nate recrystallization from 90% methanol and 90% ethanol. The recrystallization was repeated three times.

Anal. Calcd. for $C_5H_7N_3O_4$: C, 34.68; H, 4.08; N, 24.27; O, 36.97; mol. wt., 173.13. Found: C, 34.85; H, 4.36; N, 24.44; O, 37.36.

Potentiometric titration in water indicated a molecular weight of 175, a pK'a value of 8.55, and a slight inflection of the curve at pH 4.0 due to decomposition. Craig countercurrent distribution of this sample between water and liquid phenol demonstrated the presence of only one substance with a partition coefficient of 1.14. This is shown in Fig. 2 where two separate criteria, ultraviolet absorption and *anti-Kloeckera brevis* activity, are plotted against tube numbers.

Crystalline azaserine is very soluble in water, only slightly soluble in absolute methanol, absolute ethanol and acetone, but soluble in warm aqueous solutions of these solvents.[1c] It gives a positive ninhydrin (blue) and sodium β-naphthoquinone-4-sulfonate test and reduces ammoniacal silver nitrate solutions readily. These tests, as well as the ultraviolet absorption, were quite useful during the isolation studies for spotting azaserine on paper chromatographic strips.

Crystallographic Data.[5]—Azaserine crystals are biaxially positive with a moderately high birefringence. Extinction is parallel on elongated, cleavage fragments and crystals. The compound probably crystallizes in the orthorhombic system. Cleavage fragments and crystals are length fast, are elongated parallel to the β vibration and have an excellent platy cleavage parallel to the α–β plane. Since the crystals tend to lie on this flat surface, it was possible to measure only two indices. No other optical orientation was observed. Measurement of the indices gives the values: $\alpha = 1.523 \pm 0.002$; $\beta = 1.607 \pm 0.002$.

(5) We are indebted to Mrs. Alice S. Corey, University of Michigan, Ann Arbor, for this information.

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DETROIT 32, MICHIGAN

[CONTRIBUTION FROM THE RESEARCH LABORATORIES, PARKE, DAVIS & COMPANY]

Azaserine, a New Tumor-inhibitory Substance. Structural Studies.[1]

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The constitution of azaserine has been established as O-diazoacetyl-L-serine. This structure has been deduced from spectroscopic evidence, functional group analyses, and acidic and basic degradative studies.

In the previous publication,[2] a new antibiotic, azaserine, has been reported which was isolated from culture broth filtrates of a *Streptomyces* and characterized as a homogeneous yellow-green crystalline compound possessing an empirical formula $C_5H_7N_3O_4$. The hydrophilic behavior of the compound as well as the positive ninhydrin and sodium β-naphthoquinone-4-sulfonate color tests strongly indicated the presence of an amino acid function in the molecule. Confirmatory evidence for this was found in the infrared spectrum. The low series of bands between 3.5 and 4.0 μ, the strong band at 6.22 μ with the shoulders on the high and low wave length sides, and the strong band at 6.63 μ are typical of amino acids.[3] One of the strongest and perhaps the most diagnostic band in the spectrum is that occurring at 4.66 μ. Pronounced absorption in this region is characteristic of a cumulative double bond type structure such as that occurring in an azide, aliphatic diazo compound, or a ketene. The doublet at 5.89 and 5.96 μ could be a conjugated ester carbonyl while the band at 8.42 μ was thought probably to be associated with an ester group.

(1) Presented before the Division of Medicinal Chemistry at the 125th Meeting of the American Chemical Society, Kansas City, Mo., March 26, 1954.

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As was mentioned in the previous publication,[2] acidification of aqueous solutions of the antibiotic with mineral acids resulted in the immediate destruction of the ultraviolet absorbing properties as well as in the disappearance of the 4.66 μ band in the infrared region. Since this acid decomposition was accompanied by vigorous gas evolution, it was suspected that an aliphatic diazo group was present in the intact antibiotic.

The gas evolved on acidification proved to be pure nitrogen. When the decomposition was accomplished with dilute sulfuric acid and the liberated gas collected in an azotometer, a value of 16.18% or two-thirds of the total nitrogen was obtained. A ninhydrin–carbon dioxide determination on the intact antibiotic gave a value of 7.97% carboxyl nitrogen corresponding to the remaining one-third of the total. A similar value was also obtained by the Kjeldahl procedure. These data precluded an azide type structure.

