Microdegradation Procedure for Radioactive Peptide Ergot Alkaloids

G. BASMADJIAN and H. G. FLOSS

Abstract □ A degradation procedure for ^{14}C-labeled peptide ergot alkaloids is described; it allows the determination of the specific activities of all the products obtained in the hydrolysis of these alkaloids from a sample of not more than 10 μmoles.

Keyphrases □ Ergot alkaloids, radioactive peptides—biosynthesis, degradation, isotope distribution □ Peptide ergot alkaloids, radiolabeled—degradation, distribution □ TLC—separation, analysis

In the course of biosynthetic studies on ergot alkaloids, the need arose for a procedure that would permit the determination of the radioactivity distribution within the various portions of the molecules of ^{14}C-labeled peptide-type ergot alkaloids. To be useful, the degradation procedure had to meet the following criteria:

1. It should allow the determination of the specific radioactivity of all hydrolytic fragments of peptide ergot alkaloids.
2. The specific radioactivity of all the fragments should be determined directly, rather than by difference, in order to establish a radioactivity balance.
3. The degradation should be universally applicable to all peptide ergot alkaloids and even to simple mixtures of alkaloids like ergotoxine.
4. It should be possible to carry out the entire degradation starting with a sample of not more than 10 μmoles of radioactive alkaloid, since this is usually the amount available after extensive purification of the alkaloids from one experiment and since nonlabeled carrier material is often difficult to obtain in quantity.

The degradations used in previous biosynthetic studies on peptide ergot alkaloids (1–5) required rather large samples of alkaloid and/or were not complete since only some fragments of the molecule were isolated. This article describes a degradation procedure which meets essentially all the above criteria and which has been successfully employed in studies on the biosynthesis of ergotamine and ergotoxine (6). This degradation is applicable to all peptide ergot alkaloids containing the cyclol structure, it can be carried out with samples of 10 μmoles, and it allows the direct determination of the specific radioactivity of all hydrolytic fragments of these alkaloids.

EXPERIMENTAL

Pure samples of the alkaloids ergotamine, ergocornine, and ergocryptine were used[1]. Radioactive alkaloids, degraded, were samples obtained from feeding experiments[2] which had been purified to constant specific radioactivity. D,L-Valine-1-^{14}C was purchased[3]. Radioactivity determinations were carried out in a Beckman LS 100 liquid scintillation spectrometer, using 10 ml. of a toluene scintillator solution (7 g. 2,5-diphenyloxazole and 0.3 g. dimethyl-1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene/1000 ml. toluene) for compounds dissolved in organic solvents or 10 ml. of Bray's solution (7) for aqueous samples.

The counting efficiency for each sample was determined by recounting the sample after the addition of a known amount of toluene-^{14}C as internal standard. Colorimetric determinations were carried out in a Zeiss PMQ II spectrophotometer using cells with a 1-cm. lightpath. Standard curves were determined for all the compounds to be analyzed using analytically pure, crystalline materials; from these, factors were obtained for the conversion of extinction readings into concentrations. Ergonovine maleate[4] was used to standardize the quantitative determination of ergot alkaloids and lysergic acid by reaction with *p*-dimethylaminobenzaldehyde (8, 9). The reagent used (van Urk's reagent) consists of 200 mg. *p*-dimethylaminobenzaldehyde and 0.15 ml. of 10% $FeCl_3$ solution in 100 ml. of 65% (v/v) sulfuric acid. TLC was carried out on 0.25-mm. thick Merck precoated silica gel plates, unless stated otherwise.

Degradation of Alkaloids—*Alkaline Hydrolysis*—Ten micromoles of the alkaloid, containing between 5×10^3 and 10^5 d.p.m. of ^{14}C, is placed in a 10-ml. round-bottom flask equipped with a gas inlet and a reflux condenser which can be closed by a stopcock. The alkaloid is dissolved in 0.5 ml. of methanol (ergotamine) or chloroform (ergocornine, ergocryptine, ergotaminine, and ergotoxine), and 2 ml. of 8% aqueous KOH is added. The system is flushed with nitrogen and heated to 100° in an oil bath. After 10 min., the stream of nitrogen is stopped, the system is closed, and refluxing is continued for a total of 1 hr. The flask is then cooled and its contents acidified to pH 1–2 with concentrated HCl.

