ride in 300 ml. of pyridine, at 0°, was added, in small portions, 49 g. (0.35 mole) of sublimed isonicotinyl chloride. The mixture was stirred two hours and kept overnight at room temperature. It was then poured into 700 ml. of water, the mixture was cooled and the solid filtered. This material was recrystallized from water to give 19 g. (30% yield) of product, m.p. 241–242°.

Pyrido(3,4-d)pyridazine-1,4-diol, Hydrazine Salt.—To a solution of 48 g. (0.24 mole) of dimethyl cinchomeronate in 960 ml. of methanol was added dropwise 360 ml. (6.0 moles) of 85% hydrazine hydrate. Subsequently, the mixture was refluxed for six hours, cooled and the solid filtered. The yield was 58.5 g., m.p. > 300°. An analytical sample was recrystallized from 95% ethanol to give the hydrazine salt, m.p. > 300°.

Anal. Calcd. for $C_7H_9N_5O_2$: C, 43.07; H, 4.65; N, 35.90. Found: C, 43.40; H, 4.98; N, 34.57.

The hydrazine salt was dissolved in warm water and the solution acidified with acetic acid. The solid which separated was filtered and recrystallized from dimethylformamide to give 31.5 g. (80% yield) of product, m.p. > 300.

3-(4-Pyridyl)-5-pyrazolone.—To 19.3 g. (0.1 mole) of ethyl isonicotinylacetate in 25 ml. of *n*-propyl alcohol was added 6 ml. (0.1 mole) of 85% hydrazine hydrate. When the mixture was warmed slightly a vigorous reaction occurred. The mixture was refluxed for four hours and cooled. The solid was filtered and recrystallized from glacial acetic acid to give 14.5 g. (90% yield) of product, m.p. 286–287° (dec.).

Streptomycin A Hydrazone.—A solution of 69 g. (0.1 mole) of streptomycin A trihydrochloride in 500 ml. of water was treated with a solution of 6 g. (0.1 mole) of 85% hydrazine hydrate in 100 ml. of water. The combined solutions were kept overnight, clarified and freeze-dried to give 60 g. (85% yield) of product, m.p. 180–185°.

Methyl 4-Pyridyl Ketone Hydrazone.—A mixture of 9.2 g. (0.08 mole) of methyl 4-pyridyl ketone and 18.0 ml. (0.3 mole) of 85% hydrazine hydrate was refluxed for seventy minutes and cooled. The precipitated solid was filtered and recrystallized from benzene–hexane to give 9 g. (59% yield) of product, m.p. 114–116°.

NEW BRUNSWICK, N. J.

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, PITMAN-MOORE CO.]

Hypotensive Alkaloids from *Veratrum album* Protoveratrine A, Protoveratrine B and Germitetrine B[1a]

BY HAROLD A. NASH AND ROBERT M. BROOKER[1b]

RECEIVED NOVEMBER 1, 1952

An investigation of the hypotensive alkaloids of *Veratrum album* has resulted in the finding that protoveratrine prepared from this source is a mixture of two alkaloids and in the isolation of a new hypotensive alkaloid, named germitetrine B, from the "amorphous alkaloid" fraction. One of the alkaloids, protoveratrine A, was found to conform to the accepted structure of protoveratrine except that it yielded two instead of one mole of acetic acid on hydrolysis. Protoveratrine B was found to yield protoverine, 2-methylbutyric acid, 2,3-dihydroxy-2-methylbutyric acid and two moles of acetic acid on hydrolysis. Germitetrine B was found to yield germine, 2-methylbutyric acid, 2,3-dihydroxy-2-methylbutyric acid and two moles of acetic acid.

The original purpose of the work reported here was to prepare protoveratrine for clinical testing and then to search for alkaloids responsible for the hypotensive activity of the so-called "amorphous alkaloid" fraction from *Veratrum album*. Paper chromatographic methods developed to aid in following the fractionation of the "amorphous alkaloids" soon revealed that protoveratrine, itself, as prepared by the technique of Craig and Jacobs,[2,3] was, in fact, a mixture of two alkaloids. This necessitated a reinvestigation of the chemistry of protoveratrine.

Such mixtures of two alkaloids were encountered in protoveratrine from all six lots of *Veratrum album* roots and rhizomes examined. The proportion of protoveratrine B varied from 0.36 to 0.58 among these six different lots. No significant variation was noted among different preparations from the same lot of roots and rhizomes. Attempts to separate the two alkaloids by fractional crystallization were unsuccessful. To purify protoveratrine for analysis, Craig and Jacobs[2,3] had used crystallization by addition of ammonia to a solution of the acetate in alcohol and crystallization from chloroform–ether. Carrying out serial fractional crystallizations by these two techniques and examining the successive crops and mother liquors by paper chromatography at each step, we obtained no evidence of separation of the two alkaloids. Fractional crystallization from acetone likewise gave no indication of separation. In their paper, Jacobs and Craig[3] call attention to their dissatisfaction with the analytical results they obtained. A mixture of alkaloids such as we have found would explain their apparently low carbon analyses.

Separation of protoveratrine into protoveratrines A and B[4] on a macro scale was accomplished by a

(1) (a) Paper read before the Medicinal Chemistry Division of the American Chemical Society at the Atlantic City Meeting, September, 1952. (b) Indiana Central College, Indianapolis, Indiana.

(2) L. C. Craig and W. A. Jacobs, *J. Biol. Chem.*, **143**, 427 (1942).

(3) W. A. Jacobs and L. C. Craig, *ibid.*, **149**, 271 (1943).

(4) Following the suggestion of Dr. W. A. Jacobs of Rockefeller Institute for Medical Research these alkaloids have been given names to indicate their being part of the recognized clinical entity "protoveratrine" instead of following the alternative procedure of modifying the parent trivial name to indicate partially the structure. In the paper read at the American Chemical Society meeting protoveratrine A was referred to simply as "protoveratrine" and protoveratrine B was termed "oxyprotoveratrine X."

