Tumor Inhibitors XXI

Active Principles of *Acer negundo* and *Cyclamen persicum*

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An alcoholic extract of leaves and stems of *Acer negundo* L. was found to show significant inhibitory activity against sarcoma 180 in mice. Systematic fractionation of the extract led to concentration of the activity and separation of two chromatographically homogeneous active acidic saponin fractions. One of the latter saponin fractions also showed significant inhibitory activity against the Walker intramuscular carcinosarcoma 256 tumor in rats. An alcoholic extract of the tubers of *Cyclamen persicum* Mill. was found to show significant inhibitory activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (KB). Systematic fractionation led to separation of a chromatographically homogeneous saponin fraction with significant inhibitory activity against the Walker 256 tumor in rats. Preliminary characterization of the active saponin fractions is described.

In the course of the continuing search for tumor inhibitors from plant sources, an alcoholic extract of *Acer negundo* L.[1] (*Aceraceae*) was found to show significant inhibitory activity against sarcoma 180 in mice,[2] and an alcoholic extract of *Cyclamen persicum* Mill.[3] (*Primulaceae*) was found to show significant cytotoxic activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (KB).[2] The present report describes the systematic fractionation and the isolation and preliminary chemical characterization of growth-inhibitory saponin fractions from each extract. The observations confirm and extend earlier reports of the tumor-inhibitory (1, 2) and cytotoxic (3) activity of saponins.

The fractionation of the alcoholic extract of *Acer negundo* is summarized in Schemes I and II. Fraction H material, dissolved in 10% methanol in chloroform, was subjected to chromatography on adsorbent magnesium silicate,[4] and the active fractions from this column were further purified on silicic acid columns, to yield two one-spot but noncrystalline acidic saponin materials. Attempts to further purify these materials by chromatography and countercurrent distribution were unsuccessful.

The *in vivo* assay data for fractions obtained in a typical experiment are reported in Table I. The evaluation of assay results by the CCNSC on a statistical basis in sequential testing is such that a material is considered active if it causes reduction of tumor weight to 42% or less.[2] The inhibitory activity of fraction P against the Walker carcinosarcoma 256 tumor in rats over a wide dosage range is noteworthy.

The preliminary fractionation of the alcoholic extract of *Cyclamen persicum* is summarized in Scheme III. Fraction X material dissolved in 10% methanol in chloroform was subjected to chromatography on silicic acid columns, to yield a one-spot but noncrystalline saponin material. Attempts to further purify this material by chromatography and countercurrent distribution were unsuccessful.

The cytotoxicity data are reported in Table II. Evaluation of the tissue culture assay results by the CCNSC is such that a material is considered active if the ED_{50} (dose inhibiting growth to 50% of control growth) equals or is less than 4 mcg./ml.[2] The Walker 256 assay data for fraction E′ are reported in Table III. It is noteworthy that the homogeneous saponin fraction E′ showed significant inhibitory activity against the Walker 256 tumor system *in vivo*, even though the cytotoxicity against KB cells was not marked; no explanation is readily apparent.

EXPERIMENTAL

Melting points were determined on a Hoover Uni-Melt melting point apparatus and were corrected. Infrared spectra were determined in chloroform solution on a Beckman IR5A infrared spectrophotometer. Specific rotations were measured on a Zeiss-

