appear to be reversible. Chloroanilines are readily produced during the metabolism of halogenated formyl- and acetanilides by *F. oxysporum* (Kaufman *et al.*, 1972c). In identical experiments with 4-chloronitrobenzene as a substrate, we have observed the production of small amounts (3–4%) of 4-chloroaniline (Figure 7). 3,4-Dichloroaniline was also detected as a metabolite of 3,4-dichloronitrobenzene (Kaufman *et al.*, 1972a). The reduction of nitro aryl compounds to the corresponding arylamine by *F. oxysporum* (Madhosingh, 1961) and other microorganisms (Chacko *et al.*, 1936) has also been observed by others. The apparent reversibility of these reactions has made stoichiometric measurement of individual metabolic reactions difficult. Although such a dynamic relationship exists among the various metabolites, in experiments with ^{14}C-phenyl-labeled anilines the percentage of labeled carbon that can no longer be extracted from the aqueous medium increases with the length of incubation (Kaufman *et al.*, 1972b). Studies are continuing to characterize the microbial production and degradation of water-soluble metabolites of chloroanilines.

LITERATURE CITED

Bartha, R., *Science* **166**, 1299 (1969).
Bartha, R., Pramer, D., *Science* **156**, 1617 (1967).
Bartha, R., Linke, H. A. B., Pramer, D., *Science* **161**, 582 (1968).
Booth, J., Boyland, E., *Biochem. J.* **91**, 362 (1964).
Bordeleau, L. M., Bartha, R., *Bull. Environ. Contam. Toxicol.* **5**, 34 (1970).
Bordeleau, L. M., Rosen, J. D., Bartha, R., *J. Agr. Food Chem.* **20**, 573 (1972).
Boyland, E., Nery, R., *Analyst* **89**, 95 (1964).
Casida, J. E., Augustinsson; I., *Biochim. Biophys. Acta* **36**, 411 (1959).
Chacko, C. I., Lockwood, J. L., Zabik, M. L., *Science* **154**, 893 (1966).
Chisaka, H., Kearney, P. C., *J. Agr. Food Chem.* **18**, 854 (1970).
Farrow, M. D., Ingold, C. K., *J. Chem. Soc. London* **125**, 2543 (1924).
Gunner, H. B., *Nature (London)* **197**, 1127 (1963).
Ingold, C. K., *J. Chem. Soc. London* **127**, 513 (1925).
Iwasaki, I., Utsumi, S., Ozawa, T., *Bull. Chem. Soc. Jap.* **25**, 226 (1952).
Kaufman, D. D., Plimmer, J. R., Iwan, J., 162nd National Meeting of the American Chemical Society, Washington, D. C., September 1971.
Kaufman, D. D., Plimmer, J. R., Iwan, J., Klingebiel, U. I., *J. Agr. Food Chem.* **20**, 916 (1972a).
Kaufman, D. D., Plimmer, J. R., Iwan, J., Klingebiel, U. I., 163rd National Meeting of the American Chemical Society, Boston, Mass., April 1972b.
Kaufman, D. D., Plimmer, J. R., Iwan, J., Klingebiel, U. I., unpublished data, 1972c.
Kearney, P. C., Plimmer, J. R., Guardia, F. B., *J. Agr. Food Chem.* **17**, 1418 (1969).
Knowles, C. O., Gupta, A. K. S., Hassan, T. K., *J. Econ. Entomol.* **62**, 411 (1969).
Lieb, H. B., Still, C. C., *Plant Physiol.* **44**, 1672 (1969).
Madhosingh, C., *Can. J. Microbiol.* **7**, 553 (1961).
Neuberg, C., Reinfurth, E., *Biochem. Z.* **139**, 561 (1923).
Neuberg, C., Wilde, E., *Biochem. Z.* **67**, 18 (1914).
Pease, H. L., *J. Agr. Food Chem.* **10**, 279 (1962).
Plimmer, J. R., Kearney, P. C., Chisaka, H., Yount, J. B., Klingebiel, U. I., *J. Agr. Food Chem.* **18**, 859 (1970).
Rosen, J. D., Siewerski, M., Winnett, G., Abstr 24, Division of Pesticide Chemistry, 158th National Meeting of the American Chemical Society, New York, N. Y., September 1969.
Still, G. G., *Org. Mass Spectrom.* **5**, 977 (1971).
Taylor, T. W. J., Baker, M. A., "Sidgwick's Organic Chemistry of Nitrogen," Oxford, 1942.
Vogel, A. I., "A Textbook of Practical Organic Chemistry," Longmans, Green and Co., New York, N. Y., 1956.