NOTE ADDED IN PROOF.—Since the submission of this paper for publication, two pertinent articles have appeared in print. M. W. Klohs, *et al.*, THIS JOURNAL, **74**, 5107 (1952), describe an alkaloid which they call neoprotoveratrine and which they isolated from *Veratrum viride*. W. L. Glen, G. S. Meyers, *et al.*, *Nature*, **170**, 932 (1952), have announced the separation of a crude crystalline fraction from *Veratrum album* and its separation by countercurrent distribution into alkaloids they describe as protoveratrine, veratetrine, and germitetrine. We have now exchanged samples with both groups and find protoveratrine B, veratetrine and neoprotoveratrine chromatographically identical. In personal communications, Dr. G. S. Meyers reports protoveratrine B and veratetrine identical (including infrared), and Dr. M. W. Klohs reports protoveratrine B and neoprotoveratrine identical. The "protoveratrine" reported by both of these groups is presumably protoveratrine A. By countercurrent distribution techniques, they separated the alkaloids corresponding to protoveratrines A and B before attempting to obtain pure crystalline materials.

Craig countercurrent distribution procedure using a chloroform–water–acetic acid system. The distribution was carried out in separatory funnels. A typical distribution curve using 14 plates is shown in Fig. 1. Protoveratrine A is seen to show a peak concentration at funnel 12 and protoveratrine B, a peak concentration at funnel 3. The distribution of each of these alkaloids between the aqueous and chloroform phases and, therefore, the exact rate of movement of each through a countercurrent distribution series depends markedly on the total concentration.

Protoveratrine B, as obtained from this gross separation of protoveratrines A and B, showed the presence of a small amount of an additional alkaloid when subjected to paper chromatography using ethylene chloride as the developing agent. This contaminant was evidenced as a faint spot moving just ahead of the protoveratrine B zone. Protoveratrine B was purified of this contaminant by a 32-plate countercurrent distribution procedure using chloroform and 1 *N* sodium acetate buffer of *p*H 3.25. In this distribution, the peak concentration of protoveratrine B was found in funnel 13. The contaminating alkaloid was found in a pool of funnels 14–19 but not in a pool of funnels 7–13.

Protoveratrines A and B were found to be remarkably alike in many of their properties as may be seen from data listed in the Experimental section. The two alkaloids melted with decomposition at essentially the same temperature (267–270°) (the exact decomposition point depending on the rate of heating and the temperature of the melting bath on insertion of the samples) and mixtures showed no depression of decomposition point. On alkaline hydrolysis, both yielded protoverine, identified on the bases of melting point, optical rotation, carbon–hydrogen analyses, and melting point of the hydrochloride of the acetonyl derivatives. Following the course of the alkaline hydrolysis by means of paper chromatography showed that some isoprotoverine[3] appeared before all the ester alkaloid had been hydrolyzed. A further series of changes resulting in alkamines derived from isoprotoverine could be noted on longer time action of alkali. It was determined that these secondary changes do not result in the release of volatile acid fragments.

Examination of the alkaline hydrolysates of protoveratrine A showed the presence of three acids which were identified as acetic acid, 2-methylbutyric acid, and 2-hydroxy-2-methylbutyric acid by means of their *p*-phenylphenacyl esters.

The alkaline hydrolysates of protoveratrine B yielded acetic acid and 2-methylbutyric acid, identified as their *p*-phenylphenacyl esters, and an additional acid. This additional acid was non-volatile with steam. It was isolated by a 20-hour continuous extraction of the acidified hydrolysate with ether. The ether extract was subjected to steam distillation to remove the volatile acids. After reducing the residue from the steam distillation to dryness *in vacuo* and drying over desiccant, crystals of the acid were obtained. Recrystallization from chloroform, benzene or ether yielded crystals melting at 102–103°. Analysis and equivalent weight

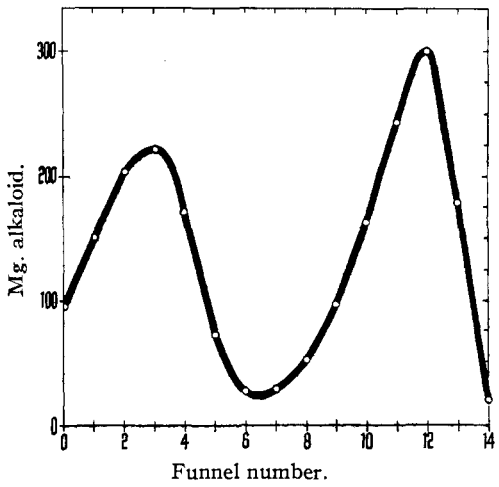

Fig. 1.—Countercurrent distribution of protoveratrine.

determinations indicated a formula of $C_5H_{10}O_4$. The acid gave a positive iodoform test. Identification as 2,3-dihydroxy-2-methylbutyric acid was accomplished by periodic acid oxidation under acidic conditions followed by preparation of the 2,4-dinitrophenylhydrazones of the oxidation products. The 2,4-dinitrophenylhydrazones of acetaldehyde and pyruvic acid were obtained in good yields.

During the earlier part of this work, volatile acid determinations indicated two moles of volatile acid in protoveratrine B and two moles of readily volatile acid plus one mole of the less volatile 2-hydroxy-2-methylbutyric acid in protoveratrine A. These results were difficult to reconcile with equivalent weight determinations which indicated equivalent weights 30 to 60 units higher than those calculated for esters containing one mole of protoverine and one mole of each of the identified acids. It was finally found that the use of *p*-toluenesulfonic acid and a 20-minute hydrolysis period[5] is inadequate for the hydrolysis of these ester alkaloids and that three moles of volatile acid are obtainable on complete hydrolysis. This is illustrated in Table I for the case of protoveratrine B. Similar results were obtained for each of the three ester alkaloids studied.

TABLE I

EFFECT OF HYDROLYTIC CONDITIONS ON THE APPARENT NUMBER OF VOLATILE ACIDS IN PROTOVERATRINE B

Hydrolytic conditions Acid	Time, min.	Moles of volatile acid[a]
p-Toluenesulfonic	20	2.24, 2.34
p-Toluenesulfonic	30	2.44[b]
p-Toluenesulfonic	60	2.61[c]
p-Toluenesulfonic	30	
followed by NaOH	20	2.98, 2.96, 3.04

[a] A molecular weight of 810 was used in calculating these figures. [b] Alkaline hydrolysis of the residue yielded 0.66 mole additional volatile acid. [c] Alkaline hydrolysis of the residue yielded 0.41 mole additional volatile acid.