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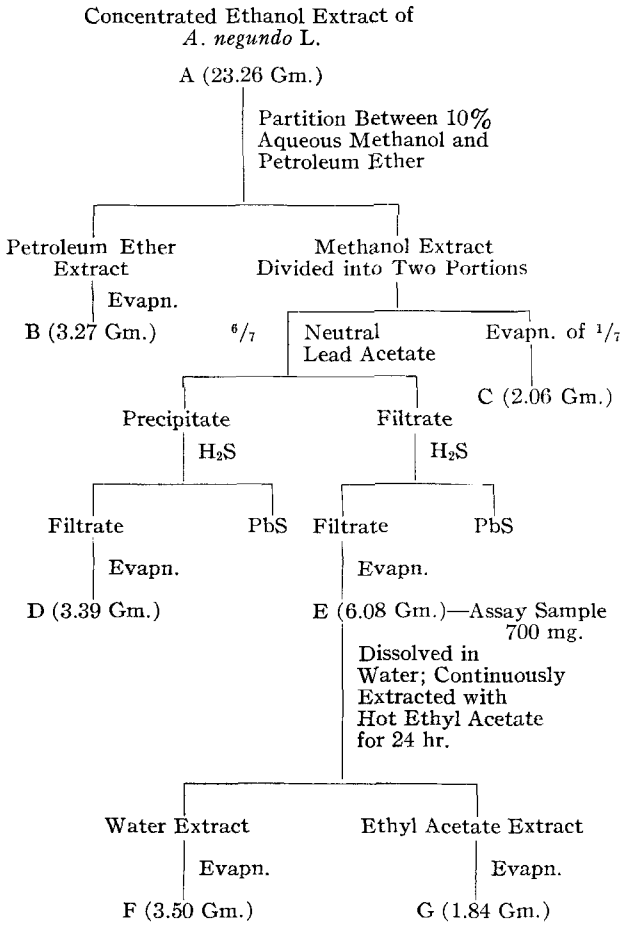
[1] Leaves and stems, gathered in the vicinity of Madison, Wis., July 1961. The authors thank Professor H. H. Iltis, University of Wisconsin, for confirming the identity of the plant. A voucher specimen is deposited in the University of Wisconsin Herbarium.

[2] Assays were performed under the auspices of the Cancer Chemotherapy National Service Center. The procedures were those described in *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

[3] Tubers, gathered in Israel in April 1962. The authors acknowledge with thanks receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the USDA by the Cancer Chemotherapy National Service Center.

[4] Marketed as Florisil by the Floridin Co., Tallahassee, Fla.



Flow Sheet for Preliminary Fractionation of Tumor-Inhibitory Extract from A. negundo *L.*

Scheme I

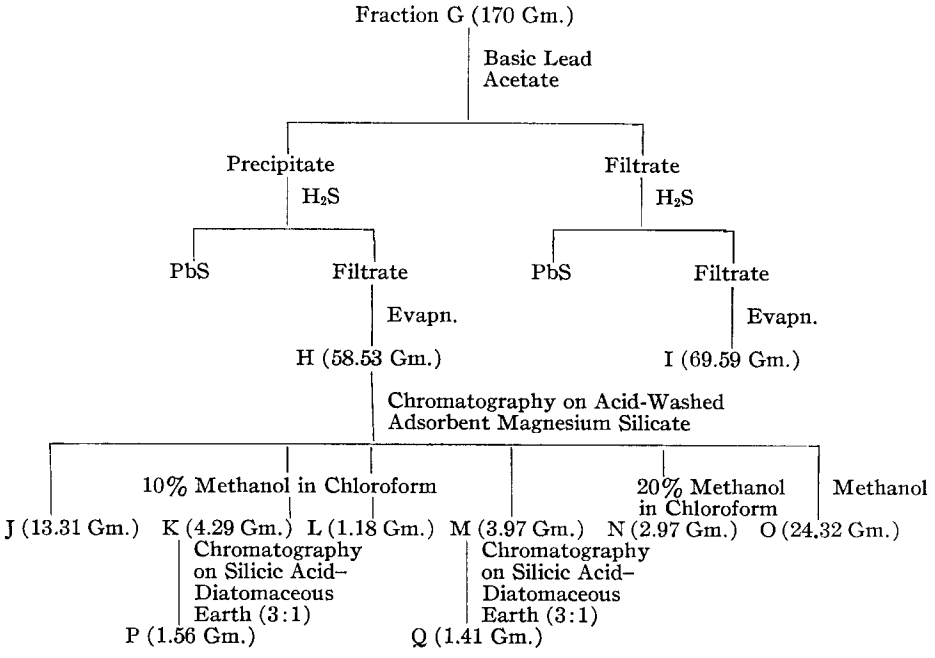
Winkel polarimeter. The petroleum ether used was Skellysolve B (b.p. 60–68°). Diatomaceous earth[5] was used for chromatography.