Although the infrared absorption bands at 5.89, 5.96 and 8.42 μ were indicative of an ester linkage, the possibility of a diazoketone group existing in the molecule could not be overlooked. Studies on the reactivity of the antibiotic and model compounds with hydroxylamine hydrochloride were therefore undertaken to establish this point. When an aqueous solution of azaserine at pH 3.4 was allowed to react with hydroxylamine hydrochloride solution, the pH increased gradually, reaching a value of 4.2 after two and one-half hours. A small

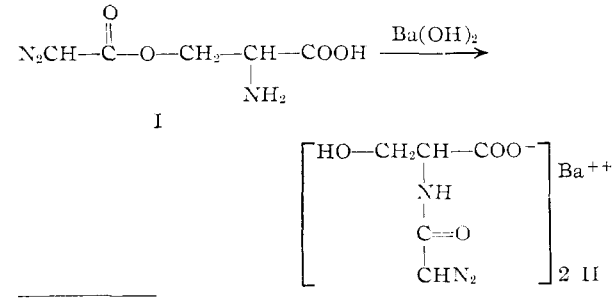
amount of nitrogen gas was liberated during the reaction, accounting for the uptake of acid and increase in *p*H. An aqueous alcoholic solution of ethyl diazoacetate under the same conditions behaved in exactly the same manner. However, when 1,4-bisdiazoacetylbutane[4] was subjected to the above conditions, a drop in *p*H resulted, and back titration with standard alkali indicated that oxime formation had occurred to the extent of 70% of theory.

Confirmatory evidence for a diazo ester type linkage was obtained by examining the ultraviolet absorbing properties of the two above-mentioned model compounds. Whereas 1,4-bisdiazoacetylbutane exhibited two maxima at *p*H 7.0, $E^{1\%}_{1cm.}$ 634 and 1078 at λ 245 and 275 mμ, respectively, ethyl diazoacetate possessed but one sharp maximum with $E^{1\%}_{1cm.}$ 1600 at λ 250 mμ. This corresponds to an ε of 18240 as compared with 19722 for azaserine.

It was therefore concluded that azaserine was a diazo ester of an α-amino acid and the only structure compatible with the empirical formula and analytical data was O-diazoacetylserine (I).

Acid hydrolysis using hot 2 *M* formic acid liberated L-serine and glycolic acid. The serine was isolated from a synthetic cation exchange column (Dowex 50) and characterized by microanalyses, by infrared spectra, by optical rotation determinations, and as the N-2,4-dinitrophenyl derivative. Glycolic acid isolated from the column effluent was characterized by R_f value[5] and as the *p*-bromophenacyl ester. When the hydrolysis was carried out at room temperature at *p*H 2.0, crystalline O-glycolyl-L-serine was isolated and identified by microanalyses. Catalytic reduction[6] of azaserine with palladium-on-charcoal yielded crystalline O-acetyl-L-serine which was isolated by partition chromatography on cellulose and characterized by microanalyses, infrared spectrum and mixed melting point with an authentic sample.

Azaserine under mild alkaline conditions undergoes acyl migration to the amino nitrogen although at a much slower rate than do the O-acetyl and glycolyl derivatives. Whereas the latter two underwent rearrangement almost instantaneously upon addition of one equivalent of base (negative ninhydrin color), 43% of the azaserine, as measured against *K. brevis*, remained after two hours with barium hydroxide at *p*H 12.0. After five hours at this *p*H, about 10% of the microbiological activity remained while the extinction had been lowered by about 50%. The barium salt of N-diazoacetyl-L-serine (II) was isolated as a crude yellow-green powder, $E^{1\%}_{1cm.}$ 448 at λ 252 mμ (*p*H 7.0). A paper chromatographic strip run on this material gave two very faint ninhydrin zones indicating relatively complete migration with a small amount of hydrolysis to free serine. This salt possessed little or no microbiological activity.