Received for review June 16, 1972. Accepted October 12, 1972.

COMMUNICATIONS

Insecticidal and Anticholinesterase Activity of Benzotriazolyl Methyl and Dimethylcarbamates

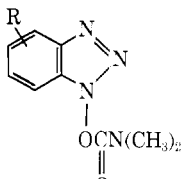
A new series of heterocyclic carbamates based on 1*H*-1-benzotriazol have been synthesized and evaluated as insecticides and as inhibitors of fly and bovine cholinesterases. Only in the dimethylcarbamates do regression analyses show high correlation between cholinesterase inhibition and chemical reactivity of the inhibitors as expressed by Hammett's σ constant. Anticholinesterase activity of the monomethylcarbamates increases and that of the dimethylcarbamates decreases with an increase in the σ value of the substituents.

Several groups of heterocyclic carbamate esters have been shown to possess insecticidal properties. Weismann *et al.* (1951), followed by Gysin (1954), were the first to study the pyrazolyl dimethylcarbamates. Their extensive work led to the development of insecticides such as isolan and dimetilan. In a search for safe and hydrolytically stable carbamates, the same group developed, later on, the hydroxyquinoline derivatives such as the 2-methylquinolyl-8 methylcarbamate (Gubler *et al.*, 1968). Kilsheimer *et al.* (1969) describe yet another class of heterocyclic methylcarbamates based on benzothiophene; preeminent among these is benzo[*b*]thien-4-yl methylcarbamate (Mobam). This communication presents data on a new group of heterocyclic carbamates with interesting anticholinesterase properties.

EXPERIMENTAL SECTION

Chemicals. The benzotriazolyl dimethylcarbamates were prepared by reaction of the corresponding 1-hydroxybenzotriazoles (0.05 mol) in dry pyridine (40 ml) with dimethylcarbamoyl chloride (0.06 mol) at room temperature for 24 hr. Addition of water precipitated the crude prod-

Table I. Physical Properties and Biological Activities of Substituted Benzotriazolyl Dimethylcarbamates



R	Hammett's sigma $\Sigma\sigma$	mp, °C	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N	I_{50} M Fly	I_{50} M Bovine
6-CH_3	−0.17	99–101	54.5	5.5	25.4	54.7	5.5	25.5	4×10^{-7}	1×10^{-5}
[4,5-b]Py[a]	−0.16	106–108	46.6	4.4	33.8	46.5	4.2	33.7	7×10^{-7}	1.2×10^{-5}
H	0.0	87–88	52.4	4.9	27.2	52.3	4.8	27.2	5×10^{-7}	8×10^{-6}
4-CH_3; 5-Cl	0.2	143–144	47.2	4.4	22.0	47.3	4.4	22.0	2.3×10^{-6}	1×10^{-4}
4-Cl	0.23	106–107	44.9	3.8	23.3	45.1	3.8	23.2	1×10^{-6}	3×10^{-5}
6-Cl[b]	0.23	129–131	44.9	3.8	23.3	45.0	3.8	23.2	1.5×10^{-6}	4×10^{-5}
5-Cl	0.37	134–135	44.9	3.8	23.3	45.0	3.7	23.3	6×10^{-6}	2×10^{-4}
6-CF_3	0.55	98–99	43.8	3.3	20.4	43.8	3.1	20.4	1.3×10^{-5}	5×10^{-4}
5,6-$(Cl)_2$	0.59	183–185	39.3	2.9	23.8	39.2	2.8	24.0	1×10^{-5}	5×10^{-4}
4-NO_2; 6-CH_3	0.61	219–220	45.3	4.2	26.4	45.2	4.2	26.2	3.3×10^{-6}	5×10^{-5}
6-$CONHC_6H_5$	0.63	170–171	59.1	4.6	21.5	59.2	4.5	21.5	1.7×10^{-5}	5×10^{-4}
6-NO_2	0.78	152–153	43.0	3.9	27.9	42.9	3.8	28.0	2×10^{-5}	4×10^{-4}

[a]Py = pyridyl. [b]LD_{50} = 0.07% against *Boophilus microplus* larvae in Petri dish test.

uct which was isolated by filtration or extraction with chloroform. Analytically pure material was obtained by crystallization from a suitable solvent. The physical properties and analytical data for these chemicals are given in Table I.

