Hydrolytic Behavior of Isoalloxazines Related to Riboflavin II

Kinetics of Degradation of 9-Methylisoalloxazine in Alkaline Media

By DEODATT A. WADKE and DAVID E. GUTTMAN

The kinetics of hydrolysis of 9-methylisoalloxazine under aerobic conditions was investigated at hydroxide-ion concentrations both below and above its pKa. The nature of the pH profile indicated that a specific base-catalyzed reaction was responsible for the degradation and that the unionized species was much more susceptible to hydrolysis than the ionized form. The hydrolysis in pH 9.0 borate buffer and in strongly alkaline medium was investigated rather extensively. Here quantitative separation of the reaction intermediates and products was achieved by the use of ion-exchange chromatography. Rate studies showed that under strongly basic conditions the parent isoalloxazine disappeared faster than the intermediate anil, while the reverse was true at pH 9.0. These studies also showed that the rate of disappearance of the anil paralleled the rate of appearance of its decomposition products. Evidence was obtained to indicate that the degradation of the isoalloxazine involved an initial hydroxide-ion attack at the C_α position to yield a carbinolamine which underwent further reaction to yield the anil or the ureide and then the final products.

A PREVIOUS COMMUNICATION (1) summarized the results of some preliminary studies on the hydrolytic degradation of 9-methylisoalloxazine. These studies showed that at least three different products could be formed from the parent compound and that these were 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid, 1,2,3,4 - tetrahydro - 1 - methyl - 2,3 - dioxoquinoxaline, and 1,2-dihydro-1-methyl-2-oxoquinoxaline. The relative concentrations of the products formed were seen to depend on the pH of the reaction medium. It was further shown that 5-(6-methylaminophenylimino)barbituric acid anil was formed from the parent isoalloxazine and subsequently disappeared from the reaction mixture. Evidence was presented to suggest that the anil existed in equilibrium with 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxy ureide, and it was postulated that the ureide could further decompose by at least three mechanisms to yield three different products.

In the present investigation the kinetics of various reactions involved in the transformations of the isoalloxazine to products were investigated. Studies were restricted to reactions occurring in solutions which were exposed to air. Anaerobic systems which were shown to exhibit more complex behavior (1) were not investigated. The pH profile for the disappearance of the isoalloxazine in the alkaline range of pH was determined and extensive kinetic studies were conducted on reactions which occurred in strongly basic solution and in pH 9.0 buffer. These quantitative studies showed that in strongly alkaline medium 80% of the parent isoalloxazine was transformed into the keto acid, that the anil was formed and disappeared at the same rate as the formation of the keto acid, and that another transient intermediate, possibly a carbinolamine, was initially formed from the parent isoalloxazine. The data also revealed that the rate of disappearance of the suspected carbinolamine closely paralleled that of the anil and suggested the possibility that the anil and the suspected carbinolamine were in equilibrium in solution. Studies at pH 9.0, where the major product was the dioxo compound, showed that the rate of appearance of this compound paralleled the rate of disappearance of the parent compound and indicated that the rate-determining step in the formation of the dioxo compound was one involving the loss of 430 mμ absorbance of the isoalloxazine.

EXPERIMENTAL

Materials.—9 - Methylisoalloxazine (I), 5 - (6-methylaminophenylamino)barbituric acid anil (III), 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid (V), and 1,2,3,4-tetrahydro-1-methyl-2.3-dioxo-quinoxaline (VI) were obtained as described previously (1, 2).

All other materials used in this study were obtained from commercial sources.

Procedure.—*Kinetic Studies.*—The experimental method used was the same as that described previously (3). The rate of disappearance of 9-methylisoalloxazine was determined by following the decrease in absorbance at 430 mμ. The rates of hy-

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drolysis were investigated over the pH range 7.9 to 14.0 and at 35° ± 0.01°. In addition, systems in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide were also studied at 45° ± 0.01°.

The degradation of the anil (III) was investigated in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide. This study was conducted at 45°. Samples were withdrawn at various time intervals and complete spectra were determined using a Beckman DB spectrophotometer.