I II

Experimental

Acid Hydrolysis of Azaserine.—A solution of 525 mg. of crystalline azaserine in 40 cc. of 2 *M* formic acid was heated on a steam-bath for 2 hours. The hydrolysate was concentrated to dryness *in vacuo*, distilled water was added to the residue and the concentration repeated. The concentration was repeated twice more using ethanol as the diluent.

A. Isolation of L-Serine.—The residue was dissolved in 10 cc. of deionized water and added to a small chromatographic column containing 5 g. of Dowex 50 (100–200 mesh, H^+ cycle). The column was washed with 100 cc. of water and then eluted with 0.1 *M* ammonium hydroxide solution. The effluent was collected until the ninhydrin positive component broke through. This solution was used for the glycolic acid isolation.

The fraction which was ninhydrin positive proved to be homogeneous and possessed the same R_f as serine[7] when examined by paper chromatography. The solution was concentrated to dryness *in vacuo* and the white residue after recrystallization from aqueous ethanol yielded 125 mg. of white needles, $[\alpha]^{26}_D$ −7.3° (*c* 5.5% in water), $[\alpha]^{26}_D$ +14.8° (*c* 6.5% in 2 *N* hydrochloric acid); reported for L-serine[8] −6.8° and +14.8°, respectively. The infrared absorption spectrum (in Nujol mull) was identical with authentic L-serine.

Anal. Calcd. for $C_3H_7O_3N$: C, 34.28; H, 6.71; N, 13.33. Found: C, 34.52; H, 6.96; N, 13.29.

The N-2,4-dinitrophenyl derivative,[9] m.p. 178° uncor., was characterized by analysis and mixed melting point with authentic L-N-2,4-dinitrophenylserine, which showed no depression.

Anal. Calcd. for $C_9H_9O_7N_3$: C, 39.86; H, 3.35; N, 15.49. Found: C, 39.68; H, 3.58; N, 15.52.

B. Isolation of Glycolic Acid.—The Dowex 50 column effluent was concentrated to dryness *in vacuo* leaving a viscous gummy residue. This residue was extracted twice with 20-cc. portions of ether and decanted. Upon evaporation of the ether a semi-solid gummy residue remained. This was extracted with ether and the extract on evaporation yielded 107 mg. of crystalline residue, m.p. 68–70°; reported for glycolic acid 79°.[10] Paper chromatograms using amyl alcohol–formic acid solvent[5] indicated identical R_f values (0.44) for both glycolic acid and the crystalline residue; furthermore a mixed paper chromatogram showed but one zone. The crystalline compound was further characterized by derivatization to the *p*-bromophenacyl ester, m.p. 138–139°; glycolic acid *p*-bromophenacyl ester melts at 138°[10] and a mixed melting point showed no depression.

Anal. Calcd. for $C_{10}H_9O_4Br$: C, 43.98; H, 3.32; Br, 29.26. Found: C, 44.30; H, 3.66; Br, 29.29.

The ether-insoluble residue gave two zones when chromatographed with the amyl alcohol–formic acid solvent,[5] R_f 0.25 and 0.44, the latter being glycolic acid. The slower-moving component was not characterized further.

Isolation of O-Glycolyl-L-serine.—A solution of 100 mg. of azaserine in 40 cc. of water was adjusted to *p*H 2.2 with 5 *M* formic acid and allowed to remain at room temperature for 2.5 hours. A spectrophotometric assay indicated that over 99% of the azaserine had been decomposed. The solution was then freeze-dried and the residue crystallized

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from ethanol–water to give colorless needles, m.p. 149–150° uncor.

Anal. Calcd. for $C_5H_9O_5N$: C, 36.81; H, 5.56; N, 8.59. Found: C, 36.90; H, 5.78; N, 8.74.

Catalytic Hydrogenation of Azaserine.—Thirty mg. of palladium black[11] was suspended in 8 cc. of water and one drop of 0.1 *N* sodium hydroxide solution added. The stirred mixture was then washed three times with water by centrifugation. The catalyst was transferred to a solution of 500 mg. of azaserine in 50 cc. of 50% aqueous ethanol and hydrogenated at 32 p.s.i. for 4 hours. The catalyst was removed by filtration and the filtrate on lyophilization gave 364 mg. of white powder. Ascending paper chromatography using 70% isopropyl alcohol–water showed three zones, R_f 0.22, 0.31 and 0.45.

