells at timed intervals. Rate of sedimentation would be reflected by the decreasing cell counts with time. Cell counts were obtained by utilizing three different threshold settings on the instrument to give a size distribution of the viable population at each counting interval. The protozoa were separated into three size groups: small, large, and very large, and the relative sedimentation rates were obtained for each group.

The line graph (Fig. 4) shows the profound toxic effect of acronycine upon the motility of the total cell population. The bar graphs (Fig. 5) illustrate the relative sedimentation rates of the sized cells. It is interesting to note that the rate and degree of cell immobilization is less with the very large cells than in the large and small groups. No firm explanation can be given for this phenomenon at this time.

SUMMARY

Acronycine has been shown to be a very potent alkaloid against a multiplicity of mouse neoplasms.

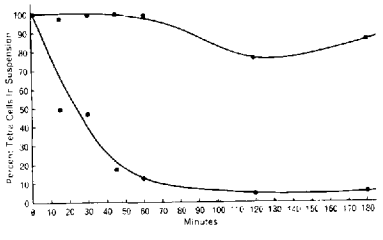


Fig. 4.—Tetrahymena motility studies. Key: upper curve with control c 1% EtOH; lower curve, acronycine, 50 mcg./ml.

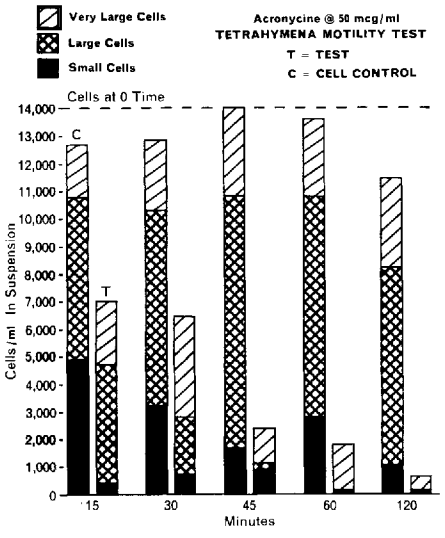


Fig. 5.—Tetrahymena motility test.

It possesses the broadest antitumor spectrum of any alkaloid isolated to date in these laboratories, being significantly active against 12 of 17 experimental neoplasms. Furthermore, it is not only effective when administered by a variety of routes, but it has demonstrated significant activity in delayed therapy experiments.

Unlike the alkaloids derived from *Catharanthus roseus* G. Don (*Vinca rosea* Linn.) (18), whose most striking experimental feature is their activity against the P-1534 leukemia in DBA/2 mice, acronycine is inactive against this neoplasm. Those systems of special interest which are responsive to this alkaloid are: the X-5563 plasma cell myeloma, a model system of multiple myeloma in man; the Shionogi carcinoma 115, an androgen-dependent tumor, potentially a model system for prostatic cancer; the C-1498 myelogenous leukemia which is nonresponsive to any of the clinically useful chemotherapeutic agents.

Other biological parameters include activity against certain protozoa and tissue culture activity against human epidermoid cancer cells and human amniotic cells. Acronycine has been shown to be an effective agent in blocking DMBA-induced adrenal apoplexy. Although some degree of leukopenia has been seen in certain experiments, the correlative weight loss associated with leukopenia was not in evidence.

No apparent mitotic arrest was seen in tissue culture studies utilizing either diploid Chinese hamster lung cells or the polyploid HeLa cells. This observation can be construed as lending credence to the theory that metaphase arrest is not a necessary function of an active oncolytic agent.

Acronycine, being an *N*-methyl acridone, possesses structural features unrelated to the *Catharanthus roseus* alkaloids or to any of the other presently known antitumor agents. Therefore, it represents a new lead toward chemotherapeutic management of a variety of neoplasms.

Studies are continuing in an attempt to ascertain as thoroughly as possible the character of the isolatable ingredients of this plant. The preparation of various derivatives and the determination of their biological activities, in an attempt to establish structure-activity relationships, are currently under way, and the results of these efforts will be reported at a later date.

REFERENCES

(1) "The Royal Horticultural Society Dictionary of Gardening," vol. 1, 2nd ed., Clarendon Press, Oxford, England, 1956, p. 33.
(2) Audas, J. W., "Native Trees of Australia," Whitcombe and Tombs, Melbourne, Australia, p. 136.
(3) Engler, A., in "Die Natürlichen Pflanzenfamilien," Engler, A., and Prantl, K., eds., vol. 19a, Wilhelm Engelmann, Leipzig, East Germany, 1931, pp. 211, 309–310.
(4) Hughes, G. K., Lahey, F. N., Price, J. R., and Webb, L. J., *Nature*, **162**, 223(1948).
(5) Crow, W. D., and Price, J. R., *Australian J. Sci. Res.*, **2A**, 282(1949).
(6) Lahey, F. N., and Thomas, W. C., *ibid.*, **2A**, 423 (1949).
(7) Brown, R. D., Drummond, L. J., Lahey, F. N., and Thomas, W. C., *ibid.*, **2A**, 622(1949).
(8) Lahey, F. N., Lamberton, J. A., and Price, J. R. *ibid.*, **3A**, 155(1950).
(9) Brown, R. D., and Lahey, F. N., *ibid.*, **3A**, 593(1950).
(10) Lamberton, J. A., and Price, J. R., *Australian J. Chem.*, **6**, 66(1953).
(11) Farnsworth, N. R., Henry, L. K., Svoboda, G. H., Blomster, R. N., Yates, M. J., and Euler, K. L., *Lloydia*, to be published.
(12) Johnson, I. S., Wright, H. F., Svoboda, G. H., and Vlantis, J., *Cancer Res.*, **20**, 1016(1960).
(13) Puck, T. T., and Steffen, J., *Biophysics (USSR) (Engl. Trans.)*, **3**, 279(1963).
(14) Boyland, E., Sims, P., and Huggins, C., *Nature*, **207**, 816(1965).
(15) Johnson, I. S., Simpson, P. J., and Cline, J. C., *Cancer Res.*, **22**, 617(1962).
(16) Guttman, H. N., and Friedman, W., *Trans. N. Y. Acad. Sci., Ser. II*, **26**, 75(1963).
(17) Van Eys, J., and Warnock, L. G., *J. Protozool.*, **10**, 467(1963).
(18) Svoboda, G. H., "Proceedings First Symposium European Cancer Chemotherapy Group (G.E.C.A.)," Excerpta Medica Foundation, Amsterdam, The Netherlands, 1966, p. 1.
(19) Macdonald, P. L., and Robertson, A. V., *Australian J. Chem.*, **19**, 275(1966).