Isolation and Quantitation of Keto Acid—The hydrolysate is transferred to a 15-ml. conical centrifuge tube containing 1 ml. of a freshly prepared solution of 2,4-dinitrophenylhydrazine in 2 *N* HCl (5 mg./ml.). The mixture is kept at room temperature for 20 min. with occasional shaking. Three milliliters of ethyl acetate is then placed on top of the aqueous solution, and the mixture is shaken on a vibromixer. The organic phase is removed with a Pasteur pipet and transferred to a 125-ml. separator. This extraction is repeated until the ethyl acetate layer is almost colorless. The combined extracts are washed with 3×3 ml. of water, the washings are combined with the original aqueous phase and freeze-dried in a 25-ml. round-bottom flask, and the residue is saved for further processing. Petroleum ether (b.p. 30–60°) is added to the organic phase to beginning cloudiness, and the solution is extracted with 5×10 ml. of a carbonate solution (5.3 g. Na_2CO_3 and 0.84 g. $NaHCO_3$ in 1000 ml. of distilled water).

The extract is washed with 3×5 ml. of ethyl acetate–petroleum ether (1:1), which is discarded, then acidified to pH 1–2 with concentrated HCl, and extracted with ethyl acetate until the organic layer is colorless. The ethyl acetate extract is washed with 3×5 ml. of water, dried over anhydrous Na_2SO_4, and taken to dryness in a rotary evaporator. The residue is dissolved in 3 ml. of ethanol, filtered through a coarse sintered-glass funnel into a centrifuge tube, and concentrated to 1 ml. in a stream of nitrogen; 2 *N* HCl is added until cloudiness results. The mixture is left in the refrigerator overnight. After centrifugation, the supernatant is removed with a Pasteur pipet; the yellow-orange crystals are washed with 2×1 ml. of distilled water and dried *in vacuo* at 50° over P_2O_5. TLC at this point [chloroform–benzyl alcohol–acetic acid (70:30:3)] always showed a single spot in the case of the dimethylpyruvate 2,4-dinitrophenylhydrazone and two spots (*cis*- and *trans*-isomers) for the derivative of pyruvate.

The crystalline dinitrophenylhydrazone is dissolved in 2 ml. of

[1] Provided by Farmitalia S.A. through the courtesy of Dr. C. Spalla.
[2] Carried out by Dr. D. Gröger, Institute for Biochemisty of Plants, Halle, Germany, or by Dr. C. Spalla, Institute for Basic Research, Farmitalia S.A., Milan, Italy.
[3] Volk Radiochemical Co.
[4] Provided by Eli Lilly and Co. through the courtesy of Dr. Gordon Svoboda.

Table I—Degradation of Two Samples of Ergotoxine (Mixture of Ergocornine and Ergocryptine from Identical, Duplicate Biosynthetic Experiments)

Degradation Number	Calculated Specific Activity of Alkaloid, d.p.m./μmole	Dimethylpyruvate	Valine	Leucine	Proline	Lysergic Acid
		d.p.m./μmole = % of Total				
1	Ergocornine 3920	58% 2280	37% 1450	— 54	3% 130	1.5% 60
	Ergocryptine 2520	90%	—	2%	5%	3%
2	Ergocornine 456	54% 247	40% 184	— 16	4% 18.5	1% 6.5
	Ergocryptine 288	86%	—	6%	6%	2%