The benzotriazolyl methylcarbamates were prepared by reaction of the corresponding 1-hydroxybenzotriazoles (0.02 mol) in dry tetrahydrofuran with methyl isocyanate (0.02 mol) in the presence of catalytic amounts of triethylamine at room temperature for 24 hr. Removal of solvent *in vacuo* gave the crude product, which was purified by crystallization from a suitable solvent. The physical properties and analytical data for these chemicals are given in Table II.

The starting 1-hydrobenzotriazoles were prepared by the method of Müller and Zimmermann (1925) from the appropriate chloronitrobenzenes and hydrazine hydrate in refluxing ethanol.

Compounds not previously reported in the literature follow.

4-Chloro-1-hydroxy-1H-benzotriazole, mp 160° dec (aqueous ethanol).

Anal. Calcd for $C_6H_4ClN_3O$: C, 42.50; H, 2.38; N, 24.78. Found: C, 42.33; H, 2.39; N, 24.87.

5-Chloro-1-hydroxy-4-methyl-1H-benzotriazole, mp 189° dec (aqueous ethanol).

Anal. Calcd for $C_7H_6ClN_3O$: C, 45.79; H, 3.30; N, 22.87. Found: C, 45.67; H, 3.20; N, 23.04.

6 - (α,α,α-Trifluoromethyl)-1-hydroxy-1H-benzotriazole, mp 143–145° (aqueous ethanol).

Anal. Calcd for $C_7H_4F_3N_3O$: C, 41.39; H, 1.98; N, 20.69. Found: C, 41.17; H, 1.93; N, 20.53.

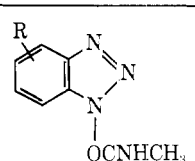
1-Hydroxy-1H-benzotriazole-6-carboxanilide, mp 234° dec (aqueous ethanol).

Anal. Calcd for $C_{13}H_{10}N_4O_2$: C, 61.41; H, 3.96; N, 22.04. Found: C, 61.20; H, 4.14; N, 21.91.

1-Hydroxy-1H-v-triazolo[4,5-b]pyridine, mp 215° dec (ethanol).

Anal. Calcd for $C_5H_4N_4O$: C, 44.12; H, 2.96; N, 41.17. Found: C, 43.97; H, 3.07; N, 40.99.

Table II. Physical Properties and Biological Activities of Substituted Benzotriazolyl Methylcarbamates



R	Hammett's sigma $\Sigma\sigma$	mp, °C	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N	I_{50} M, fly ChE
6-CH_3	−0.17	174–176	52.4	4.9	27.2	52.5	5.0	27.3	2.5×10^{-5}
H	0.0	164–166	50.0	4.2	29.1	49.9	4.2	28.9	5×10^{-5}
4-CH_3; 5-Cl	0.2	174–175	44.9	3.8	23.3	44.7	3.6	23.3	5×10^{-5}
4-Cl	0.23	148–149	42.2	3.1	24.7	42.6	3.0	24.8	8×10^{-5}
5-Cl	0.37	195–197	42.4	3.1	24.7	42.6	3.2	24.8	4×10^{-6}
6-CF_3	0.55	189–191 (dec)	41.6	2.7	21.5	41.6	2.6	21.3	1.1×10^{-5}
5,6-$(Cl)_2$	0.59	187 (dec)	36.8	2.3	21.5	37.0	2.2	21.6	9×10^{-6}
4-NO_2; 6-CH_3	0.61	232–234	43.0	3.6	27.9	43.8	3.6	27.7	6×10^{-5}
6-$CONHC_6H_5$	0.63	174–175	57.9	4.2	22.5	58.0	4.0	22.3	6×10^{-7}
6-NO_2	0.78	179–181 (dec)	40.5	3.0	29.5	40.7	3.1	29.7	1.5×10^{-6}