To account for the extra mole of volatile acid thus found, the chromatographic technique of Marvel and Rands[6] was adapted to the quantitative deter-

(5) J. B. Niederl and V. Niederl, "Micromethods of Quantitative Organic Analysis," John Wiley and Sons, Inc., New York, N. Y., 1942, pp. 252–256.

(6) C. S. Marvel and R. D. Rands, THIS JOURNAL, **72**, 2642 (1950).

mination of the individual acids yielded on hydrolysis. It was found that protoveratrine A yields two moles of acetic acid, one mole of 2-methylbutyric acid and one mole of 2-hydroxy-2-methylbutyric acid per mole of protoverine. This gives an empirical formula of $C_{41}H_{63}O_{14}N$ for this compound. Analytical figures and equivalent weight determinations support this formula. By the same quantitative chromatographic technique, two moles of acetic acid, one of 2-methylbutyric acid and one of 2,3-dihydroxy-2-methylbutyric acid were found in protoveratrine B, corresponding to an empirical formula of $C_{41}H_{63}O_{15}N$. Analytical data and equivalent weight determinations support this formula.

After removal of "protoveratrine" from the total alkaloids, considerable hypotensive activity remained in the residual "amorphous alkaloid" fraction. Application of paper chromatographic methods to the examination of this fraction showed the presence of at least fifteen alkaloids. There are undoubtedly many more than fifteen alkaloids in the mixture since each of the two paper chromatographic zones which were examined in detail was found resolvable into several alkaloids. The proportion of the different alkaloids varied considerably from one lot of root and rhizomes to another.

To isolate individual alkaloids from this fraction, a 19-plate countercurrent distribution was carried out using a methylene chloride–water–acetic acid system. Paper chromatography showed that some degree of separation had been achieved although each funnel still contained several alkaloids. Biological assays showed high hypotensive activity associated with an alkaloid peak in funnels 11–14. Combination of the material recovered from funnels 11–16 and crystallization from butyl chloride gave an alkaloid which after repeated recrystallization from butyl chloride and recrystallization from aqueous acetone melted at 233–234° (slight decomposition on melting) ($[\alpha]^{25}D$ in pyridine −70°). This alkaloid showed hypotensive activity and was named germitetrine B.[7] Germitetrine B, on alkaline hydrolysis, yielded germine and acetic, 2-methylbutyric, and 2,3-dihydroxy-2-methylbutyric acids. The germine was identified by means of its melting point and mixed melting point with an authentic sample of germine,[8] and on the bases of analytical figures and melting point of the hydrochloride of the acetonyl derivative. Acetic and 2-methylbutyric acids were identified as their *p*-phenylphenacyl esters.

The 2,3-dihydroxy-2-methylbutyric acid obtained from germitetrine B melted at 66°, some 36° below the melting point of the corresponding acid from protoveratrine B; furthermore it was extremely hygroscopic. Even so, it was chromatographically indistinguishable from the dihydroxy acid from protoveratrine B and yielded acetaldehyde and pyruvic acid by periodic acid oxidation. Its equivalent weight was found to be 135.5 (134 calcd. for $C_5H_{14}O_4$). It is hypothesized that these two acids may exist in a *threo–erythro* relationship to each other.

Quantitative determination of the individual acids chromatographically showed the presence of two moles of acetic acid and one mole of 2-methylbutyric acid. By isolation, 0.81 mole of the dihydroxy acid was found. The empirical formula following from these findings and supported by carbon-hydrogen analyses and equivalent weight determinations is $C_{41}H_{63}O_{14}N$.

Determinations of hypotensive activity carried out by Dr. Benedict E. Abreu, Mrs. Alice B. Richards and Mr. William M. Alexander of these laboratories will be reported in a separate publication. Protoveratrine A, protoveratrine B and germitetrine B all show hypotensive activity, protoveratrine B being slightly less active than the other two.

Special Methods

Paper Chromatography of Alkaloids.—The principal paper chromatographic technique used in this work made use of an ethylene chloride–water–acetic acid system (49.5:49.5:1 by volume). After equilibration, the aqueous phase was used to saturate the atmosphere of the chromatographic chamber and the ethylene chloride phase was used in descending development of the strips.

The position of the alkaloids on the developed strips was ascertained by placing the dried strips on a glass plate and covering them with concd. sulfuric acid. After varying lengths of time, the position of the alkaloids was detectable by the appearance of colors and/or fluorescence.[9] This procedure therefore offered several criteria for the identification of the alkaloids besides R_f values. These were (1) the particular color developed and the sequence of color changes (2) rate of appearance of color (3) the presence or absence of fluorescence (4) color of fluorescence and (5) rate of appearance of fluorescence. A disadvantage is impermanency of record, the strips themselves dissolving after about 12 minutes.

Protoveratrines A and B were detectable as zones of bluish fluorescence appearing after two to three minutes. Germitetrine B was detectable as a bluish fluorescence appearing after four to five minutes and as a pink color appearing after six to ten minutes. Approximate R_f values were: Protoveratrine A, 0.8; protoveratrine B, 0.2; germitetrine B, 0.75; neogermitrine 0.75; germidine, 0.3.[10] R_f values were dependent on the amount of alkaloid used, increasing as more alkaloid was applied to the strips. Amounts of the order of 250 micrograms were used in obtaining the figures cited above.

Other solvent systems found useful were: chloroform–water–acetic acid (49.5:49.5:1 by volume) for examination of the more polar alkaloids of the amorphous alkaloid fraction; ethyl acetate–water–acetic acid (49.5:49.5:1), amyl acetate–water–acetic acid (49.5:49.5:1), and mixed pentanols–water–acetic acid (49.5:49.5:1) for examination of the homogeneity of protoveratrine B.

Paper Chromatography of Alkamines.—A *n*-butanol–water–acetic acid system (49.5:49.5:1 by volume) was found useful in examining the alkamines yielded by hydrolysis of the alkaloids. Using

(7) In the paper read at the American Chemical Society Meeting this alkaloid was termed "oxygermitrine W."

(8) Germine was made available through the kindness of Dr. W. A. Jacobs.

(9) Fluorescence was examined using an ultraviolet source.

(10) Neogermitrine and germidine were made available through the kindness of Dr. J. Fried of the Squibb Institute for Medical Research.

this system and descending development, protoverine moved with an R_f of about 0.29; isoprotoverine, 0.19; and germine, 0.45. Further degradation products of protoverine traveled at lesser R_f values. On treatment of the dried strips with concd. sulfuric acid, protoverine gave a pink color and a bluish fluorescence within one to two minutes; isoprotoverine, an immediate deep red color with very little fluorescence; and germine, a bluish fluorescence and a pink color within one to two minutes.