Ion-Exchange Chromatography.—One and one-half grams of a strongly basic, anion-exchange resin,[1] 100–200 mesh, was made into a slurry with distilled water and packed in a 25-ml. buret using a glass wool plug as a support. Prior to use, the column was washed with 100 ml. of 2 *M* sodium chloride followed by distilled water until the washings were free from chloride. Preliminary studies with the synthetic mixtures of the isoalloxazine (I), the anil (III), and the keto acid (V) showed that when such a mixture at about pH 5 was introduced on the column and eluted with water, the eluate (which will be subsequently referred to as the neutral fraction) contained the isoalloxazine. When the solvent was changed to 0.01 *M* acetic acid, the eluate (which will be referred to as the acidic fraction) contained the anil. Finally, elution with 2 *M* sodium chloride effected removal of the keto acid. These studies also showed that the recovery of the three compounds was quantitative within experimental limits.

This technique was utilized to effect quantitative separation of the residual isoalloxazine, the anil, and the keto acid at various stages of degradation of the isoalloxazine. Forty-five-milliliter samples were withdrawn from the reaction mixture at different times and mixed with 1 ml. of glacial acetic acid in the case of pH 9.0 samples and with 2 ml. in the case of high pH (0.5 *N* base) samples in a 50-ml. volumetric flask and the volume was adjusted to the mark with distilled water. The pH of the resulting solution was approximately 5. Forty milliliters of the acidified sample was introduced on the ion-exchange column and elution carried out in three stages to obtain the neutral fraction, the acidic fraction, and the salt fraction. Each fraction was collected in a 100-ml. volumetric flask. After adjusting to volume, the spectrum of each was recorded using a Beckman DB spectrophotometer.

RESULTS

pH Profile for the Hydrolysis of 9-Methylisoalloxazine.—Loss of absorbance at 430 mμ was used to indicate the disappearance of the isoalloxazine from solution. When the logarithm of the absorbance at 430 mμ was plotted as a function of time, a typical pseudo first-order plot resulted. Figure 1 illustrates such a plot for 9-methylisoalloxazine which was hydrolyzed in 0.5 *N* sodium hydroxide. As is seen, some deviation from linearity became apparent beyond approximately three half-lives. Similar behavior was also observed by Song *et al.* (4) in their study on photochemical degradation of flavins. As a result, rate constants were determined from initial straight line portions of the curves. The results of the studies conducted

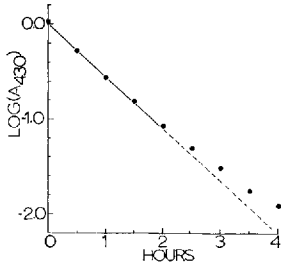


Fig. 1.—A plot illustrating the first-order disappearance of 9-methylisoalloxazine in 0.5 *N* sodium hydroxide and at 45°.

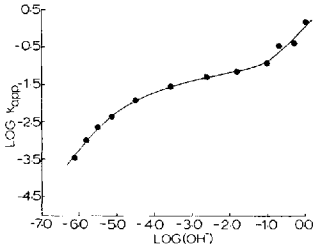


Fig. 2.—The pH profile for the hydrolysis of 9 - methylisoalloxazine at 35°.

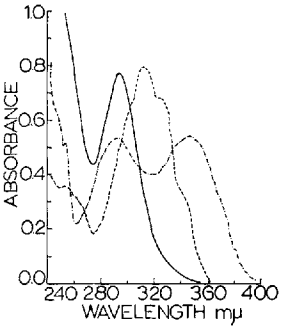


Fig. 3.—Spectra of the anil at a concentration of 1.8 × 10^{-4} *M* (——), the keto acid at a concentration of 8 × 10^{-5} *M* (- · - · -), and the dioxo compound at a concentration of 7.3 × 10^{-5} *M* (- - - -).

over a range of hydroxide-ion concentrations are summarized in Fig. 2, where logarithm of the observed rate constant was plotted as a function of the logarithm of the hydroxide-ion concentration. This pH profile shows that the reaction was approximately first order with respect to hydroxide ion at pH values below and above the pKa (9.82), but that in the region of the pKa, the order was changing.