ethyl acetate, and an aliquot of 0.5 or 1.0 ml. is counted for its radioactivity. Another aliquot, 20–50 μl. depending on the quantity, is evaporated to dryness and used for the quantitation. In the case of pyruvate dinitrophenylhydrazone, the residue is dissolved in 5 ml. of the carbonate solution and the extinction is measured at 355 nm. against a blank of carbonate solution. For dimethylpyruvate dinitrophenylhydrazone, 2 ml. of carbonate solution and 2 ml. of 2 *N* NaOH are added to the residue, and the extinction at 430 nm. is measured within 10 min. The micromoles of pyruvate or dimethylpyruvate derivative in the aliquot taken are calculated as $0.269 \times E_{355}$ or $0.252 \times E_{430}$, respectively. This figure is then used in calculating the micromoles of substance in the aliquot counted to obtain the specific radioactivity in disintegrations per minute per micromole. The remainder of the ethyl acetate solution of the dinitrophenylhydrazone is evaporated to dryness, and the residue is recrystallized from benzene–petroleum ether. The crystals are washed with 2 × 1 ml. of petroleum ether, dried, and used for another specific activity determination as described previously. The figures obtained in these two determinations in this study usually agreed within ±5%.

Formation of Dinitrophenyl Dipeptide—The freeze-dried residue from the aqueous solution obtained after the extraction of the keto acid dinitrophenylhydrazone is dissolved in 5 ml. of water; 100 mg. of $NaHCO_3$ is added, followed by a freshly prepared solution of 50 mg. of fluorodinitrobenzene in 8 ml. of ethanol. The clear solution is kept at room temperature with frequent shaking for 3 hr. in the dark, during which time its color changes from pale yellow to deep orange. All subsequent steps are performed under subdued light. The solution is evaporated to dryness in a rotory evaporator below 40°. Five milliliters of water and 50 mg. of Na_2CO_3 are added to the residue, the solution is extracted with ether until the ether layer is colorless, and the extracts are discarded. The solution is then acidified to pH 2 with concentrated HCl and again extracted with ether until the organic phase is colorless. The latter extracts are combined, dried over Na_2SO_4, and evaporated *in vacuo*. The extracted aqueous solution is freeze dried, and the residue is kept for the isolation of lysergic acid.

Isolation and Quantitation of Dinitrophenyl Amino Acids—The residue of the ether phase is dissolved in 0.5 ml. of methanol, 3 ml. of concentrated HCl is added, and the mixture is refluxed at 110° for 5 hr. After cooling, 3 ml. of water is added and the solution is extracted with ether until the ether layer is colorless. The extract is dried over Na_2SO_4 and evaporated to dryness to give a crude dinitrophenyl amino acid, *e.g.*, dinitrophenylphenylalanine from ergotamine, or a mixture of dinitrophenyl amino acids if an alkaloid mixture is degraded, *e.g.*, dinitrophenylleucine and dinitrophenylvaline from ergotoxine. The aqueous solution, which contains free proline, is freeze dried and the proline is dinitrophenylated exactly as described for the dipeptide. The crude dinitrophenylproline is obtained as described after extraction with ether. The crude dinitrophenyl amino acids are purified by TLC on 0.5-mm. thick silica gel G plates using chloroform–benzyl alcohol–acetic acid (70:30:3) as the solvent, which is allowed to travel 20 cm. (R_f values of dinitrophenyl derivatives: proline, 0.37; phenylalanine, 0.51; valine, 0.59; and leucine, 0.66).

For a better resolution of dinitrophenylvaline and dinitrophenylleucine obtained in the degradation of the alkaloid mixture ergotoxine, the solvent benzene–pyridine–acetic acid (80:20:2) is used and the plate is usually dried for 1 hr. at room temperature in the dark and developed a second time in the same system. The bands of the individual dinitrophenyl amino acids are scraped off into centrifuge tubes, 3 ml. of a 1% $NaHCO_3$ solution is added, the mixture is shaken well, and 3 ml. of ether is placed on top of it. Then 2–3 drops of concentrated HCl is added, and the contents of the tube are mixed on a vibromixer. After separation of the phases, the ether is removed with a Pasteur pipet and the extraction is repeated until a colorless ether layer results. The combined ether extracts are dried with Na_2SO_4 and evaporated to dryness in a stream of nitrogen. The residue is crystallized from methanol–water and the crystalline product is dried *in vacuo* over P_2O_5.