Quantitative Determination of the Individual Acids.—Marvel and Rands[6] have described an excellent technique for the separation and determination of the individual acids of a mixture using a moist silicic acid column developed with chloroform–butanol mixtures. The amounts of the acids are determined by titration of effluent fractions. Before this procedure could be applied to hydrolysis mixtures containing volatile acids, it was necessary to devise techniques for concentrating the acids and introducing them onto the column quantitatively. This problem was solved by concentrating the slightly alkaline hydrolysis mixtures to dryness *in vacuo* (we used lyophilization in the final stage of concentration), adding a measured amount of water to dissolve the dry sodium salts, adsorbing the aqueous solution on silicic acid, and transferring the silicic acid onto the previously packed silicic acid column using chloroform freshly saturated with hydrogen chloride. The amount of silicic acid used to adsorb the solution of sodium salts was enough to keep the ratio of silicic acid to water 20:12 by weight as in the packed column. The amount of chloroform freshly saturated with hydrogen chloride used was calculated to be more than sufficient to free all the acids from their sodium salts. An additional amount of chloroform was used as a wash.

To prevent mixing of the added silicic acid, containing the organic acids, with that already present in the packed column a plug of glass wool was placed on the packed column. Chloroform (3 ml.) was then added and all trapped air bubbles worked out of the glass wool. The silicic acid containing the adsorbed acids was then washed in. It was necessary to allow this silicic acid to settle without disturbance. Any irregularities in this added silicic acid were found to permit by-passing of part of the organic acids by the developing solvent and resulted in low recovery. The column was developed with chloroform–butanol mixtures of increasing polarity as described by Marvel and Rands.

A second modification of procedure was necessitated by the presence of both acetic acid and 2-hydroxy-2-methylbutyric acid in protoveratrine A. These two acids were not separated by chloroform–butanol development of silicic acid chromatograms. It was found that separation could be effected using as a developing agent *n*-butyl chloride (technical grade, Carbide and Carbon) containing 5% of *n*-butanol. Figure 2 shows separation of a known mixture of acids on such a column.

These chromatographic procedures for the quantitative determinations of the individual acids were adaptable to the determination of the acids in the volatile acid fractions as well as those in total hydrolysates. In the case of the volatile acids the total amount of acid introduced on the column was known, thus giving an internal check on chromatographic recovery.

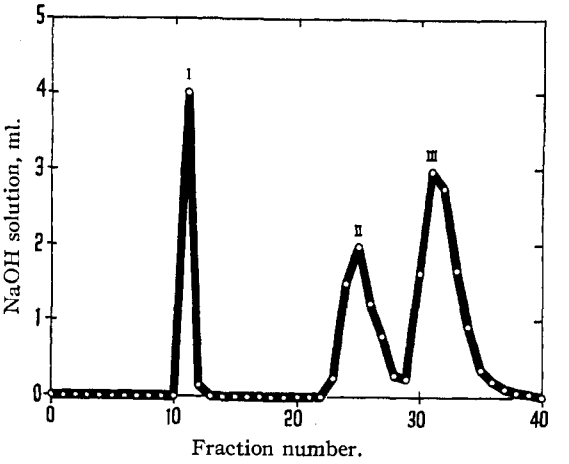

Fig. 2.—Chromatography of a known mixture of acids on a modified Marvel and Rands silicic acid column. The acids were carried through a simulated hydrolysis with *p*-toluenesulfonic acid, neutralized, lyophilized and introduced on the column adsorbed on silicic acid. Five milliliter effluent fractions were titrated: I, 2-methylbutyric acid, 94.2% recovery; II, 2-hydroxy-2-methylbutyric acid, 99.2% recovery; III, acetic acid, 98.7% recovery.

When *p*-toluenesulfonic acid was used to hydrolyze the alkaloids and the total hydrolysate was introduced in the column, the *p*-toluenesulfonic acid interfered only with the determination of the dihydroxy acids.

Experimental[11,12]

Isolation of Protoveratrine A and Protoveratrine B.—Crude protoveratrine was isolated by the procedure described by Craig and Jacobs.[2] In a typical preparation, 8 kg. of *Veratrum album* roots and rhizomes yielded 4.1 g. of crude protoveratrine and 45.1-g. of "amorphous alkaloids." After repeated recrystallization from chloroform–ether and recrystallization from alcohol (by addition of ammonia to a solution of the acetate in alcohol) there was obtained 2.1 g. of protoveratrine melting with dec. at 266–267°, $[\alpha]^{25}D$ −38.6° in pyridine.

To separate protoveratrines A and B, 2.01 g. of protoveratrine was partitioned between chloroform and 2% acetic acid solution by the Craig countercurrent distribution technique. Funnels numbered 0–14 were used and the alkaloid was introduced into funnel 0. Twenty milliliters of each solvent phase was used at each step. The chloroform phase was moved. Results are shown in Fig. 1. Funnels 0–6 were pooled as protoveratrine B and funnels 10–14, as protoveratrine A. The alkaloids were recovered in each case by making the solutions alkaline with ammonia and extracting with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate and evaporated nearly to dryness *in vacuo*.

Purification of Protoveratrine B.—To remove traces of other alkaloids from protoveratrine B, a 32 plate countercurrent distribution employing a chloroform–1 *N* sodium acetate buffer system at *p*H 3.25 was used. The chloroform phase was moved. The distribution curve shown in Fig. 3 was obtained using 20 ml. of each solvent phase at each step and 2 g. of protoveratrine B. (Amounts were determined in this case by use of a quantitative colorimetric reaction.) The curve does not show the presence of the contaminating alkaloid but when an arbitrary division was made, harvesting the contents of funnels 7–13 and 14–19 as separate pools, paper chromatography showed the ab-

(11) All melting points are corrected.

(12) Analyses by Dr. Adalbert Elek. Alkaloid and alkamine samples were dried at 120°, using his special technique.