A chromatographic column containing 30 g. of powdered cellulose (Solka Floc) was then prepared.[12] The cellulose powder was prewashed with 0.1 *N* hydrochloric acid, acetone and water. Metallic cations were removed with 8-hydroxyquinoline.[13] A solution of 150 mg. of reduction product in 5 cc. of 70% isopropyl alcohol–water, was applied to the column and developed with the solvent. The eluate was collected in 5-cc. fractions automatically every 20 minutes and paper strips were run on the ninhydrin-positive ones. The solvent system was *t*-butyl alcohol, acetic acid and water (50:25:25) and the following results were obtained.

Fractions 13-20, one zone of R_f = 0.58 – 0.62
26-29, one zone of R_f = 0.36 – 0.41
46-49, one zone of R_f = 0.29 (yellow)

Synthetic O-acetyl-L-serine, prepared by the method of Sakami and Toennies,[14] had an R_f of 0.62 and mixed paper chromatograms with fractions 13–20 showed but one zone. Fractions 14–18 were concentrated to dryness *in vacuo* and the resulting product was recrystallized twice from ethanol–water to give 53.5 mg. of white needles, m.p. 151.5–151.8°. A mixed melting point with an authentic sample of O-acetyl-L-serine showed no depression. The infrared absorption spectrum was identical with the synthetic L-isomer but distinct from that of the racemate.

Anal. Calcd. for $C_5H_9O_4N$: C, 40.81; H, 6.17; N, 9.52. Found: C, 40.75; H, 6.25; N, 9.54; Van Slyke amino N, 9.55.

Inactivation of Azaserine with Barium Hydroxide.—An aqueous solution of azaserine was adjusted to *p*H 12.0 with barium hydroxide solution (about 2.5 equiv. required) and the solution diluted to give a final concentration of 1 mg./cc. Aliquots were withdrawn at various time intervals, diluted ten times with *p*H 7.8 phosphate buffer solution, and assayed spectrophotometrically and microbiologically. The results are expressed in Table I. The ultraviolet absorption values were determined at *p*H 7.0, and *Kloeckera brevis* was used as the assay organism.

TABLE I

Time, min.	Optical units/cc.[2]	λ_{max} at mμ	Bioassay, μg./cc.
0	11.5	250	107
1	11.5	250	111
5	10.75	250	114
10	12.1	250	103
30	11.8	250	78
60	12.2	250	78
120	12.9	251	46
300	6.1	252	11

Isolation of Crude Barium N-Diazoacetyl-L-serinate.—To 53 mg. of azaserine in 15 cc. of water was added 1 cc. of 0.6 *M* barium hydroxide solution to a final *p*H of 12.1; the mixture was diluted to 22 cc. with water. After standing for 5 hours at room temperature in a closed cylinder, a microbiological assay indicated that about 90% of the material had been inactivated. After standing an additional 1.75 hours, a piece of Dry Ice was added and the yellow solution centrifuged. The supernatant was lyophilized and the yellow powder obtained was dissolved in 1.5 cc. of water and the white precipitate separated by centrifugation. To the yellow supernatant was added 15 volumes of absolute ethanol followed by 15 volumes of ether. The precipitate obtained after standing overnight at 5° was removed by centrifugation and washed with 50% ethanol–ether followed by absolute ether. The hygroscopic yellow-green powder was dried *in vacuo*, yield 52 mg., $E^{1\%}_{1\,cm.}$ 448 at λ_{max} 252 mμ (*p*H 7.0); microbiological assay: 5 μg./mg. based on crystalline standard. A paper chromatographic strip using *t*-amyl alcohol, acetone and water (9:2.5:7.2) as the solvent system and applying 100 μg./strip showed very faint ninhydrin zones with R_f values 0.16 and 0.32. Serine and azaserine gave values of 0.16 and 0.29, respectively, under these conditions.

Although ultraviolet analysis indicates the salt to be about 50% pure, paper chromatographic studies show that acyl migration has occurred, accompanied by only a small amount of hydrolysis to free serine.

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