Extraction and Preliminary Fractionation of A. negundo—Coarsely ground leaves and twigs of *A. negundo* (2.8 Kg.) were twice extracted continuously with 95% ethanol for 5 hr., and the ethanol extract was concentrated under water pump pressure to a thick dark syrup (A, 370 Gm.). A portion of the extract (23.26 Gm.) was partitioned between petroleum ether (600 ml.) and 10% aqueous methanol (400 ml.); the petroleum ether solution and one seventh of the 10% aqueous methanol layer were evaporated under reduced pressure (B, 3.27 Gm., and C, 2.06 Gm., respectively). The remaining 10% aqueous methanol layer was treated with a methanol solution of neutral lead acetate (25% w/v) to complete precipitation. The precipitate was removed by centrifuging and washed with a little methanol before suspending in methanol and bubbling in hydrogen sulfide. The lead sulfide was filtered after precipitation had been completed, and the filtrate was concentrated to dryness under reduced pressure (D, 3.39 Gm.). The supernatant liquid from lead acetate precipitation was also treated with hydrogen sulfide, filtered, and evaporated (E, 6.08 Gm.). The material not precipitated by neutral lead acetate (after removal of an assay sample) was partitioned between water (30 ml.) and ethyl acetate (150 ml.) and the aqueous layer continuously extracted for 20 hr. with ethyl acetate. The two layers were concentrated under reduced pressure (F, 3.50 Gm., and G, 1.84 Gm., respectively).

A larger batch of fraction G (245 Gm.) was prepared from the crude extract (1450 Gm.) and a portion (170 Gm.) was dissolved in methanol (1500 ml.) and treated with a saturated methanolic solution of basic lead acetate to complete precipitation. The precipitate was removed by centrifugation and washed with a little methanol before suspending in methanol (1400 ml.) and bubbling in hydrogen sulfide. The lead sulfide was filtered after precipitation had been completed, and the filtrate was evaporated (H, 58.53 Gm.). The supernatant liquid from basic lead acetate precipitation was also treated with hydrogen sulfide, filtered, and evaporated (I, 69.59 Gm.).

Chromatography of Active Fraction H—Trial chromatographies on adsorbent magnesium silicate and silicic acid indicated that two materials giving

[5] Marketed as Celite 545 by Johns-Manville, New York, N. Y.



Flow Sheet for Final Fractionation and Chromatography of Active Principles of A. negundo *L.*

Scheme II

a brown color with 3% ceric sulfate in 3 *N* sulfuric acid were responsible for the activity of fraction H.

Fraction H (58.53 Gm.) was dissolved in methanol (100 ml.) and diluted with chloroform (900 ml.). The solubles (51.43 Gm.) were added to a column of acid-washed adsorbent magnesium silicate (3 Kg.) in chloroform. The material was eluted with 10% methanol in chloroform, and fractions were collected and examined by thin-layer chromatography on Silica Gel G using ethyl acetate–acetic acid–water (3:1:3) upper phase as developing solvent and ceric sulfate spray reagent. Fraction J (13.31 Gm.) was eluted before the first active material. Fraction K (4.29 Gm.) contained the first active material. Fraction L (1.18 Gm.) contained a mixture of the two active materials. Fraction M (3.97 Gm.) contained the second active material. The solvent was changed to 20% methanol in chloroform, fraction N (2.97 Gm.) was collected, and the remaining material was finally removed with methanol (O, 24.32 Gm.).

Fraction K (4.28 Gm.), dissolved in 25 ml. of 25% methanol in chloroform, was added to a column of silicic acid–diatomaceous earth (3:1, 800 Gm.) and the column was eluted with 7% methanol in chloroform (2.5 L.). The solvent was changed to 10% methanol in chloroform (12 L.), and fractions were collected and examined by TLC, using ethyl acetate–acetic acid–water (3:1:3) upper phase as solvent, and ceric sulfate as spray reagent. The fractions richest in the first active material were combined (P, 1.56 Gm.). Elution was continued with 20% methanol in chloroform (2 L.) and the remaining material was finally removed with methanol. Fraction P accounted for the activity of fraction K, and was essentially one-spot material on TLC.

Fraction M (3.9 Gm.), dissolved in 25 ml. of 25% methanol in chloroform, was added to a column of silicic acid–diatomaceous earth (3:1, 750 Gm.) and the column was eluted with 8.5% methanol in chloroform (2 L.). The solvent was changed to 10% methanol in chloroform (9 L.), fractions were collected and examined by TLC using ethyl acetate–acetic acid–water (3:1:3) upper phase as solvent, and ceric sulfate as spray reagent. The fractions richest in the second active material were combined (Q, 1.41 Gm.). Elution was continued with 20% methanol in chloroform (2 L.) and the remaining material was removed with methanol. Fraction Q accounted for the activity of the fraction M, and was one-spot material on TLC.

Fractions P and Q were also tested against Walker carcinosarcoma 256 (see Table II); only fraction P showed activity.