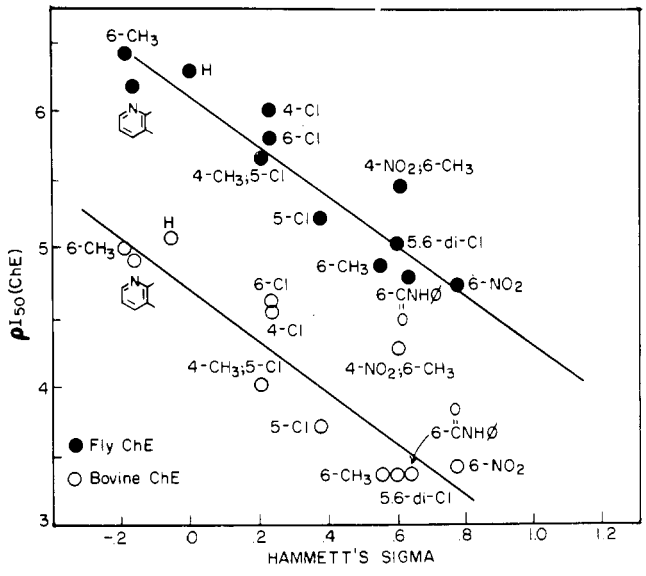


Figure 1. Relationship of pI_{50} for fly and bovine cholinesterase inhibition to σ values for substituted benzotriazolyl dimethylcarbamates.

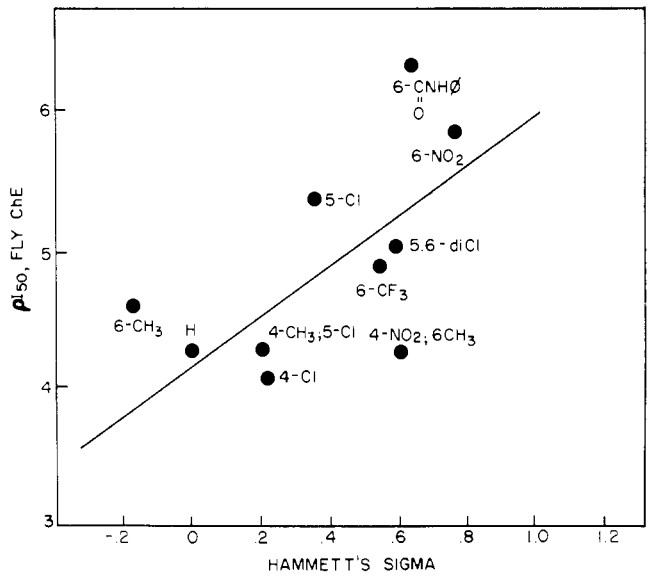


Figure 2. Relationship of pI_{50} for fly cholinesterase inhibition to σ values for substituted benzotriazolyl methylcarbamates. Regression line equation: fly $pI_{50} = 4.14 + 1.76\sigma$.

Cholinesterase Determinations. The procedure described by Sacher and Olin (1972) was used for the enzyme determinations. Briefly, the cholinesterase was incubated with the carbamates for 12 min, utilizing a Technicon Autoanalyzer apparatus (Winter, 1960). The I_{50} values, the molar concentration giving 50% inhibition of the enzyme under the test condition, were estimated graphically by using three to five inhibition measurements around this point. The I_{50} values reported are averages of at least two separate determinations.

Bovine erythrocyte cholinesterase was purchased from Sigma Chemical Company, St. Louis, Mo. For assay, the bovine enzyme was diluted to a concentration of 2.5 μM units per ml (1 μM unit will hydrolyze 1 μmol of acetylcholine per minute at pH 8.0 at 37°). Whole fly homogenates were utilized to prepare the fly cholinesterase (Darlington *et al.*, 1971) which was used in our assays.

Bioassays. Two-day-old susceptible female houseflies (*Musca domestica* L. SNAIDM strain) were utilized for the fly toxicity measurements. Flies were treated topically as described by Sacher and Olin (1972). For the synergism studies flies were treated as above with a carbamate–2,3-methylenedioxynaphthalene combination at a 1:1 ratio (w/w). The latter synergist was used because of its excellent activity with a great variety of carbamates (Metcalf *et al.*, 1966; Sacher *et al.*, 1971).

Three-day-old, early fourth instar larvae of the yellow fever mosquito (*Aedes aegypti* L.) reared on a liver powder diet (Craig and Vandehey, 1962) were used for testing. Test solutions were prepared by adding 0.05 ml of acetone solution of an appropriate concentration to 50 ml of distilled water, and 15 to 20 larvae were placed therein. Mortality was determined 24 hr later.

Correlations in the data were examined on a computer by a linear regression program.

