Spectrophotofluorometry of Reserpine, Other Rauwolfia Alkaloids, and Related Compounds*

By R. P. HAYCOCK, P. B. SHETH, and W. J. MADER

The excitation and fluorescent characteristics of a number of Rauwolfia alkaloids and related compounds are reported using a Spectrophotofluorometer. A procedure for the determination of reserpine in mixtures of reserpine and rescinnamine is described.

THE PHENOMENA of natural fluorescence of reserpine and other rauwolfia alkaloids has been noted by various investigators since the isolation of reserpine was reported, and several investigators have utilized fluorescences for the determination of reserpine in dosage form and in biological tissues (1–3). However, these investigators employed filter type fluorometers which restrict the fluorescent detection to the visible region of the spectrum and to those fluorophors that absorb light corresponding to one of the available lines of the mercury emission spectrum. The development of spectrophotofluorometers has extended the scope of fluorescent analysis to permit the excitation of compounds and the measurement of resulting fluorescence throughout the ultraviolet and visible regions.

To date, little data have been reported on scanning spectrophotofluorometric measurements of rauwolfia alkaloids. This led to a survey of the excitation and fluorescent characteristics of a number of rauwolfia alkaloids and related compounds for possible usefulness as a basis for the identification and quantitative assay of reserpine in the presences of other rauwolfia alkaloids.

EXPERIMENTAL

Apparatus.—Aminco-Bowman Spectrophotofluorometer with silica cells, 1-cm. cross section, four transparent sides. Recorded spectra were obtained, using a Mosely Model No. 3 Flat Bed X-Y Recorder, modified for use with the Spectrophotofluorometer.

Solutions.—Dissolve 25.0 mg. of the compound in 1 ml. of chloroform and dilute to 100 ml. with methanol. Dilute 3.0 ml. to exactly 50 ml. with methanol and further dilute 10 ml. to exactly 50 ml. with methanol. The diluted solution contains 3.0 μg. of alkaloid per ml. Indole, norharmane, and harmaline are prepared to contain 1.5 μg. per ml.

Technique.—The Aminco-Bowman Spectrophotofluorometer used in this study was calibrated using 0.1 μg. U. S. P. quinine sulfate per ml. in 0.1 *N* sulfuric acid in accordance with the technique of Sprince and Rowley (4). The excitation maximum was observed at 350 mμ, and the corresponding maximum fluorescent peak occurred at 450 mμ. The peaks checked within 5 mμ on repeated tests and agreed with the corrected values reported by Sprince and associate.

The fluorescent spectrum for each solution was scanned on the oscilloscope screen, while changing the excitation wavelength until the appearance of a fluorescent band. At the fluorescent maximum, the wavelength of maximum excitation was determined by manipulation of the excitation monochromator. Under these conditions, excitation and fluorescent maxima were obtained for each compound tested, as shown in Table I, together with the structural formula and relative fluorescent sensitivity. The relative fluorescent sensitivity is the product of the meter reading on the transmission scale and meter multiplication factor. Excitation and fluorescent spectra of reserpine, rescinnamine, 3-dehydroreserpine, and tetrahydroreserpine are presented in Figs. 1–4. These spectra are typical of the other alkaloids and related compounds.

METHOD DEVELOPMENT

An adaptation of the Szalkowski-Mader colorimetric reaction of reserpine with nitrous acid is the official (5) and preferred procedure for determining reserpine in dosage form. In a prior publication (6) from this laboratory, it was established that the 7-methoxy-β-carboline group, i.e., the 11-methoxy group and the AB and C ring skeleton of reserpine, is the functional group responsible for the yellow pigment which is formed. Of the alkaloids containing this similarity to reserpine, methyl reserpate and reserpic acid are eliminated in the extraction procedure and dehydroreserpine and tetradehydroreserpine do not interfere. The other principal alkaloid found in various species of rauwolfia which contains this common structural similarity to reserpine and reacts with nitrous acid is rescinnamine. A preliminary examination of the fluorescent spectra of reserpine and rescinnamine showed that they exhibit different excitation and fluorescent spectra and led to a study of the practicability of determining reserpine in the presence of rescinnamine by spectrophotofluorometry.