At this stage, the material is homogeneous as judged by TLC in the two systems mentioned previously. For the determination of the specific radioactivity, the material is dissolved in 2 ml. of ethanol, one aliquot is used for counting and another aliquot (20 μl.) for colorimetry. The latter aliquot is evaporated, the residue is dissolved in 5 ml. of a 1% $NaHCO_3$ solution, and the extinction is measured at 385 nm. (dinitrophenylproline) or 360 nm. (dinitrophenylvaline, dinitrophenylleucine, and dinitrophenylphenylalanine) against a blank of 1% $NaHCO_3$ solution. The factors for the conversion of E_{385} or E_{360} into micromoles of dinitrophenyl amino acid in the aliquot analyzed are 0.280 (dinitrophenylproline), 0.302 (dinitrophenylphenylalanine), 0.327 (dinitrophenylleucine), and 0.331 (dinitrophenylvaline). The remainder of the ethanol solution of the dinitrophenyl amino acid is taken to dryness, the residue is recrystallized from ether–ligroin, and the determination of the specific radioactivity is repeated.

Isolation and Quantitation of Lysergic/Isolysergic Acid—The freeze-dried residue of the aqueous phase obtained after the extraction of the dinitrophenyl amino acids is dissolved in 1 ml. of water and quantitatively streaked on a 46- × 57-cm. water-washed sheet of Whatman 3MM chromatography paper. The chromatogram is developed descendingly with distilled water as the solvent for 4–5 hr. (R_f values: lysergic acid, 0.30; and isolysergic acid, 0.40; varying somewhat depending on the amount of salts present). The bands of lysergic and isolysergic acid are marked quickly under UV light, cut out, and eluted together with distilled water. The eluate is evaporated to dryness *in vacuo* below 50°, and the residue is dissolved in 2 ml. of water. One aliquot is used for counting and another aliquot (usually 50 μl.) for colorimetry. To the latter, 1 ml. of a 2% succinic acid solution and 2 ml. of van Urk's reagent are added, and the extinction at 580 nm. is read after 10–20 min. against a blank of 1 ml. of 2% succinic acid and 2 ml. of van Urk's reagent. The micromoles of lysergic and isolysergic acids in the aliquot analyzed are calculated as $0.195 \times E_{580}$.

Check on Consistency of Specific Radioactivity Determinations—A sample of D,L-valine-^{14}C of an approximate specific radioactivity of 10 μc./mmole was prepared by appropriate dilution of D,L-valine-1-^{14}C (20 mc./mmole) with nonlabeled carrier material. Ten micromoles of this sample was dinitrophenylated as outlined previously, and the specific radioactivity of the dinitrophenylvaline was determined by the method used in the degradation. The average of two determinations was 1.83×10^4 d.p.m./μmole. Thirty micromoles of the same sample was dissolved in 1 ml. of 0.1 *M* phosphate buffer (pH 7.0); 10 mg. of catalase and 5 mg. of L-amino acid oxidase

were added, as well as a drop of *n*-octanol as antifoam; and oxygen was passed through the solution at 37° for 3 hr. One drop of concentrated HCl and 1 ml. of dinitrophenylhydrazine solution were then added, and the dimethylpyruvate dinitrophenylhydrazone was isolated and purified as described previously. Determination of its specific radioactivity by the method used in the degradation gave 1.76×10^4 d.p.m./μmole as the average of two determinations.

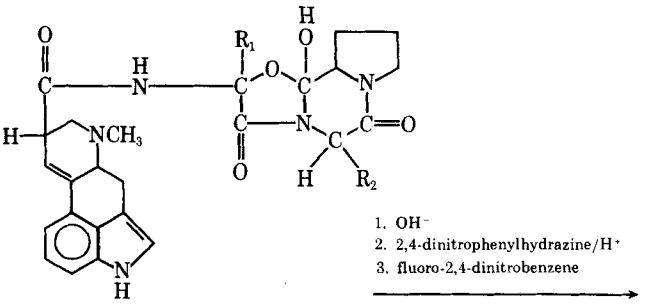
RESULTS AND DISCUSSION

Several degradations have been used in the structure elucidation of the peptide ergot alkaloids (*cf.*, 10), but some of them, *e.g.*, the cleavage with hydrazine (11), the reductive cleavage with $LiAlH_4$ (12), or the pyrolytic cleavage (12), *a priori* seemed not very suitable for this study. The requirement that it should be possible to carry out the entire degradation on a sample of not more than 10 μmoles severely limits the choice of procedures, because it requires reasonably good yields for all the products and it excludes weighing as the method for quantitating the compounds in the determination of the specific radioactivity. Rather, all the products have to be isolated in the form of a colored derivative suitable for colorimetry or have to be quantitated by a suitable color reaction.