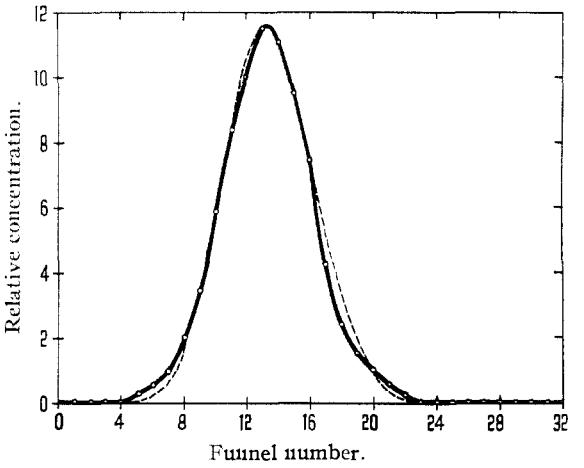

Fig. 3.—Countercurrent distribution of protoveratrine B: —O—, found; – – – – –, theoretical (K = 0.71).

sence of the contaminating alkaloid in the 7–13 pool and its presence in the 14–19 pool.

Protoveratrine B.—Protoveratrine B obtained from funnels 7–13 was recrystallized by dissolving 400 mg. in 50 ml. of boiling acetone and reducing the volume to 20 ml. on a water-bath; first crop, 220 mg. Properties were: m.p. 268–270° (dec.)[13]; $[\alpha]^{25}D$ −37° (c, 1 in pyridine); $[\alpha]^{25}D$ −3.5° (c, 1 in chloroform).

Anal. Calcd. for $C_{41}H_{63}O_{16}N$: C, 60.80; H, 7.84. Found: C, 60.84, 61.11; H, 8.12, 7.85.

In potentiometric equivalent weight determinations, 95.5 mg. of protoveratrine B (after drying at 120° at < 1 mm.) in 3 ml. of chloroform required 2.38 ml. of 0.0490 N p-toluenesulfonic acid in chloroform; 85.8 mg. required 2.15 ml.; equiv. wt. 815, 817; calcd. for $C_{41}H_{63}O_{15}N$, 810.

In a volatile acid determination, 31.0 mg. of protoveratrine B was heated 30 minutes with 1 ml. of p-toluenesulfonic acid reagent[5] on a boiling water-bath, 0.5 ml. of 1 N sodium hydroxide was added, and steam distillation was carried out; volatile acids consumed 8.42 ml. of 0.0111 N base. Two ml. of 1 N base was then added to the residue and heating on the water-bath continued 20 minutes; 2 ml. of p-toluenesulfonic acid was added and steam distillation again was performed. Volatile acids consumed 2.29 ml. of 0.0111 N base for a total of 10.71 ml. of base; calcd. for ester of protoverine with two moles of acetic acid and one mole each of 2-methylbutyric and 2,3-dihydroxy-2-methylbutyric acids: 10.35 ml. In a second determination, 39.9 mg. hydrolyzed with p-toluenesulfonic acid and then with base before steam distillation yielded acids equivalent to 15.04 ml. of 0.00995 N base; calcd.: 14.86 ml. Blanks were run on protoverine, to make certain that protoverine itself did not yield volatile acids under conditions of alkaline degradation, and on the reagents.

Hydrolysis of Protoveratrine B.—Protoveratrine B (200 mg.) was allowed to stand 48 hours at room temperature with 6 ml. of methanol, 1.3 ml. of 1 N sodium hydroxide and 3.3 ml. of water. The methanol was then removed *in vacuo*, the pH adjusted to 5.0 with hydrochloric acid and then to 8.0 with sodium carbonate. The alkamine was removed by 12 hours continuous extraction with chloroform. The aqueous solution was lyophilized and the p-phenylphenacyl esters prepared by the technique of Fried, White and Wintersteiner,[14] using chromatographically purified p-phenylphenacyl bromide. The esters were separated on a silicic acid chromatogram developed with benzene.[15] In order of their appearance in the effluent and identified by failure to depress the melting point of authentic samples, three substances were isolated: unchanged p-phenylphenacyl bromide; p-phenylphenacyl ester of 2-methylbutyric acid, m.p. after recrystallization 71.0–71.5°; and p-phenylphenacyl ester of acetic acid, m.p. after recrystallization 109.5°.

Sufficient alkamine for further work was isolated in a separate hydrolysis of 3.0 g. of protoveratrine B using essentially the conditions for hydrolysis of the 200 mg. After 22 hours of continuous chloroform extraction of the carbonate buffered hydrolysate (at pH 8.0) 1.075 g. of material melting with slow effervescence at 204–206° was obtained. An additional 380 mg. of lower melting material shown chromatographically to contain both protoverine and isoprotoverine was obtained. After recrystallization of the 1.075 g. from methanol, material melting with slow effervescence at 208–210° was obtained. Jacobs and Craig[3] report m.p. 195–200° (uncor.) for protoverine recrystallized from methanol. Converting the methanolate to the hydrate by adding water to a methanol solution and boiling gave a material melting at 210–215°; Jacobs and Craig[3] report 210–216° (uncor.).

Anal. Calcd. for $C_{27}H_{43}O_9N$: C, 61.67; H, 8.25; equiv. wt., 525. Found: C, 61.53, 61.42; H, 8.14, 8.15; equiv. wt. (potentiometric titration in chloroform), 528.

$[\alpha]^{25}D$ of material recrystallized from methanol −13.5° (c, 1.0 in pyridine); Jacobs and Craig[3] report −12°.

The hydrochloride of the acetonyl derivative prepared according to Jacobs and Craig[3] melted at 275° with decomposition. They report 278–281° with preliminary discoloration and sintering.

2,3-Dihydroxy-2-methylbutyric Acid from Protoveratrine B.—One gram of protoveratrine B was heated to 50° for one hour with 33 ml. of methanol, 16.5 ml. of water and 6.6 ml. of 1 N sodium hydroxide. The methanol was removed in partial vacuum at 40°. The residue was acidified with sulfuric acid and extracted continuously with ether for 16 hours. The ether was evaporated and the residue steam distilled until the volatile acids were removed. The remaining water was then removed *in vacuo* and the distillation flask placed in a desiccator. After overnight drying, 166.1 mg. of crystals was obtained, m.p. 94°. The crystals were dissolved in butanol and chloroform and chromatographed on a Marvel and Rands[6] silicic acid column. Acid equivalent to 13.08 ml. of 0.00882 N sodium hydroxide was obtained on 1-ml. aliquots of effluent fractions 28–37 (10 ml. fractions).