Procedure.—A solution of reserpine in methanol was prepared as outlined under Experimental to give a range of 1 μg. to 5 μg. per ml. The excitation monochromator and fluorescent monochromator were set at 280 mμ and 360 mμ, respectively, at the maximal excitation and fluorescent response of reserpine. The relationship between concentration of reserpine and fluorescence is shown in Fig. 5. All measurements were made using $^1/_{16}$ inch defining slit (band pass = 6 mμ) and a 1P21 photomultiplier. The blank reading served as the fiducial point, and the instrument was set at an arbitrary point with the highest standard. The fluorescence is proportional to concentration in the range of 1 to 5 μg. per ml.

The above method has been successfully applied to mixtures of reserpine containing as much as 80%

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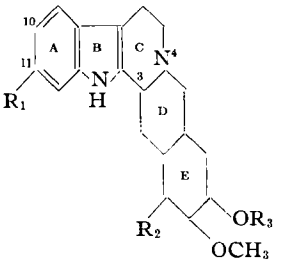
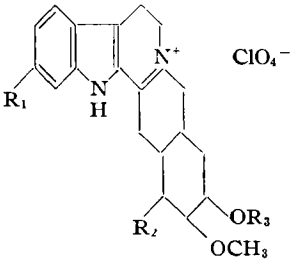
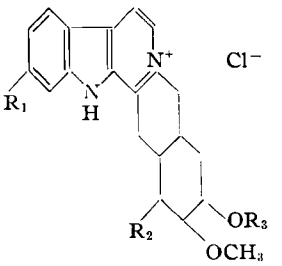
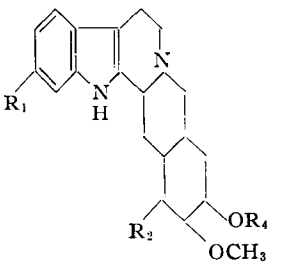
TABLE I.—SENSITIVITY AND MAXIMUM EXCITATION AND FLUORESCENT WAVELENGTHS FOR SOME RAUWOLFIA ALKALOIDS AND RELATED INDOLES[a]

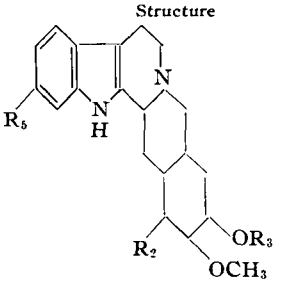
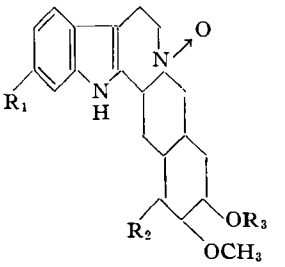
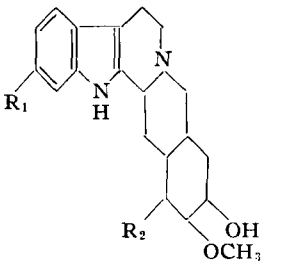
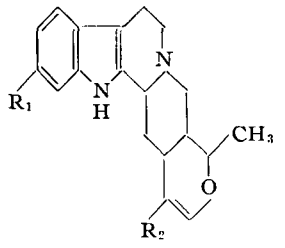
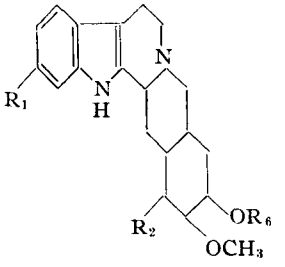
Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Reserpine		280	360	6
Isoreserpine	.....	390	510	18
Dehydroreserpine perchlorate		390	510	75
Tetradehydroreserpine chloride		340	440	50
Rescinnamine		310	440	2

rescinnamine with recoveries within 2 to 3% of the theoretical reserpine content as shown in Table II. The reproducibility of replicate determinations was within 3%. This difference is not more than can be accounted for by analytical errors.