Kinetic Studies on the Hydrolysis of the Anil (III).—The kinetics of transformations involving the anil was also followed spectrophotometrically. Figure 3 shows the spectra of the anil (III), the keto acid (V), and the dioxo compound (VI). It is seen that the anil possesses negligible absorbance at 340 mμ, where both the keto acid and the dioxo compound exhibit significant absorbance. The increase in absorbance at this wavelength was thus used to follow the progress of the reactions. Although it was shown in a previous study (1) that the anil probably existed in solution in equilibrium with the ureide (IV), the predominant species, under the pH conditions of this study, was the anil. The concentration of the ureide was apparently so small that it did not contribute to the absorbance at this wavelength. Figure 4 illustrates that, in 0.5 *N* sodium hydroxide solution, the rate of appearance

[1] Marketed as Amberlite CG-400 by the Rohm & Haas Co., Philadelphia, Pa.

of the keto acid exhibited a first-order dependency on anil concentration. The rate constant was calculated to be 0.18 hr.$^{-1}$ at 45°. In a pH 9 system it was found that rate of appearance of the major product here, the dioxo compound, was also first order with respect to anil and that the rate constant for the reaction at 45° was 5.0×10^{-2} hr.$^{-1}$.

Quantitative Ion-Exchange Chromatography.—Figure 5 illustrates the results of kinetic studies which were conducted with 9-methylisoalloxazine in 0.5 *N* sodium hydroxide. It is seen that there occurred in this system a rapid disappearance of the parent isoalloxazine, appearance and disappearance of the anil, and appearance of the keto acid. From these data, rate constants were estimated for the various processes and are tabulated in Table I.

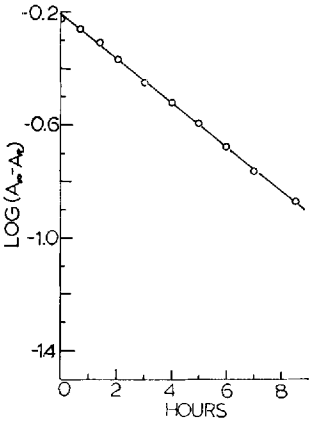


Fig. 4.—A plot illustrating the first-order dependency of keto acid formation on the concentration of the anil compound in 0.5 *N* sodium hydroxide and 45° ± 0.01°. A_∞ represents the maximum absorbance achieved at 340 mμ, while A_t represents the absorbance observed at this wavelength at time *t*.

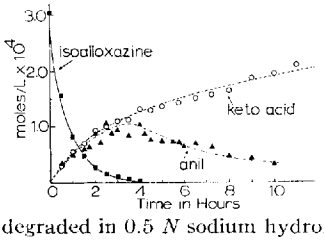


Fig. 5.—A plot of concentration *versus* time for the isoalloxazine, the anil, and the keto acid as observed in the reaction mixture that was degraded in 0.5 *N* sodium hydroxide and at 45°.

TABLE I.—RATE CONSTANTS FOR APPEARANCE AND DISAPPEARANCE OF VARIOUS COMPOUNDS IN 0.5 *N* NaOH AT 45° ± 0.01°

Reaction	Rate Constant, hr.$^{-1}$
Disappearance of isoalloxazine	1.3
Disappearance of the anil in the degrading soln. of isoalloxazine	0.19
Appearance of the keto acid from isoalloxazine	0.17
Disappearance of the suspected carbinolamine	0.22