After some trials with an acid hydrolysis method using a cation-exchange resin (13), this procedure was abandoned because of poor yields in favor of the milder alkaline hydrolysis (14). The latter makes use of the fact that the cyclol structure incorporating an α-hydroxy amino acid is much more sensitive to base hydrolysis than normal peptide bonds. Thus, hydrolysis of normal peptide ergot alkaloids like ergotamine and ergocornine with 8% KOH for 1 hr. at reflux temperature produces lysergic and isolysergic acid, an α-keto acid from the α-hydroxy amino acid, and a dipeptide containing proline at the carboxy terminal end. The latter can be further hydrolyzed with acid to give the two constituent amino acids. This procedure was adopted as the general degradation scheme (Scheme I), and the various products were isolated in the form of suitable derivatives. Treatment of the reaction mixture obtained in the alkaline hydrolysis with acidic 2,4-dinitrophenylhydrazine solution converts the keto acid into its dinitrophenylhydrazone. Control experiments showed that under these conditions no significant decomposition of the lysergic acid takes place. The keto acid dinitrophenylhydrazone can be extracted into ethyl acetate and is purified and quantitated essentially by the method of Katsuki *et al.* (15) and Kawano *et al.* (16), which consists of measuring the extinction at 355 nm. (pyruvate dinitrophenylhydrazone) or 430 nm. (dimethylpyruvate dinitrophenylhydrazone) in alkaline solution. The relation between extinction and concentration is linear over a range of 1–13 mcg./ml., and concentrations are usually adjusted to obtain extinctions between 0.3 and 0.4.

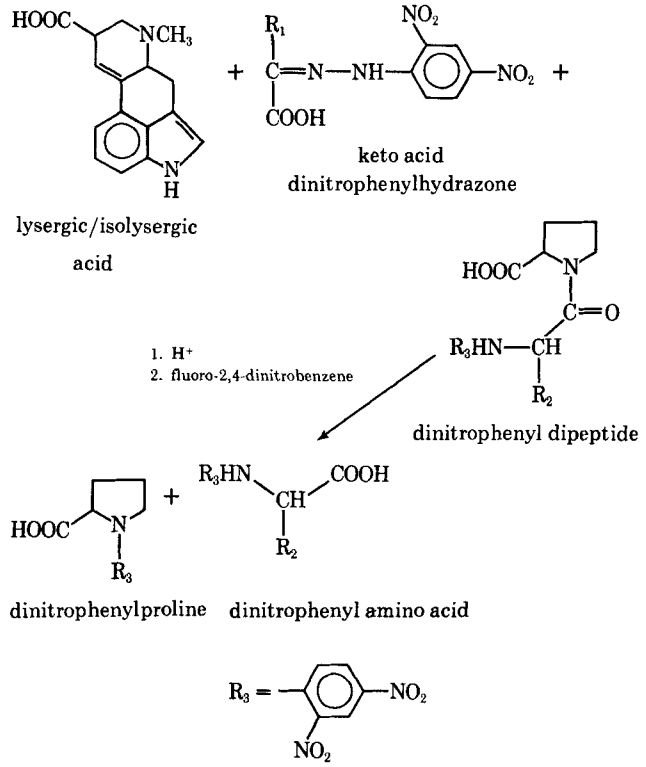
Separation of the dipeptide from the lysergic acid mixture is achieved by dinitrophenylation of the free amino group of the dipeptide, followed by extraction of the resulting dinitrophenyl dipeptide into ether at an acidic pH. From the aqueous phase remaining behind, lysergic and isolysergic acid are isolated by preparative paper chromatography. Their quantity is determined by the color reaction with van Urk's reagent, which is widely used for the quantitative determination of ergot alkaloids (8, 9). The dinitrophenyl dipeptide is further subjected to acid hydrolysis, which cleaves the peptide bond without affecting the dinitrophenyl group, giving rise to free proline and a dinitrophenyl amino acid. The two are separated by ether extraction of the latter and, subsequently, the proline is also converted into its dinitrophenyl derivative. All the dinitrophenyl amino acids are purified by preparative TLC followed by recrystallization and are quantitated colorimetrically as described by Lucas *et al.* (17). If a mixture of alkaloids is degraded, a mixture of dinitrophenyl amino acids is obtained in the acid hydrolysis, which is resolved in the TLC purification step, and the individual amino acid derivatives are then determined separately.