Fraction P was a yellow powder, m.p. 198–205° dec., $[\alpha]_D^{28} = +28.5°$ (c 1.79, MeOH).

Fraction Q was a beige powder, m.p. 230–237° dec., $[\alpha]_D^{27} = +8.6°$ (c 1.45, MeOH).

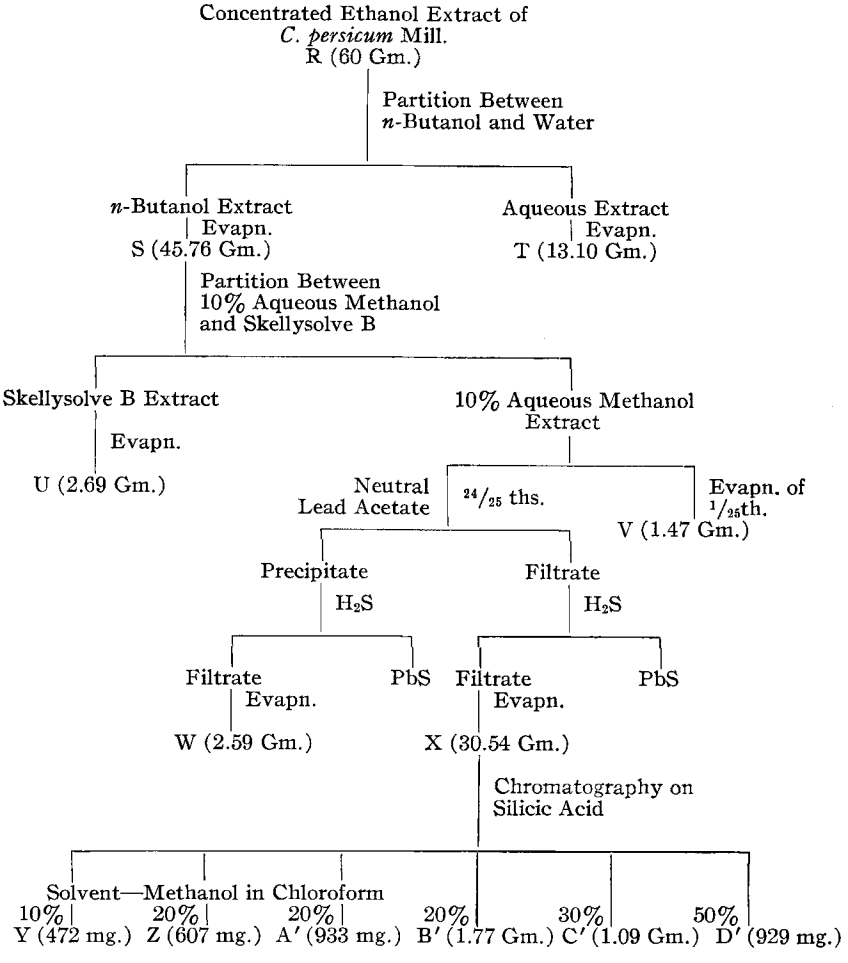
The acidic nature of fractions P and Q was indicated by their solubility properties. Both were readily soluble in dilute aqueous sodium bicarbonate, from which they were precipitated upon acidification with dilute hydrochloric acid.

Extraction and Preliminary Fractionation of C. persicum—Coarsely ground tubers of *C. persicum* (500 Gm.) were twice extracted continuously with 95% ethanol for 5 hr., and the ethanol extract was concentrated under water pump pressure to a brown resinous solid (R, 175 Gm.). A portion of the extract (60 Gm.) was partitioned between *n*-butanol (1 L.) and water (500 ml.), and the two solutions were evaporated separately under reduced pressure (S, 45.76 Gm., and T, 13.10 Gm., respectively). The *n*-butanol extract was further partitioned between

petroleum ether (1 L.) and 10% aqueous methanol (500 ml.), and the petroleum ether solution and one twenty-fifth part of the 10% aqueous methanol were evaporated under reduced pressure (U, 2.69 Gm., and V, 1.47 Gm., respectively). The remaining 10% aqueous methanol layer was treated with a saturated

TABLE I—ACTIVITY OF FRACTIONS FROM *A. negundo* L.