It is pertinent to note that the rate constant characterizing the appearance of the keto acid is approximately the same as that found for the disappearance of the anil and that these constants are close to that obtained when pure anil was used as the starting material under similar conditions of temperature and hydroxide-ion concentration. The study also showed that approximately 80% of the parent isoalloxazine was eventually transformed to the keto acid. It is also apparent from the figure that the relatively rapid disappearance of 9-methylisoalloxazine from solution was not accompanied by as rapid an appearance of the anil. This was rather surprising in view of the evidence that the anil is an intermediate in the transformation of the parent compound to products. It was also interesting to observe that, in the initial stages of the reaction, the final product, the keto acid, was found in concentrations which were approximately the same as those formed for the anil. This behavior strongly suggested that another reactive intermediate was formed and that it was in equilibrium with the anil. The existence of such a species in the reaction mixture was also indicated by spectral examination of the neutral fractions obtained in the chromatographic procedure. The spectrum of such a fraction is shown in Fig. 6. It appears to be that of a mixture. The spectrum of 9-methylisoalloxazine alone is also shown for comparative purposes. It was assumed, based on the first-order decay of absorbance at 430 mμ, that absorbance at this wavelength was due solely to 9-methylisoalloxazine and its concentration in the fraction was calculated. Absorbance values at other wavelengths, resulting from this concentration, were then determined using appropriate molar absorptivities. When these values were subtracted from those obtained for the fraction, a difference spectrum was generated which is also shown in Fig. 6. The difference spectrum has a maximum at 350 mμ and is apparently due to a transient intermediate. Difference spectra were obtained for a number of fractions representing different reaction times and the logarithm of the absorbance at 350 mμ of the difference spectrum was plotted as a function of time. Figure 7 represents this plot and shows that this unknown intermediate initially formed rather rapidly and as time progressed disappeared from the medium at a rate which was slower than the rate of formation. The rate content for the disappearance was estimated from the later linear portion of the curve to be 0.22 hr.$^{-1}$ and is tabulated with other rate constants in Table I. This value is reasonably close to that obtained for the disappearance of anil and further supports the possibility of the existence of a rapidly achieved equilibrium between the unknown intermediate and the anil. The absolute concentration of the unknown compound at any time cannot be determined. However, if it was in equilibrium with the anil and if the equilibrium was established fairly rapidly then, at any time [unknown] = $K_{eq.}$[anil], where $K_{eq.}$ = equilibrium constant and the brackets represent concentrations. From the knowledge of the absorbance at 350 mμ due to the unknown compound and the concentration of the anil at the same time, one can calculate a term equivalent to the product of the molar absorptivity of the unknown at 350 mμ and the equilibrium constant.

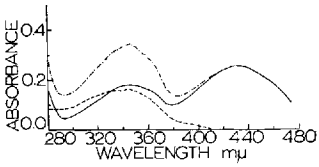


Fig. 6.—Spectrum of the aqueous fraction isolated from the reaction mixture that was partially degraded in 0.5 *N* sodium hydroxide (– · – · –) and that of the pure isoalloxazine (———). The dotted line represents the difference spectrum (see text).

Table II shows the values of this parameter calculated from data obtained at different times. The concentrations of the anil used in these calculations were obtained from the smooth curve represented in Fig. 5. It can be seen that the values agree fairly well among themselves and further support the hypothesis concerning the equilibrium. It is rather surprising that this supporting evidence was obtained since the analytical method employed does physically separate the two compounds which are thought to be in equilibrium, *i.e.*, the anil was held on the column while the unknown compound was passed through. Apparently, the forward and the reverse rates for the equilibrium are quite slow at the pH of the acid-quenched samples as compared to the rate of passage of solutions through the column.