RESULTS AND DISCUSSION

Tables I and II list the compounds with their appropriate substituent R, the corresponding Hammett's σ values (Jaffe, 1953), their melting points, elemental analyses, and the pertinent biological data. The correlation of fly and bovine pI_{50} (the negative logarithm of I_{50}) with σ is given in Figures 1 and 2.

The equations for the regression lines of ChE *vs.* σ in the dimethylcarbamates are fly $pI_{50} = 6.10 - 1.77\sigma$ and bovine $pI_{50} = 4.70 - 1.82\sigma$. The correlation coefficients are 0.93 and 0.88 for the fly and bovine regression lines, respectively, and the corresponding standard deviations are 0.24 and 0.34. This high correlation between the chemical reactivity and the anticholinesterase action of the dimethylcarbamates indicates that σ values alone can account for most of the differences in activity of these compounds.

Metcalf and Fukuto (1965) have shown that in phenyl and xylenyl methylcarbamates, in which the para substituents are small, there is an inverse relationship between the Hammett σ coefficient and anticholinesterase. Therefore, the results obtained with the monomethylcarbamates (Table II and Figure 2) were somewhat surprising. Even though the correlation coefficient is low (0.60), there is a definite increase in anticholinesterase activity with an increase in the electron withdrawing of the substituents. A somewhat better fit (0.67) was attained when σ minus was used for the correlation. The use of σ minus constant has been proposed for reactions where a negative charge is developed in the reaction center. Since these carbamate esters are derivatives of phenolic compounds, this improved correlation with σ minus is expected.

The 15–49× selectivity between fly and bovine cholinesterase in these carbamates compares favorably with the 10–15× enzyme selectivity reported for a series of ethylphosphonates (Darlington *et al.*, 1971). This difference might be attributed to the better fit of bulky cholinesterase inhibitors to the insect enzyme (Hollingworth *et al.*, 1967) or to the presence of other esterases in the whole fly as compared to the bovine erythrocyte cholinesterase preparation.

All these compounds showed little or no activity against mosquito larvae at 2.0 ppm. The chemicals listed in Table I were practically inactive when tested against houseflies at doses as high as 2.0 μg/female. Furthermore, attempts to synergize these chemicals with 2,3-methylenedioxynaphthalene (1:1 w/w) did not improve their performance. Some of these carbamates, however, have shown appreciable activity against other insect species and some acarina (*e.g.*, 6-chlorobenzotriazolyl dimethylcarbamate, Table I).

LITERATURE CITED

Craig, G. B., Jr., Vandehey, R. C., *Ann. Entomol. Soc. Amer.* **55**, 47 (1962).

Darlington, W. A., Partos, R. D., Ratts, K. W., *Toxicol. Appl. Pharmacol.* **18**, 542 (1971).
Gubler, K., Margot, A., Gysin, H., *J. Sci. Food Agr. Suppl.* 13 (1968).
Gysin, H., *Chemia (Buenos Aires)* **8**, 205 (1954).
Hollingworth, R. M., Metcalf, R. L., Fukuto, T. R., *J. Agr. Food Chem.* **15**, 242 (1967).
Jaffe, H. H., *Chem. Rev.* **53**, 191 (1953).
Kilsheimer, J. R., Kaufman, H. A., Foster, H. M., Driscoll, P. R., Glick, L. A., Napier, R. P., *J. Agr. Food Chem.* **17**, 91 (1969).
Metcalf, R. L., Fukuto, T. R., *J. Agr. Food Chem.* **13**, 220 (1965).
Metcalf, R. L., Fukuto, T. R., Wilkinson, C. F., Fahmy, M. H., El-Aziz, S. A., Metcalf, E. R., *J. Agr. Food Chem.* **14**, 555 (1966).
Müller, E., Zimmermann, G., *J. Prakt. Chem.* **111**, 293 (1925).
Sacher, R. M., Metcalf, R. L., Metcalf, E. R., Fukuto, T. R., *J. Econ. Entomol.* **64**, 1011 (1971).
Sacher, R. M., Olin, J. F., *J. Agr. Food Chem.* **20**, 354 (1972).
Weismann, R., Gasser, R., Grab, M., *Experientia* **7**, 117 (1951).
Winter, G. D., *Ann N. Y. Acad. Sci.* **87**, 575 (1960).

Reuven M. Sacher*
Gerhard H. Alt
Walter A. Darlington[1]

Agricultural Division
Monsanto Commercial Products Co.
St. Louis, Missouri 63166

[1]Deceased.