DISCUSSION

The following discussion is not meant to be a complete evaluation of the findings, rather it is intended only to point out the more evident results to emerge from the investigation. The excitation and fluorescent spectra of reserpine, reserpinine, deserpidine, renoxidine (reserpine N-oxide), methyl reserpate, methyl O-(3,5-dimethoxy-4-hydroxybenzoyl) reserpate, syrosingopine, and syrosingopine N-oxide generally showed peaks at approximately 280 mμ with a corresponding fluorescent maximum at approximately 360 mμ. The order of magnitude of fluorescence depends upon certain groups in various positions of the basic alkaloid structure. For example, the 11-methoxy moiety impedes fluorescences as is evident by a comparison of reserpine with deserpidine, which does not possess an 11-methoxy group and produces an increase in fluorescences equivalent to $2^1/_2$ above that of reserpine. Reserpinine, a tertiary indole alkaloid with a heterocyclic ring E and possessing an 11-methoxy substituent emits fluorescent light below that of reserpine. The introduction of oxygen at position 4, however, is an enhancing group as is evident by an increase in the fluoresence emission

TABLE I—*Continued*

Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Deserpidine		280	360	15
Renoxidine (reserpine N-oxide)		280	360	8
Methyl reserpate		300	360	19
Reserpinine		300	360	2
Methyl O-(3,5-dimethoxy-4 hydroxybenzoyl) reserpate		300	360	8

of renoxidine (reserpine N-oxide) and syrosingopine N-oxide. The esterified alcoholic function of reserpine, i. e., trimethoxybenzoic acid decreases the fluorescences considerably as can be seen by a comparison of the fluorescent emission of reserpine with methyl reserpate. The substitution of a hydroxyl group in the esterified function, i.e., 3,5-dimethoxy-4-hydroxybenzoic acid has an enhancing effect upon the fluorescence displayed by reserpine as is evident by the increased fluorescences of methyl O-(-3,5-dimethoxy-4-hydroxybenzoyl) reserpate.

The esterified alcoholic function of rescinnamine, i. e., trimethoxycinnamic acid, is responsible for a shift in the excitation maximum to 310 mμ and a corresponding shift in the fluorescent emission to 440 mμ. In addition to the shift, the fluorescent response is considerably weaker than that produced by the reserpine nucleus. This qualitative and quanti-

TABLE I—*Continued*

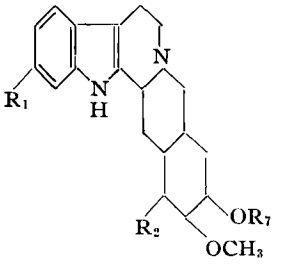
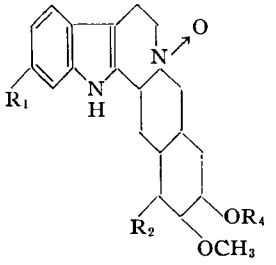
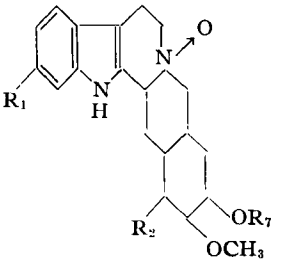
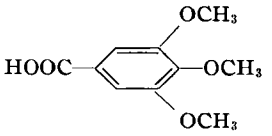
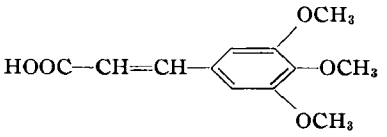
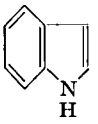
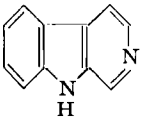
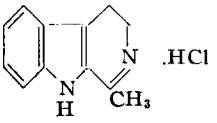
Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Syrosingopine		300	360	4
Rescinnamine N-oxide		310	440	2–3
Syrosingopine N-oxide		290	350	6
Trimethoxybenzoic acid		280	360	2
Trimethoxycinnamic acid		300	400	<1
Indole		280	340	36
Norharmane		350 285	380 380	36 40
Harmaline hydrochloride		390	490	150

TABLE I—*Concluded*

Compound	Structure	Wavelength, mμ Excitation, max.	Wavelength, mμ Fluorescence, max.	Relative Fluorescent Sensitivity
Key:				
$R_1 = CH_3O$	$R_5 = H$			
$R_2 = COOCH_3$				
$R_3 = OC$—(3,4,5-trimethoxyphenyl)	$R_6 = OC$—(3-methoxy-4-hydroxy-5-methoxyphenyl)			
$R_4 = OC$—CH=CH—(3,4,5-trimethoxyphenyl)	$R_7 = OC$—(3,5-dimethoxy-4-$OCOOC_2H_5$-phenyl)			

[a] The authors wish to acknowledge the assistance of their associates, R. A. Lucas and P. R. Ulshafer, who supplied us with some of the alkaloids.