Solutions of 9-methylisoalloxazine which were maintained at a pH of 9 did not contain measurable concentrations of the anil or the keto acid. Consequently, quantitative studies here were restricted to the determination, as a function of reaction time, of the concentrations of residual isoalloxazine and dioxo compounds. Both compounds appeared in the neutral fraction of the chromatographic elution scheme. Figure 8 shows the spectrum of such a fraction. Since the dioxo compound does not absorb in the visible region of the spectrum, a simultaneous spectrophotometric analysis of the two component mixture was readily made. Figure 9 shows the time course for the disappearance of isoalloxazine and appearance of the dioxo compound. It was determined that 70% of the parent compound was eventually converted to dioxo compound. The data shown in Fig. 9 were used to estimate rate constants for the processes and these are tabulated in Table III. The rate constant describing the disappearance of the isoalloxazine is sufficiently close to that found for the appearance of dioxo compound to suggest that the rate-determining step in the formation of the product from the parent compound was that involving the ion of the 430 mμ bond of the isoalloxazine. Intermediates such as the anil, the ureide, or the unknown species referred to earlier were apparently present in steady-state concentrations which were too low to detect. It is apparent from the data of Table III that the anil, under these conditions, degraded more rapidly than the parent isoalloxazine. This is in agreement with results obtained in the earlier study (1) where the anil could not be detected in degrading solutions of 9-methylisoalloxazine maintained at a pH of 9.

DISCUSSION

As is seen in Fig. 2, the pH profile for 9-methylisoalloxazine exhibits curvature over the pH range

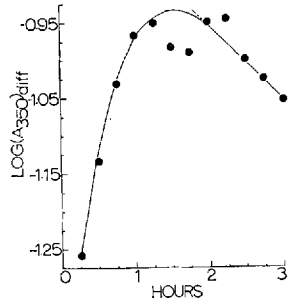


Fig. 7.—A curve showing the appearance and subsequent disappearance of the suspected carbinolamine intermediate.

TABLE II.—VALUES OF THE PRODUCT OF THE MOLAR ABSORPTIVITY OF THE SUSPECTED CARBINOLAMINE AND THE EQUILIBRIUM CONSTANT, AS CALCULATED FROM THE DATA CORRESPONDING TO DIFFERENT TIMES[a]

Time, hr.	$K_{eq.}$ × molar Absorptivity × 10^{-3}
2.25	0.93
2.50	0.91
2.75	0.81
3.00	0.81
3.25	0.82
3.50	0.80
3.75	0.84
4.00	0.82
4.25	0.82

[a] See text.

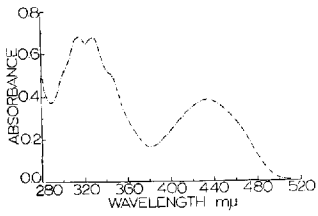


Fig. 8.—Spectrum of a neutral fraction obtained by ion - exchange chromatography of a reaction mixture which was partially degraded in pH 9.0 borate buffer at 45°.

bracketing the pKa of the compound (9.82). This was not unexpected and indicated that both ionized and unionized species underwent a base-catalyzed reaction, but at different rates. Thus, the apparent rate constant for the reaction can be defined in terms of ionization of the compound as:

$$k_{app.}(I_t) = k_1(OH^-)(I^-) + k_2(OH^-)(IH) \quad \text{(Eq. 1)}$$

where

$k_{app.}$ = the observed pseudo first-order rate constant,
I_t = the total concentration of the isoalloxazine,
IH = the stoichiometric concentration of the unionized isoalloxazine,
I^- = the stoichiometric concentration of the ionized isoalloxazine,
k_1 = the second-order rate constant for the disappearance of the ionized form,

k_2 = the second-order rate constant for the disappearance of the unionized form.

Equation 1 can be rearranged in the manner suggested by Schwartz *et al.* (5) to show:

$$k_{\text{app.}}/\text{OH}^- = k_1 + (k_2 - k_1)f_{\text{IH}} \quad \text{(Eq. 2)}$$

where, f_{IH} = the fraction unionized of the isoalloxazine. Equation 2 predicts a linear relationship between $k_{\text{app.}}/\text{OH}^-$ and f_{IH}. Such a plot for the system investigated is shown in Fig. 10. The excellent linearity observed over a wide range of hydroxide-ion concentrations supports the underlying hypothesis. The values of k_1 and k_2 can be calculated from the slope and the intercept of such a plot. These values as determined by the method of least squares were found to be 4.45 L. mole.$^{-1}$ hr.$^{-1}$ for k_1 and 5.01×10^2 L. mole^{-1} hr.$^{-1}$ for k_2. As would be expected from electrostatic considerations the rate constant characterizing the reaction of the negatively charged species is considerably smaller than that of the neutral species.