Fraction	Dose, mg./Kg.	Survivors	Animal Wt. Change, Gm. Diff. (T-C)	Tumor Wt., mg. (Test/Control)	T/C × 100
			Against Sarcoma 180		
A	500	0/4			Toxic
	250	1/4	−0.3	265/1563	Toxic
	125	4/4	−3.0	603/1563	38
B	160	4/4	+0.8	521/879	59
	80	4/4	+1.7	474/879	53
	40	4/4	+1.3	738/879	83
C	175	4/4	−2.7	290/1062	27
	87.5	4/4	+1.3	396/1062	37
	43.7	4/4	+0.3	771/1062	72
D	200	4/4	+1.5	253/879	28
	100	4/4	+1.5	655/879	74
	50	3/4	−1.2	1030/879	117
E	350	0/4			Toxic
	175	4/4	−3.1	46/879	5
	87.5	4/4	−2.1	715/879	81
F	350	4/4	+1.1	1323/1361	97
	175	4/4	+3.4	1244/1361	91
	87.5	4/4	+0.3	1045/1361	76
G	190	0/4			Toxic
	95	1/4	−1.7	205/1361	Toxic
	47.5	3/4	−1.8	330/1361	24
H	100	0/4			Toxic
	50	0/4			Toxic
	25	4/4	+1.2	561/1438	39
I	100	4/4	+0.9	1409/1438	97
	50	4/4	−1.1	1473/1438	102
	25	4/4	+1.4	1615/1438	112
J	80	4/4	+1.7	2124/2101	101
	40	4/4	+2.2	1598/2101	76
	20	4/4	+1.0	2653/2101	126
K	16	0/4			Toxic
	8	0/4			Toxic
	4	4/4	−3.4	1311/1867	70
L	12	0/4			Toxic
	6	3/4	−4.9	950/1867	50
	3	4/4	−3.0	874/1867	46
M	16	0/4			Toxic
	8	0/4			Toxic
	4	2/4	−7.2	243/1867	Toxic
N	14	3/4	−4.2	820/1867	43
	7	3/4	−2.0	932/1867	49
	3.5	3/4	+0.1	1828/1867	97
O	40	4/4	−0.7	1139/2101	54
	20	4/4	+0.2	2441/2101	116
	10	4/4	+0.7	2295/2101	109
P	10	0/4			Toxic
	5	1/4	−4.5	485/1192	Toxic
	2.5	3/4	−4.8	317/1192	26
Q	6	2/4	−3.3	568/1192	Toxic
	5	1/4	−3.8	565/1192	Toxic
	4	4/4	−5.4	270/1192	22
	3	3/4	−4.4	478/1192	40
	2	4/4	−3.9	346/1192	29
	1	4/4	−3.5	484/1192	40
			Against Walker Carcinosarcoma 256		
P	6	4/4	−24	1500/4000	37
	3	4/4	−20	1100/4000	27
	1.5	4/4	−12	2500/4000	62
	7.5	5/6	−5	2600/9100	28
	5.0	6/6	−6	3000/9100	32
	3.3	6/6	−10	2000/9100	21
	2.2	6/6	−6	3500/9100	38
Q	8	2/4	−20	1700/5800	Toxic
	4	4/4	−13	5000/5800	86
	2	4/4	0	5200/5800	89



Flow Sheet for Fractionation of Cytotoxic Extracts of C. persicum *Mill.*

Scheme III

methanol solution of neutral lead acetate to complete precipitation. The precipitate was removed by centrifuging and washed with a little methanol before suspending in methanol and bubbling in hydrogen sulfide. The lead sulfide was filtered off after precipitation had been completed, and the filtrate was evaporated (W, 2.59 Gm.). The supernatant liquid from lead acetate precipitation was also treated with hydrogen sulfide, filtered, and concentrated to dryness under reduced pressure (X, 30.54 Gm.).

TABLE II—CYTOTOXICITY OF FRACTIONS FROM *C. persicum* MILL.