Received for review June 12, 1972. Accepted October 16, 1972.

Detection of Diethylstilbestrol Contamination in Swine Feedstuff

Ten commercial preparations of pelleted swine feed associated with hyperestrogenism in swine were analyzed for the mycotoxin F-2 (zearalenone) in an effort to explain the estrogenic activity. None of the suspected fungal estrogen, F-2, produced by *Fusarium roseum* was found. Five of these samples were selected for more intensive investigation, and all five were found to contain diethylstilbestrol (DES) in concentrations of 240, 2400, 28, 90, and 1–10 ppb, respectively. All five samples, when incorporated into a nutritionally balanced rat diet and fed to 21-day-old female white rats, caused a significant increase in the uterine weight. As little as 0.8 to 1.0 mcg of authentic DES (total dose) increased the uterine weight of test rats to 150 mg, as compared to 32 mg for control rats. Final identification of DES was made by mass spectrometry and mass fragmentography.

One known cause of estrogenism in swine is F-2 (zearalenone), produced by *Fusarium roseum* Snyder & Hanson (and possibly by other species of *Fusarium*) growing in maize or in other ingredients of the feed (Christensen *et al.*, 1965; Mirocha *et al.*, 1967; Nelson *et al.*, 1970; Stob *et al.*, 1962). Over the past several years we have received numerous samples of feed from practicing veterinarians in Minnesota and other states suspected of being responsible for outbreaks of estrogenism in herds of swine. These were tested for F-2 (zearalenone), and also were fed to 21-day-old virgin female weanling white rats for 5–7 days, after which the rats were sacrificed and their uteri removed and weighed. In our experience this is a very reliable bioassay for estrogenic compounds in feedstuffs. Approximately 50% of the samples received did not contain detectable amounts of F-2, but caused an increase in the weight of uteri of rats to which they were fed. It was apparent that the hyperestrogenism was due to either an unidentified natural metabolite or to causes other than fungal contamination. We decided to look closely for the presence of minute quantities of diethylstilbestrol (DES) in such samples, although some of these samples had already been screened for DES using the standard colorimetric method as described in the AOAC manual (1971).

As judged by the increase in weight of the uteri of rats, DES is approximately 1000 times as potent as F-2. Since DES is added to many batches of feed compounded for beef steers, and since feeds for swine may be mixed and pelleted or otherwise processed in the same plant and in the same machines as those used to prepare feed for beef steers, it was thought that DES might occasionally get into swine feed as a contaminant or pollutant.

Amount of DES Necessary to Cause Enlargement of Uteri of Rats. Measured amounts of DES were incorporated into a nutritionally balanced ration developed for rats and were fed to rats. A total dose of 0.8–1.0 mcg, over a period of 5–7 days, resulted in an approximately fivefold increase in the weight of uteri of the rats (average of five controls, 32 mg; average of three rats given 0.8–1.0 mcg of DES, 150 mg). This amount is too small to be detected by the standard colorimetric method specified by the AOAC manual (1971).

Method Used to Detect Small Amounts of DES in Sample Feeds. The ground feed sample (6 kg) was moistened to about 15–20% water content and extracted with about 10 l. of ethyl acetate. The extract was dried over Na_2SO_4, concentrated under vacuum, and yielded 195.9 g of solids. The latter was partitioned between equal volumes of acetonitrile and petroleum ether (bp 30–60°) and the acetonitrile layer was extracted four times with petroleum ether. Each of the purification steps was checked for biological activity by incorporating an aliquot of the extract on a balanced ration and feeding to 21-day-old weanling female rats. The rats were sacrificed after 5 days and the degree of estrogenic activity was determined by weighing the uterus (Umberger *et al.*, 1958). The most active fraction was found in the acetonitrile (390 mg average uterine weight) as compared to the petroleum ether (75 mg).

The acetonitrile-soluble extract (19.1 g) was loaded onto a large column (55 mm o.d.) consisting of 850 g of Davison 923 silica gel. The column was developed with solvents of the eluotropic series in increasing order of polarity and starting with petroleum ether, 60–70°.

As shown in Table I, the estrogenic component was found in fractions 9 and 10 as evidenced by the rat uterus bioassay ($\frac{1}{120}$ of total fraction added to a balanced rat ration). Column fraction 9 was next chromatographed on