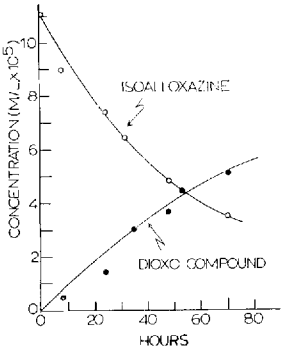


Fig. 9.—A concentration *vs.* time plot for the disappearance of 9-methylisoalloxazine and the appearance of the dioxo compound in a pH 9 borate buffer at 45°.

TABLE III.—RATE CONSTANTS FOR APPEARANCE AND DISAPPEARANCE OF VARIOUS COMPOUNDS IN pH 9.0 BORATE BUFFER AT 45° ± 0.01°

Reaction	Rate Constant, hr.$^{-1}$ × 10^2
Disappearance of isoalloxazine	1.6
Appearance of the dioxo compd. from isoalloxazine	1.8
Disappearance of the anil	5.0

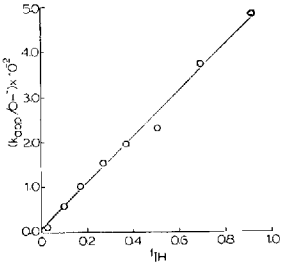
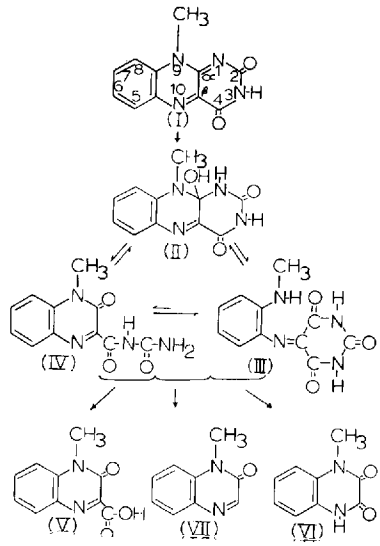


Fig. 10.—A plot showing the relationship expressed by Eq. 2 for 9-methylisoalloxazine at 35° ± 0.01°.

The reaction shown in Scheme I was presented and discussed earlier (1). It was suggested at that time that a carbinolamine intermediate could be initially formed from the isoalloxazine. It is indeed possible that the compound detected in the neutral fraction during the course of this study was this carbinolamine intermediate. It would result from the attack of hydroxide-ion at the C_α position of the isoalloxazine. Thus loss of absorbance at 430 mμ can be explained on this basis since such an attack would destroy the conjugated system $-N_{10} = C_\beta - C_\alpha = N_1-$, which is responsible for absorption of radiation in the visible region of the spectrum. The carbinolamine would be expected to possess a pKa similar to the parent compound and to appear, therefore, in the neutral fraction of the ion-exchange separation. In addition, the charge-density calculations for isoalloxazines reported by Pullman and Pullman (6) suggest that C_α is a good center for nucleophilic attack. Tetrahedral carbinolamine intermediates have been postulated by others in, for example, the hydrolysis of Schiff bases (7) and iminolactones (8). The work of Cunningham and Schmir (8) on the hydrolysis of iminolactones is of special interest here. According to their reasonings, a carbinolamine of the postulated structure (II) could undergo rupture of C_α—N_9 bond to yield the anil or breaking of C_α—N_1 bond to yield the ureide. It is conceivable then that both the anil and the ureide existed in equilibrium with the carbinolamine and the suspected ureide–anil equilibrium, reported earlier (1), occurred through the carbinolamine compound.

Results have been presented to show that the major products of alkaline hydrolysis of 9-methylisoalloxazine, in the presence of air, were the keto acid (V) and the dioxo compound (VI). In the earlier study (1) the presence of a third product—namely, 1,2 - dihydro - 1 - methyl - 2 - oxoquinoxaline (VII)—was reported. This compound could



Over-all Reaction Mechanism for the Degradation of 9-Methylisoalloxazine.