The acid was recovered by evaporating to dryness *in vacuo* the remaining 9 ml. of the acid-containing fractions. After recrystallization from chloroform and ether the acid melted at 102–103°. It was dried over phosphorus pentoxide for analysis. (It slowly sublimed when drying *in vacuo* at less than 2 mm. was attempted.)

Anal. Calcd. for $C_5H_{10}O_4$: C, 44.77; H, 7.51; neut. equiv., 134. Found: C, 44.88; H, 7.71; neut. equiv., 133.

The acid gave a positive iodoform test and negative test with 2,4-dinitrophenylhydrazine. Periodic acid oxidation was carried out using 41.2 mg. of the acid and 6 ml. of 0.1 M periodic acid. After 20 minutes at room temperature, the solution was made alkaline with sodium bicarbonate and 1.95 ml. of 5% potassium arsenite and 7.5 ml. of 10% sodium thiosulfate pentahydrate added. The solution was acidified with hydrochloric acid and added slowly to 250 ml. of 2,4-dinitrophenylhydrazine reagent (saturated solution in 2% hydrochloric acid); 101 mg. of hydrazones were obtained. The hydrazones were chromatographed on a Bentonite 625 column[16] using first ether, then ether–acetone, and finally ether–acetone–ethanol mixtures of increasing polarity to effect development and finally elution of the more tightly held zone. Two zones were apparent. The first from the column contained 48 mg. of material melting at 160°; after recrystallization from ethanol, m.p. 163°. It gave no melting point depression with an authentic sample of the 2,4-dinitrophenylhydrazone of acetaldehyde. The second contained 56.5 mg. of material which after recrystallization from ethyl acetate melted at 219°; it gave no melting point depression with authentic 2,4-dinitrophenylhydrazone of pyruvic acid.

Quantitative Determination of Individual Acids from Protoveratrine B.—Acids collected from steam distillation of a p-toluenesulfonic acid and sodium hydroxide hydrolysate of 39.9 mg. of protoveratrine B (acids equivalent to 15.04 ml. of 0.00995 N base) were lyophilized. The residue was dissolved in 0.6 ml. of water and adsorbed on 1 g. of silicic acid. This silicic acid was transferred into the pre-

(13) Inserted in the melting point bath at 200° and heated at 5° per minute to 260° and then at 2° per minute. The melting point was 266° inserting at 260° and heating 1° per minute.

(14) F. Fried, H. L. White and O. Wintersteiner, THIS JOURNAL, **72**, 4621 (1950).

(15) J. G. Kirchner, A. N. Prater and A. J. Haagen-Smit, *Ind. Eng. Chem., Anal. Ed.*, **18**, 31 (1946).

(16) J. W. White, *Anal. Chem.*, **20**, 726 (1948).

pared silicic acid column[6] with 5 ml. of chloroform saturated with hydrogen chloride plus an 8-ml. chloroform rinse. Development of the column was with the series of solvents used by Marvel and Rands.[6] Titration of 10-ml. fractions showed 2-methylbutyric acid equivalent to 4.65 ml. of 0.00995 *N* sodium hydroxide in fractions 4–8 and acetic acid equivalent to 10.40 ml. of base in fractions 19–27. These values indicated 0.93 mole of 2-methylbutyric acid and 2.10 moles of acetic acid. In two similar determinations on the total acid and base hydrolysates (omitting the steam distillation step) 0.98 and 0.95 mole of 2-methylbutyric acid and 2.18 and 1.77 mole of acetic acid were found. The *p*-toluenesulfonic acid used in hydrolysis interfered with determination of the dihydroxy acid in these hydrolysates.

Protoveratrine A.—Protoveratrine A was obtained from funnels 10–14 of the 14 funnel countercurrent distribution. After recrystallization from acetone, its properties were not changed by further recrystallization (m.p. 267–269° with dec.,[18] $[\alpha]^{25}D$ −40.5° (*c*, 1 in pyridine), $[\alpha]^{25}D$ −10.5° (*c*, 1 in chloroform)).

Anal. Calcd. for $C_{41}H_{63}O_{14}N$: C, 62.02; H, 8.00. Found: C, 61.76, 62.07; H, 8.02, 7.85.

In potentiometric equivalent weight determinations, 109.5 mg. of protoveratrine A (after drying at 120° at < 1 mm.) consumed 2.80 ml. of 0.0492 *N* *p*-toluenesulfonic acid in chloroform; 107.2 mg. consumed 2.71 ml.; equiv. wt., 794 and 804; calcd. for $C_{41}H_{63}O_{14}N$, 794.

Volatile acids were determined as in the second technique described for protoveratrine B; 3.12 mole of volatile acid per mole of alkaloid of molecular weight 794 was found. Under the conditions used, less than 0.1 mole of 2-hydroxy-2-methylbutyric acid is steam distilled.

Hydrolysis of Protoveratrine A.—Hydrolysis of 2 g. of protoveratrine A was carried out by the technique described for protoveratrine B. The alkamine was recovered by the technique of Jacobs and Craig.[3] Seven hundred milligrams of protoverine and 580 mg. of isoprotoverine[3] (m.p. 265° and chromatographically identical with the product which could be produced from pure protoverine by alkali treatment) were obtained. The protoverine after recrystallization from methanol melted with effervescence at 208–210°; no melting point depression with protoverine from protoveratrine B. The hydrate prepared from the methanolate by the technique of Jacobs and Craig[3] slowly melted at 210–216°.

Anal. Calcd. for $C_{27}H_{43}O_9N$: C, 61.67; H, 8.25. Found: C, 61.54; H, 8.62.

The hydrochloride of the acetonyl derivative prepared according to Jacobs and Craig[3] melted at 275° with sintering and decomposition; no melting point depression with the similar compound from protoveratrine B.

The aqueous phase after chloroform extraction to remove the alkamines was lyophilized. The residue from lyophilization was used for preparation of *p*-phenylphenacyl esters. After chromatography, *p*-phenylphenacyl esters of the following were obtained and identified by failure to depress the melting points of authentic samples: 2-methylbutyric acid (ester, m.p. 72.0°); acetic acid (ester, m.p. 110°); 2-hydroxy-2-methylbutyric acid (ester, m.p. 122°).