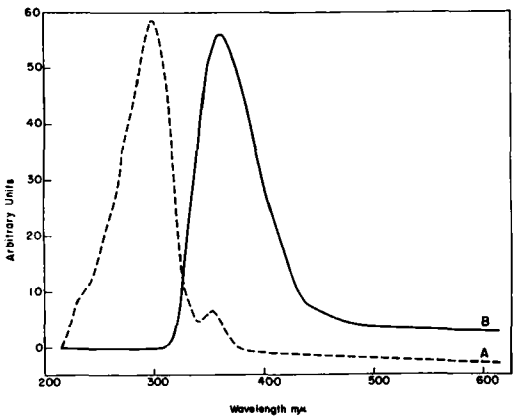


Fig. 1.—Excitation and fluorescent spectra of reserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 360 mμ. B. Fluorescent scan—excitation held at 280 mμ.

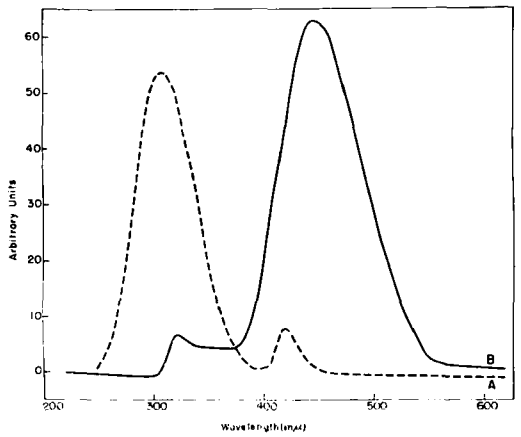


Fig. 2.—Excitation and fluorescent spectra of rescinnamine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 440 mμ. B. Fluorescent scan—excitation held at 315 mμ.

TABLE II.—ANALYSES OF RESERPINE-RESCINNAMINE MIXTURES

Reserpine Added, %	Rescinnamine Added, %	Reserpine Found, %
99.2	0.8	98.5
99.2	0.8	99.2
95.2	4.8	95.2
95.2	4.8	94.2
80.0	20.0	80.0
80.0	20.0	81.5
40.0	60.0	40.9
25.0	75.0	25.8
16.7	83.3	17.2

tative difference can be utilized in the determination of reserpine in the presence of rescinnamine. However, it is not applicable when applied to preparations containing other rauwolfia alkaloids having identical fluorescent characteristics to that of reserpine unless used in conjunction with other techniques. For example, methyl reserpate and reserpic acid may be removed by preliminary extraction with acid and base as outlined in the U S. P. nitrite procedure (5). The presence of deserpidine, a possible contaminant of commercial reserpine obtained from *Rauwolfia canescens*, may be detected by chromatography (7) on paper and on columns or may be determined by difference between the spectrophotofluorometric technique and the U. S. P. nitrite procedure (5), since deserpidine does not react with nitrous acid. In spite of its limitations, the spectrophotofluorometry technique embodies certain advantages not inherent in other procedures and, in particular, may be applied to the determination of micro quantities of reserpine.

Dehydroreserpine and 3-isoreserpine display a high visible fluorescence at 510 mμ, which is maximally excited at 390 mμ. This shift in fluorescences of isoreserpine from the ultraviolet is not accompanied by a noticeable change in the absorption spectrum of the basic reserpine nucleus. The fluorescence of dehydroreserpine is equivalent to twelvefold that of reserpine, and the fluorescent emission of iso-

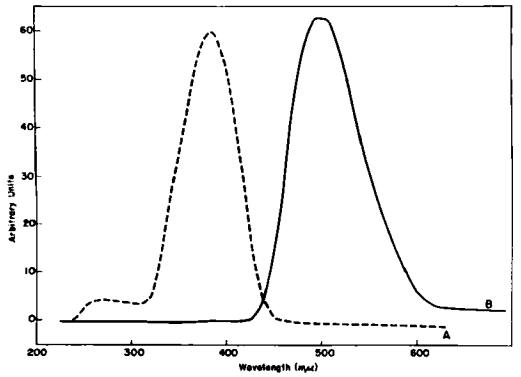


Fig. 3.—Excitation and fluorescent spectra of 3-dehydroreserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 510 mμ. B. Fluorescent scan—excitation held at 390 mμ.