The recovery of the various degradation products is fairly good. The yields of the keto acid derivatives are 60–85%, lysergic/isolysergic acid 30–50%, and the dinitrophenyl amino acids 50–90%. The specific radioactivities of the products are determined by counting aliquots of the same solutions that are used for the colorimetry. No problems are encountered with the color quenching of these compounds in samples of up to 5 μmoles, and counting efficiencies between 45 and 70% are usually obtained. The radioactivity balance is very good. Thus, in four complete degradations of ergotamine-^{14}C



	R_1	R_2
ergotamine	$-CH_3$	$-CH_2-C_6H_5$
ergocornine	$-CH(CH_3)_2$	$-CH(CH_3)_2$
ergocryptine	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$

ergotoxine = mixture of ergocornine and ergocryptine



Scheme I—Degradation of peptide ergot alkaloids

from biosynthetic experiments, the sums of the specific radioactivities of the products amounted to 103, 98, 100, and 82%, respectively, of the measured specific radioactivity of the alkaloid. The low figure in the last degradation seems to be due to an impurity in the alkaloid sample degraded. These data indicate that the specific radioactivity determinations by the method employed are quite accurate.

As an additional check on this point, a control experiment was carried out. Of a sample of valine-1-^{14}C, one aliquot was converted to the dinitrophenyl derivative and its specific activity was determined by the method used in the degradation. Another aliquot was oxidized to dimethylpyruvate with L-amino acid oxidase, and its specific radioactivity was determined by colorimetry of the dimethylpyruvate dinitrophenylhydrazone. The figures obtained by the two determinations agreed within 4%, showing that the methods used for the specific activity determination of valine and dimethylpyruvate give values consistent with each other.

The reproducibility of the degradation procedure is documented by the data given in Table I. This table shows the good agreement between the results of degradations carried out on two samples of

ergotoxine, a mixture of the alkaloids ergocornine and ergocryptine, obtained from different biosynthetic experiments with the same labeled precursor, which were done under as near identical conditions as possible. In the degradation of such alkaloid mixtures, a radioactivity balance usually cannot be established. The percentage distribution of radioactivity can, however, be calculated if the identical moieties in the alkaloids have the same specific radioactivities.

The procedure described here has been used routinely for the degradation of labeled ergotamine and ergotoxine samples from biosynthetic experiments. It has been found to be reliable and easy to carry out. It requires only small quantities of material, and the amount of time needed is reasonable. One complete degradation requires about 3–4 days, but the working time per sample can be reduced considerably by degrading several samples simultaneously. Although this has not been done, the degradation procedure can undoubtedly be applied to other cyclol-type peptide ergot alkaloids.

REFERENCES

(1) W. A. Taber and L. C. Vining, *Chem. Ind.*, **1959**, 1218.

(2) L. C. Vining and W. A. Taber, *Can. J. Microbiol.*, **9**, 292 (1963).

(3) F. Majer, F. Kybal, and I. Komersova, *Folia Microbiol.*, **12**, 489(1967).

(4) A. Minghetti and F. Arcamone, *Experientia*, **25**, 926(1969).

(5) D. Gröger and D. Erge, *Z. Naturforsch. B*, **25**, 196(1970).

(6) G. Basmadjian, Ph.D. thesis, Purdue University, Lafayette, Ind., 1970.