Scheme I

be detected only under anaerobic conditions at pH 9 where the competing reactions leading to the formation of the dioxo compound and the keto acid were suppressed. Quantitative studies of the kinetics of the anaerobic hydrolysis are presently being conducted and will be reported at a later time.

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Proteinaceous Antitumor Substances from Plants II

Mirabilis multiflora

By AYHAN ULUBELEN* and JACK R. COLE

Preliminary screening of the aqueous extract of *Mirabilis multiflora* (*Nyctaginaceae*) has shown antitumor activity against Sarcoma 180 in mice. The isolation, purification, and partial characterization of proteinaceous material is reported. This material has shown activity against Lewis lung carcinoma, Walker carcinosarcoma 256 (intramuscular), and lymphosarcoma.

As a result of a routine screen of southwestern plants for potential antitumor activity, the aqueous extract of *Mirabilis multiflora*[1] (*Nyctaginaceae*) Britton and Rusby demonstrated activity toward the Sarcoma 180 test system in mice.[2] This screening program was carried out by the Cancer Chemotherapy National Service Center (CCNSC), Bethesda, Md. Further fractionation has resulted in activity in the Lewis lung carcinoma, Walker carcinosarcoma 256 (intramuscular), lymphosarcoma, and Sarcoma 180 test systems.

The plant is an herbaceous perennial with a large tuberous root. The above-ground portion of the plant dies during freezing weather or extended drought and regrowth occurs from subterraneous parts of the plant.

Part of the collection used in this study was obtained on rocky slopes about 5 miles south of St. David, Ariz., at an elevation of about 3,700 ft., May 31, 1962. Another part of the collection was obtained at the Boyce Thompson Southwestern Arboretum near Superior, Ariz., on July 1, 1964. It was in cultivation in a sandy loam soil within a grove of eucalyptus.

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Previous paper: Ulubelen, A., Caldwell, M., and Cole, J. R., *J. Pharm. Sci.*, **54**, 1214(1965).
* Visiting Research Associate Professor. Present address: College of Pharmacy, University of Istanbul, Istanbul, Turkey.
[1] Identification confirmed by Robert Barr, Research Associate, College of Pharmacy, and Dr. Charles Mason, Curator of the Herbarium, Botany Department, University of Arizona, Tucson. A reference specimen was also deposited.
[2] Preparation of the preliminary extraction was carried out by Dr. M. E. Caldwell.

EXPERIMENTAL

Fresh roots of *M. multiflora* (3.5 Kg.) were extracted with approximately 15 L. of petroleum ether followed by 15 L. of water at room temperature. The water solution was then washed with benzene and chloroform and lyophilized in a Repp Industries model 15 sublimator. The yield was 320 Gm. One-hundred grams of this crude material was dissolved in 1000 ml. of water at room temperature. The insoluble part was separated by centrifuge and discarded (32 Gm.). The remaining aqueous portion was extracted with 5 × 200 ml. of ether to remove the remaining fatty materials. The aqueous solution was increased in volume threefold by the addition of 95% ethanol. A light-colored precipitate formed and was separated by centrifuge. It was dissolved in water and lyophilized. Twenty-one grams of the material was obtained. This crude material showed an activity against the Sarcoma 180 test system of the CCNSC. The requirement for activity in this system is two successive dose response tests showing a reduction of at least 56% in tumor size. The crude material showed a decrease in tumor size of 84% at a dose of 22 mg./Kg. The elementary analysis of this material showed 13.4% inorganic material which consists mostly of magnesium and phosphate ions and also the presence of sulfur and nitrogen. Upon hydrolysis of the material a number of amino acids were obtained. Two grams of the crude material was dissolved in water and dialyzed against distilled water, using a dialyzing tube of $^5/_8$ in. diameter (Arthur H. Thomas Co., No. 4465-A2, Philadelphia, Pa.). After a period of 7 days, the precipitate in the dialysis tube was separated from