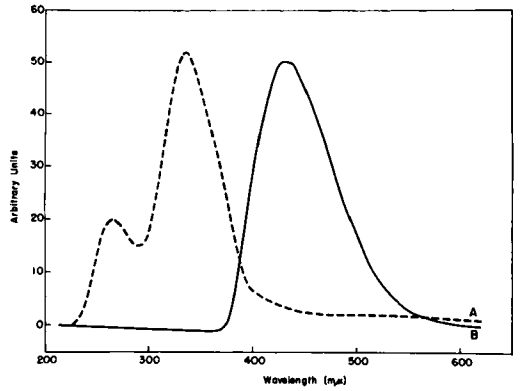


Fig. 4.—Excitation and fluorescent spectra of tetradehydroreserpine in methanol (3 μg./ml.). A. Excitation scan—fluorescence held at 440 mμ. B. Fluorescent scan—excitation held at 340 mμ.

reserpine is increased by a factor of three over that of reserpine. Presumably, this shift in fluorescences is related to epimerization at C-3 and unsaturation in ring C. Although the significance of this phenomenon is not understood, it can be useful as a method for assaying reserpine, as the visible fluorescence of dehydroreserpine is increased 12 times that of the natural fluorescences of reserpine and offers the added advantage of the possibility of using filter type fluorometers. Details of a procedure based on the conversion of reserpine to dehydroreserpine to produce a fluorescent peak at 510 mμ will be the basis of a subsequent publication.

An examination of tetradehydroreserpine, another oxidative degradation product of reserpine, likewise displays a sizeable increase of fluorescence over that of reserpine. In addition to the order of magnitude of fluorescence, the excitation and fluorescence emission are unlike those of either reserpine or dehydroreserpine. In this case the maximal fluorescent emission is found at 440 mμ when excited at 340 mμ.

CONCLUSIONS

1. A large number of rauwolfia alkaloids and related compounds have been subject to a spectrophotofluorometric study, and for each compound the wavelength of maximal excitation and fluorescence and relative fluorescent sensitivity are reported.

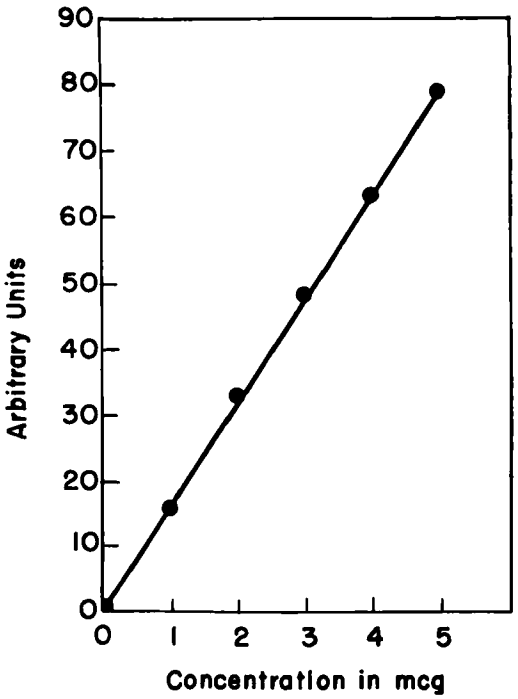


Fig. 5.—Relationship between concentration of reserpine and fluorescence.

2. The totality of the results of this survey allows several generalizations as to structural groups and fluorescence. The methoxy moiety and esterified substituent, trimethoxybenzoic acid, produces a decrease in the emitted light. The degree of fluorescence of indole alkaloids with a heterocyclic ring E is less than that of the reserpine nucleus. The introduction of oxygen at position 4 enhances the fluorescent emission. The substitution of trimethoxycinnamic acid as the esterified alcoholic function in place of trimethoxybenzoic acid is responsible for a shift as well as a weaker fluorescent response. Epimerization at C-3 and unsaturation in ring C shifts the fluorescence from the ultraviolet to the visible with an increase in the fluorescent intensity of several magnitudes.

3. A fluorometric procedure based on the conversion of reserpine to dehydroreserpine has been suggested. Details will be published later.

4. A practical spectrophotofluorometric procedure for the determination of reserpine in mixtures of reserpine and rescinnamine has been described that is based on the natural fluorescence of reserpine at 360 mμ when maximally excited at 280 mμ. Rescinnamine exhibits different excitation and fluorescent spectra and does not interfere.