Quantitative Determination of the Individual Acids of Protoveratrine A.—Acids collected from steam distillation of a *p*-toluenesulfonic acid and sodium hydroxide hydrolysate of 35.5 mg. of protoveratrine A (acids consumed 12.56 ml. of 0.0111 *N* sodium hydroxide) were lyophilized and placed on a Marvel and Rands[6] column as described for acids from protoveratrine B. The column was developed with *n*-butyl chloride (technical grade) containing 5% butanol. Titrating 5-ml. fractions of the effluent, 2-methylbutyric acid equivalent to 3.48 ml. of 0.0111 *N* base was found in fractions 6–11; 2-hydroxy-2-methylbutyric acid equivalent to 0.12 ml. of base was found in fraction 24; and acetic acid equivalent to 8.36 ml. of base was found in fractions 27–34. To determine acids not steam distilled, the residue from the steam distillation was lyophilized and placed on a similar column. No 2-methylbutyric acid was found; 2-hydroxy-2-methylbutyric acid equivalent to 4.24 ml. of 0.0111 *N* base and acetic acid equivalent to 0.36 ml. of base were found. This totals, for both fractions, 2.16 moles of acetic, 0.87 mole of 2-methylbutyric and 1.08 moles of 2-hydroxy-2-methylbutyric acid per mole of alkaloid (of mol. wt. 794).

In a similar determination on 38.0 mg. of protoveratrine A hydrolyzed with *p*-toluenesulfonic acid and then with sodium hydroxide and lyophilized directly without steam distilling the volatile acids, 1.88 moles of acetic, 0.92 mole of 2-methylbutyric and 0.93 mole of 2-hydroxy-2-methylbutyric acids were found.

Salts of Protoveratrines A and B.—Protoveratrine A thiocyanate was prepared by the technique of Poethke[17]; m.p. 226–227° (dec.).[18] Protoveratrine B thiocyanate was prepared by the same technique, but three days standing at 4° was required before crystals formed; m.p. on recrystallizing from methanol and water 224–225° (dec.).[18] A mixture of the pure thiocyanates melted at 220–221° (dec.); thiocyanate prepared from a mixture of the two alkaloids melted at 220–221° (dec.).

The hydrochloride of protoveratrine A was prepared by adding a slight excess of 0.05 *N* hydrochloric acid to 1.08 g. of protoveratrine A. This solution was evaporated to dryness *in vacuo* and the residue dissolved in hot 1,4-dioxane and filtered immediately. A first crop of 318 mg. of crystals quickly formed in the filtrate; m.p. 238–239° (dec.)[19]; equivalent weight by titration with base, 840, calcd. 830.

The hydrochloride of protoveratrine B was prepared in the same manner; m.p. 242–244° (dec.).[19] A mixed melting point of protoveratrine A and protoveratrine B hydrochlorides showed an 8° depression.

Isolation of Germitetrine B.—The total alkaloid fraction (180 g.) remaining after removal of protoveratrine by the technique of Craig and Jacobs[2] was subjected to a 19-plate countercurrent distribution process using methylene chloride–water–acetic acid (49.5:49.5:1 by volume). Four hundred fifty milliliters of each phase was used at each step. Funnels were numbered 0–19 and the alkaloid was introduced into funnel 0. The methylene chloride phase was moved. The material in funnels 11–16 was combined (42.6 g.). This was dissolved in 225 ml. of hot *n*-butyl chloride and 60 ml. of petroleum ether (b.p. 30–60°) was added. The solution was immediately filtered to remove a flocculant precipitate and 150 ml. of additional petroleum ether was added very slowly with stirring. After cooling, 2.8 g. of crystals of germitetrine B formed on the sides of the vessel. Additional crops totaling 2.2 g. were obtained.

Germitetrine B.—Germitetrine B isolated from the 19-plate countercurrent distribution was recrystallized four times from *n*-butyl chloride and once from acetone–water; m.p. 233–234°, $[\alpha]^{25}D$ −70° (*c*, 1 in pyridine), $[\alpha]^{25}D$ −17° (*c* 1 in chloroform). Figure 4 gives evidence of the homogeneity of this material. Germitetrine B gave 11° melting point depression with neogermitrine.[10]

Anal. Calcd. for $C_{41}H_{63}O_{14}N$: C, 62.02; H, 8.00. Found: C, 62.01; H, 8.01.

In an equivalent weight determination, 117.8 mg. of germitetrine B (dried *in vacuo* over P_2O_5 at 127°) consumed 2.98 ml. of 0.0492 *N* *p*-toluenesulfonic acid in chloroform (end-point determined potentiometrically); in a second determination 117.2 mg. consumed 2.86 ml. of 0.0512 *N* hydrochloric acid (end-point determined potentiometrically); found values were therefore 804 and 800; calcd. for $C_{41}H_{63}O_{14}N$, 794.

In a volatile acid determination, using *p*-toluenesulfonic acid and sodium hydroxide hydrolysis, 33.2 mg. of germitetrine B yielded volatile acids consuming 12.88 ml. of 0.00995 *N* base. In a second determination 31.9 mg. yielded volatile acids equivalent to 12.34 ml. of base. Found values were, therefore, 3.05 and 3.06 moles of volatile acid per mole of alkaloid of molecular weight 794.

Hydrolysis of Germitetrine B.—Alkamine was isolated from 254 mg. of germitetrine B by proceeding in the same manner as Fried, White and Wintersteiner[14] in working with germidine. After recrystallization from methanol–ether, 119 mg. of alkaloid sintering at 170° and melting at 219° was obtained. Germine has been reported[17] to melt at 220° after sintering at 160–170°. The alkamine from germitetrine B gave no melting point depression with an authentic sample of germine.[8]

Anal. Calcd. for $C_{27}H_{43}O_8N$: C, 63.63; H, 8.50. Found: C, 63.73, 63.61; H, 8.74, 8.75.

The hydrochloride of the acetonyl derivative prepared according to the procedure of Craig and Jacobs[20] melted with

(17) W. Poethke, *Arch. Pharm.*, **275**, 571 (1937).

(18) Inserted in melting bath at 210° and heated 1° per minute.

(19) Inserted in the melting bath at 235° and heated 1° per minute.

(20) L. C. Craig and W. A. Jacobs, *J. Biol. Chem.*, **148**, 57 (1943).