(7) D. A. Bray, *Anal. Biochem.*, **1**, 279(1960).

(8) H. W. van Urk, *Pharm. Weekbl.*, **66**, 473(1929).

(9) M. L. Smith, *Pub. Health Rep.*, **45**, 1466(1930).

(10) A. Hofmann, "Die Mutterkornalkaloide," F. Enke Verlag, Stuttgart, Germany, 1964, pp. 74–84.

(11) A. Stoll, T. Petrzilka, and B. Becker, *Helv. Chim. Acta*, **33**, 57(1950).

(12) A. Stoll, A. Hofmann, and T. Petrzilka, *ibid.*, **34**, 1544 (1951).

(13) M. Pöhm, *Naturwissenschaften*, **48**, 551(1961).

(14) S. Smith and G. M. Timmis, *J. Chem. Soc.*, **1937**, 396.

(15) H. Katsuki, C. Kawano, T. Yoshida, H. Kanayuki, and S. Tanaka, *Anal. Biochem.*, **2**, 433(1961).

(16) C. Kawano, H. Katsuki, T. Yoshida, and S. Tanaka, *ibid.*, **3**, 361(1962).

(17) F. Lucas, J. T. B. Shaw, and S. G. Smith, *ibid.*, **6**, 335 (1963).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 30, 1970, from the *Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, Lafayette, IN 47907*

Accepted for publication February 19, 1971.

This work was supported by the National Institutes of Health (Research Grant AM 11662 and Research Career Development Award GM 42382 to H.G.F.) and by Eli Lilly and Co.

The authors are indebted to Eli Lilly and Co. and to Farmitalia S.A. for generous gifts of pure alkaloids.

COMMUNICATIONS

Evaluation of Cryogenine in Rat Sympathetic Ganglia

Keyphrases ☐ Cryogenine—ganglionic activity, rats ☐ Ganglionic activity—cryogenine effect, rats

Sir:

Previous reports indicated that the various pharmacologic actions ascribed to the plant extracts of *Heimia salicifolia* Link and Otto essentially are elicited by its major alkaloidal constituent, cryogenine (1–3). Recent studies demonstrated that orally administered cryogenine is equipotent to phenylbutazone in both acute and chronic models of inflammation at doses appearing to be unrelated to any systemic toxic manifestation (3). In extensive gross observational studies by Robichaud *et al.* (1), the production of mydriasis, mucosal blanching, and pilomotor erection as well as decreased motor activity were noted in rats in response to intraperitoneal administration of cryogenine.

In addition to producing mild hypothermia at relatively low, nonataxic dosage levels, cryogenine has been shown to suppress conditioned behavior responses selectively in both discrete and continuous avoidance–escape situations (4). Moreover, cardiovascular studies in the anesthetized intact dog and cat have demonstrated a significant blockade of the pressor response to exogenous epinephrine, with doses of cryogenine having no apparent effect on resting blood pressure (2, 5). Kinetic experiments on various isolated tissue preparations have demonstrated, however, an apparent lack of specificity for the commonly employed cholinergic and adrenergic receptor systems (3, 5, 6). In view of the foregoing, it was of interest to determine whether the anti-inflammatory properties attributed to cryogenine might be mediated through alterations in ganglionic activity.

Young Charles River Wistar rats of either sex were anesthetized with a mixture[1] of allobarbital (100 mg./kg.), urethan (400 mg./kg.), and monoethylurea (400 mg./kg.) administered intraperitoneally. The surgical and recording procedures employed in this superior cervical ganglion preparation were described in detail by Hancock and his coworkers (7, 8). Unless indicated otherwise, the sympathetic trunk was stimulated at a rate of 0.3 Hz. (0.2 msec.). Ganglionic potentials evoked by drugs or preganglionic stimulation were recorded from the surface of the ganglion by means of silver–silver chloride bipolar electrodes. All drugs were administered by close intraarterial injection through a 27-gauge needle inserted into the common carotid artery. The constant volume of injection was 0.05 ml., and all drugs were dissolved in a solution of 0.9% NaCl. With the exception of cryogenine (used as the acetate),

[1] Dial with Urethane, Ciba.