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A Note on N-Ethoxy-N-ethyl-*m*-toluamide Insecticide*

By RANDOLPH T. MAJOR and HANS-JURGEN HESS†

Based on structural relationships it was postulated that N-ethoxy-N-ethyl-*m*-toluamide would prove to be an effective insect repellent as is N,N-diethyl-*m*-toluamide. It has been shown that N-ethoxy-N-ethyl-*m*-toluamide is an insecticide, particularly against mosquitoes.

RECENTLY, investigators in the U. S. Department of Agriculture have reported that N,N-diethyl-*m*-toluamide is an active insect repellent (1–3).

Based on analogies between the pharmacological activity of substituted amines and substituted hydroxylamines (4) it seemed likely that N-ethoxy-N-ethyl-*m*-toluamide, *m*-$CH_3C_6H_5CON(C_2H_3)OC_2H_5$, would also be an insecticide or insect repellent.

N-Ethoxy-N-ethyl-*m*-toluamide was prepared by the interaction of *m*-toluyl chloride (5) and N-ethoxy-ethylamine (6, 7).

EXPERIMENTAL

N-Ethoxy-N-ethyl-*m*-toluamide.—To 10.6 Gm. (0.069 mole) of *m*-toluyl chloride in 10 cc. of benzene was added, with external cooling, 13 Gm. (0.146 mole) of N-ethoxy-ethylamine[1] in 10 cc. of benzene. After the mixture had stood, overnight, it was treated with an aqueous solution of potassium carbonate and extracted with ether. The ether solution was dried over sodium sulfate and distilled, b.p. of the amide, 137–142° at 4.5 mm. Redistilled, b. p. 139° at 4.5 mm., yield, 10.6 Gm. (75%).

Anal.—Calcd. for $C_{12}H_{17}NO_2$: C, 69.51; H, 8.27; N, 6.76. Found: C, 69.60; H, 8.57; N, 7.05.

Biological Activity.—Dr. R. F. Phillips of the Merck Sharp and Dohme Research Laboratories has reported that the compound showed no antibacterial or antifungal activity against common plant pathogens. However, he has written us that Dr. Philip Granett of the Department of Entomology of Rutgers University put some mosquitoes, *Aedes aegypti*, in a five-inch long by two-inch diameter tube and tied a piece of cheesecloth which had been treated with a 5% solution of N-ethoxy-N-ethyl-*m*-toluamide in acetone on one end of the tube. The mosquitoes were knocked down, i. e., paralyzed or killed rapidly. Also, a moderate degree of repellency was noted against stable flies, *Stomoxys calcitrans*, but not against house flies, *Musca domestica*. Dr. Phillips has also written us that Dr. H. M. Peck of the Merck Institute for Therapeutic Research has carried out preliminary studies of the toxicity of N-ethoxy-N-ethyl-*m*-toluamide. These indicate that it is probably relatively nontoxic. No indication of dermal injury to the skin of the rabbit was noted when the material was tested for twenty-four hours by the Draize patch test. Toxicity tests in Carsworth CF strain mice gave the following results:

i.p. LD_{50}—approx. 800 mg./Kg.
P.O. LD_{50}—approx. 2.0 Gm./Kg.

Toxic signs at such levels were loss of righting reflex, sedation, exophthalmia, ataxia, lacrimation, and slow respiration.

SUMMARY

1. N-Ethoxy-N-ethyl-*m*-toluamide has been synthesized.

2. Preliminary toxicity tests indicate that N-ethoxy-N-ethyl-*m*-toluamide is relatively nontoxic systemically, and also to the skin.

3. N-Ethoxy-N-ethyl-*m*-toluamide shows considerable toxicity to mosquitoes, *Aedes aegypti*, and repells stable flies, *Stomoxys calcitrans*, but not house flies, *Musca domestica*.

* Received March 27, 1959, from the Cobb Chemical Laboratory, University of Virginia, Charlottesville.

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† Merck and Co., Inc., Post-Doctoral Fellow in Chemistry, 1957–1958.

[1] It should be noted that on one occasion a violent explosion occurred during the preparation of N-ethoxy-ethylammonium chloride by the method of R. T. Major and E. E. Fleck, *J. Am. Chem. Soc.*, **50**, 1479(1928). An aqueous solution of this salt was being concentrated *in vacuo* in an electric heating mantle. Apparently, the salt was overheated. Aqueous solutions of N-ethoxy-ethylammonium chloride should be concentrated to dryness on a water bath or a steam bath, preferably *in vacuo*.

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