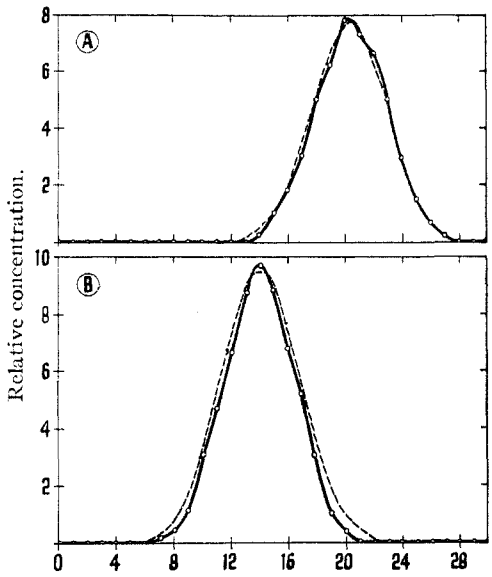

Fig. 4.—Countercurrent distribution of germitetrine B: —O—, found; - - - - -, theoretical. Curve A was obtained using an ethyl acetate–0.5 *M* acetate buffer system of *p*H 5.0. Twenty milliliters of each phase and 50 mg. of germitetrine B were used. Curve B was obtained using an ethylene chloride–0.5 *M* acetate buffer system of *p*H 3.9. Twenty milliliters of each phase and 37 mg. of germitetrine B were used. A colorimetric assay was used to determine alkaloid concentration in each case.

decomposition at 265° after previous shrinking at 254°; Craig and Jacobs reported this compound to melt at 275° after shrinking at 255°.

The volatile acids from germitetrine B were identified as their *p*-phenylphenacyl esters. Germitetrine B (1.33 g.) was hydrolyzed by 24 hours standing at room temperature with 43.4 ml. methanol, 6.6 ml. of 1 *N* sodium hydroxide and 15 ml. water. After removal of the methanol *in vacuo* the germine was extracted with chloroform. The aqueous residue was made acid with sulfuric acid and steam distilled catching the volatile acids in 0.1 *N* sodium hydroxide solution. This solution was lyophilized and the *p*-phenylphenacyl esters prepared from the residue.[14] The esters were chromatographed on silicic acid using benzene as a developing agent.[15] In order of their appearance in the effluent and identified by their failure to depress the melting point of authentic samples, three substances were isolated: unchanged *p*-phenylphenacyl bromide; *p*-phenylphenacyl ester of 2-methylbutyric acid, m.p. after recrystallization 73.5°; and *p*-phenylphenacyl ester of acetic acid, m.p. after recrystallization 109°.

2,3-Dihydroxy-2-methylbutyric Acid from Germitetrine B.—This acid was isolated from the 1.33 g. whose hydrolysis is described in the previous section. After distilling out the steam distillable acids, the aqueous residue was extracted with ether continuously for 14 hours. The ether was evaporated to dryness *in vacuo* and the residue placed over concd. sulfuric acid in a desiccator. After several days crystals appeared. These were dried *in vacuo* at 100°. The yield was 59.2 mg., m.p. 66°. Additional acid was obtained after making the aqueous residue from the ether solution alkaline and lyophilizing. The residue was dissolved in 1.2 ml. of water, adsorbed on 2 g. of silicic acid, and flushed into a Marvel and Rands[6] silicic acid column with 8 ml. of chloroform saturated with hydrogen chloride and 6 ml. of chloroform. Development of the column with the chloroform–butanol series used by Marvel and Rands[6] yielded an acid in fractions 29–68 (10-ml. fractions) with peak concentration at fraction 33. Acid equivalent to a total of 66.03 ml. of 0.0139 *N* base was obtained. The acid was recovered from the neutralized effluent solution by extraction into dilute base and extraction from this solution after acidification into ether followed by evaporation of the ether.

The acid as obtained in all isolations was extremely hygroscopic. It gave no evidence of heterogeneity either on silicic acid chromatograms or on paper chromatography using system D of Stark, Goodban and Owens.[21] The acid could not be distinguished chromatographically from the 2,3-dihydroxy-2-methylbutyric acid isolated from protoveratrine B. The acid could not be recrystallized from chloroform, benzene, alcohols or ether. Because of its extreme hygroscopicity no samples of the acid were sent for analysis.

In an equivalent weight determination, 59.2 mg. of the acid (dried several days over sulfuric acid) required 8.94 ml. of 0.0488 *N* base; equiv. wt. 135.5; calcd. for $C_5H_{10}O_4$, 134.

The acid gave a positive iodoform test and a negative test with 2,4-dinitrophenylhydrazine. A 20.4 mg. sample of the acid was oxidized with periodic acid by the same technique used for the similar acid from protoveratrine B. The 2,4-dinitrophenylhydrazones (50.2 mg.) of the oxidation products were chromatographed on a Bentonite 625 column.[16] Two zones were apparent. Material from the first to elute from the column after recrystallization from ethanol melted at 160°; no melting point depression with the 2,4-dinitrophenylhydrazone of acetaldehyde. (It gave a 22° depression with the corresponding formaldehyde derivative.) The second melted at 200–212° and after recrystallization from ethyl acetate melted at 218°; no melting point depression with an authentic sample of the 2,4-dinitrophenylhydrazone of pyruvic acid.

These oxidation products indicate the structure to be that of 2,3-dihydroxy-2-methylbutyric acid. The lower melting form of this acid is reported in the literature[22] as melting at 88°.

Quantitative Determination of Individual Acids from Germitetrine B.—Acids collected from steam distillation of a *p*-toluenesulfonic acid and sodium hydroxide hydrolysate of 33.2 mg. of germitetrine B (acids equivalent to 12.88 ml. of 0.00995 *N* base) were made alkaline, lyophilized, and transferred to a Marvel and Rands[6] column. Titration of 10-ml. fractions of effluent showed 2-methylbutyric acid equivalent to 4.36 ml. of 0.00995 *N* base in fractions 5–11 and acetic acid equivalent to 8.78 ml. of base in fractions 21–27. This is equivalent to 1.04 moles of 2-methylbutyric acid and 2.08 moles of acetic acid per mole of alkaloid.

Acknowledgment.—The authors are indebted to Dr. Earl Bockstahler for numerous helpful discussions during the course of this work.

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(22) R. Fittig and M. Penshuck, *Ann.*, **283**, 109 (1894).