Fraction	ED_{50}, mcg./ml.
R	25
S	17
T	>100
U	>100
V	2.1
W	10
X	2.7
Y	22
Z	13
A′	7.4
B′	18
C′	13
D′	18
E′	24

Chromatography of Active Fraction X—Fraction X (6.0 Gm.) dissolved in methanol (20 ml.) was diluted with chloroform (180 ml.) and the solution was introduced onto a column of silicic acid (600 Gm.) in chloroform. The column was eluted with 10% methanol in chloroform until the yellow bands were eluted (fraction Y, 472 mg.). The solvent was changed to 20% methanol in chloroform, and fractions were collected and examined by thin-layer chromatography on Silica Gel G using 50% methanol in chloroform as solvent and ceric sulfate as spray reagent. Fraction Z (607 mg.) was eluted before a one-spot active material. Fraction A′ (933 mg.) contained the one-spot active material and fraction B′ (1.77 Gm.) was eluted after the active material. The solvent was changed to 30% methanol in chloroform, when fraction C′ (1.09 Gm.) was eluted. The remaining material was removed with 50% methanol in chloroform, to yield fraction D′ (929 mg.).

TABLE III—ACTIVITY OF THE ACTIVE FRACTION FROM *C. persicum* MILL. AGAINST THE WALKER INTRAMUSCULAR CARCINOSARCOMA 256

Fraction	Dose, mg./Kg.	Survivors	Animal Wt. Change, Gm. Diff. (T-C)	Tumor Wt., mg. (Test/Control)	T/C × 100
E′	50	2/4	−14	1400/7400	Toxic
	40	4/4	−11	1900/7100	26%
	30	4/4	−7	4400/7100	61%

A larger batch of fraction A′ (9.67 Gm.), prepared from crude extract (84 Gm.), was dissolved in 5% methanol in chloroform (400 ml.) and introduced onto a column of silicic acid (1 Kg.) in chloroform. The column was eluted with 5% methanol in chloroform (4 L.), 7% methanol in chloroform (6 L.), and with 10% methanol in chloroform (10 L.), to yield fractions which were examined by TLC as before. The fractions richest in the one-spot active material were combined (E′, 4.84 Gm.) and tested in the Walker carcinosarcoma 256 assay (see Table III).

Fraction E′ was a white amorphous powder, (m.p. 229–233°); $[\alpha]_D^{28}$ = +0.8° (c 1.20 MeOH), I.R. (Nujol) 3.04 μ, 5.81 μ, 6.10 μ.

Determination of the Hemolytic Index of Fractions P, Q, and E′—The determination was performed by the method outlined in the Swiss Pharmacopoea (4) using a 1:20 dilution of blood with normal saline and Fisher standard saponin as reference. Using these conditions, fraction P had a hemolytic index of 0.8; fraction Q, 0.5; and fraction E′, 316; with reference to standard saponin at 100. Hence the *Acer negundo* active principles have low hemolytic activity.

Test for the Glycosidic Nature of Fractions P, Q, and E′—Fractions P, Q, and E′ (5 mg. of each, separately), when heated for 2 min. with a 0.5% aqueous solution of triphenyltetrazolium chloride (2 drops) and 0.5 *N* sodium hydroxide (1 drop), gave no pink color or precipitate.

Further 5 mg. portions of fractions P, Q, and E′ were hydrolyzed by refluxing for 30 min. with 0.5 *N* hydrochloric acid in 50% aqueous alcohol (0.2 ml). Each hydrolyzate was neutralized and the triphenyltetrazolium chloride test performed. The deep red solutions and red precipitates indicated the presence of reducing sugars in each of the hydrolyzates (5).

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Molecular Orbital Calculations on Sulfonamide Molecules

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The LCAO-MO method has been used to compute the electronic characteristics for a series of 50 sulfonamide derivatives. The results of the calculations have been compared with experimental parameters through correlation with pKa. For those compounds where the substituent R group was an alternate ring, the correlation of pKa and the electronic charge at the ionizing (N^1) nitrogen atom was considered significant. However, it was necessary to classify the compounds by type of R group, plotting each class separately. The simple Hückel MO treatment was inadequate for those molecules where the substituent R group attached to the N^1 nitrogen atom was a nonalternate ring or a straight chain.

IN RECENT YEARS it has become possible to study the electronic structure of large organic molecules by using the principles of quantum mechanics. This field is known as quantum chemistry, and when applied to molecules of biological interest, has been called quantum biology or quantum biochemistry (1–4). The molecular orbital (MO) method, involving a linear combination of atomic orbitals (LCAO), is ordinarily used for calculating the energies of the π or delocalized electrons in a molecule, and the abbreviation LCAO-MO is used to designate this type of calculation. In this study, a modification of the LCAO-MO method, the simple Hückel molecular orbital (HMO) approximation (3–6), has been employed.

Actually, the reactivity of drug molecules should be discussed in terms of their dynamic

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