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

Azaserine, a New Tumor-inhibitory Substance. Isolation and Characterization[1]

BY SALVATORE A. FUSARI, ROGER P. FROHARDT, ALBERT RYDER, THEODORE H. HASKELL, DORIS W. JOHANNESSEN, CAROLE C. ELDER AND QUENTIN R. BARTZ

RECEIVED JANUARY 29, 1954

Detailed methods for the isolation of crystalline azaserine from culture broth filtrates of a *Streptomyces* have been described. The purity of this compound has been established by the Craig countercurrent technique, and it has been characterized with respect to crystalline form, composition, solubility, optical properties, ultraviolet and infrared absorption and certain color reactions.

Preliminary information on isolation of azaserine, a new tumor-inhibitory substance, from laboratory culture broths produced by a *Streptomyces* and data on some of the properties of this antibiotic have been given.[2] In this paper more detailed information concerning the isolation procedure and the general properties of azaserine are presented.

Preliminary attempts to isolate azaserine from culture broth filtrates by conventional methods failed. Attempted extractions at *p*H 3, 7 and 9 with ethyl acetate, *n*-butyl alcohol and other common organic solvents were unsuccessful. The antibiotic could be partially extracted with liquid phenol, but with an unsatisfactory improvement in potency. It could be adsorbed by activated carbons (Darco G-60 and Nuchar C) in high dosage, but on elution again there was slight improvement in potency of product. Ion exchange chromatography on both acidic and basic resins proved fruitless. It appeared that the antibiotic was either destroyed or irreversibly adsorbed by Dowex 50 (H^+), Zeo-Rex (H^+), Amberlites IRA-400 (OH^-), XE-98 (OH^-) and XE-97(H^+). Passage of the culture broth filtrates through either Amberlites IRC-50(H^+) or IR-45(OH^-) revealed that only part of the activity appeared in the percolates, but none could be eluted from the column. On the other hand, passage of broth filtrates through Amberlites XE-98(Cl^-), IRA-400(Cl^-) (buffered at *p*H 7.0 with sodium acetate) and IR-45(Cl^-) showed neither adsorption nor destruction of activity.

The early stages of purification were complicated by the marked instability of azaserine. It was found that if the culture broth remained in contact with the mycelia, the biological activity disappeared rapidly. Stability studies at various temperature and *p*H levels (Table I) revealed that the antibiotic in culture broth filtrates was markedly labile except within rather narrow limits of *p*H and temperature. These data explain the failures encountered with acidic and basic ion exchange resins.

It was finally demonstrated that azaserine could be partially or completely adsorbed from 90% ethanol solutions by Nuchar C, Nuchar C-190-N, Darco G-60, Super Filtrol, Decalso, silica gel and alumina. Since it could be readily eluted from alumina by water, a system of chromatography based on adsorption from 90% ethanol followed by development with graded quantities of water in ethanol and elution with water was devised. Adsorption of the partially purified alumina products from water or very dilute aqueous acetone solution (1–2%) on a carbon column followed by elution with further quantities of dilute acetone (1–5%) gave concentrates which could be crystallized from aqueous ethanol.

TABLE I

STABILITY OF AZASERINE IN CULTURE BROTH FILTRATES AT VARIED TEMPERATURE AND *p*H

	Temperature, °C.					
	25		40		60	
Init. *p*H[a]	Loss, %	Time, hr.	Loss, %	Time, hr.	Loss, %	Time, hr.
2	100	0.25				
3	50	1				
4	50	5	100	4		
5	50	48	100	24	100	3
6	0	48	0	24	50	4
7	0	48	0	24	40	6
8	0	48	0	24	16	6
9	25	24	75	24	50	1–2
10	50	3	100	6		
11–12	100	<1	100	<1		

[a] It was noted that all samples adjusted to *p*H 2–6 tended to rise in *p*H with the passage of time whereas the more alkaline samples (*p*H 7–12) tended to drop in *p*H even though the samples were protected from atmospheric carbon dioxide by soda lime tubes.

Azaserine crystallizes as light yellow-green needles from aqueous ethanol. It does not have a sharp melting point, but undergoes decomposition over a wide range (146–162°), depending upon the rate of heating and the method of determination. It exhibits slight optical activity in aqueous solution, $[\alpha]^{27.5}D$ −0.5° (*c*, 8.46% in water at *p*H 5.18), but the rotation in 2 *N* hydrochloric acid undergoes continual change, finally becoming constant at $[\alpha]^{28}D$ +9.7°. Dissolved in *p*H 7.0 phosphate buffer, it shows characteristic absorption in the ultraviolet region with one sharp, well-defined peak of $E^{1\%}_{1\,cm.}$ 1140 at λ 250.5 mμ. The biological activity is destroyed and a hyperchromic shift to λ 252 mμ ($E^{1\%}_{1\,cm.}$ 1230) occurs in 0.1 *N* sodium hydroxide. There is a complete disappearance of ultraviolet absorption in 0.1 *N* hydrochloric acid which is associated with a total loss of biological activity and an evolution of nitrogen. The infrared absorption curve of azaserine, as shown in Fig. 1, is also quite characteristic. The sharp absorption band at 4.66 μ is distinctive and, like the ultraviolet absorption peak at 250.5 mμ, disappears completely upon acidification.

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

(2) (a) C. C. Stock, H. C. Reilly, S. M. Buckley, D. A. Clarke and C. P. Rhodes, *Nature*, **173**, 71 (1954); (b) J. Ehrlich, L. E. Anderson, G. L. Coffey, A. B. Hillegas, M. P. Knudsen, H. J. Koepsell, D. L. Kohberger and J. E. Oyaas, *ibid.*, **173**, 72 (1954); (c) Q. R. Bartz, C. C. Elder, R. P. Frohardt, S. A. Fusari, T. H. Haskell, D. W. Johannessen and A. Ryder, *ibid.*, **173**, 72 (1954).

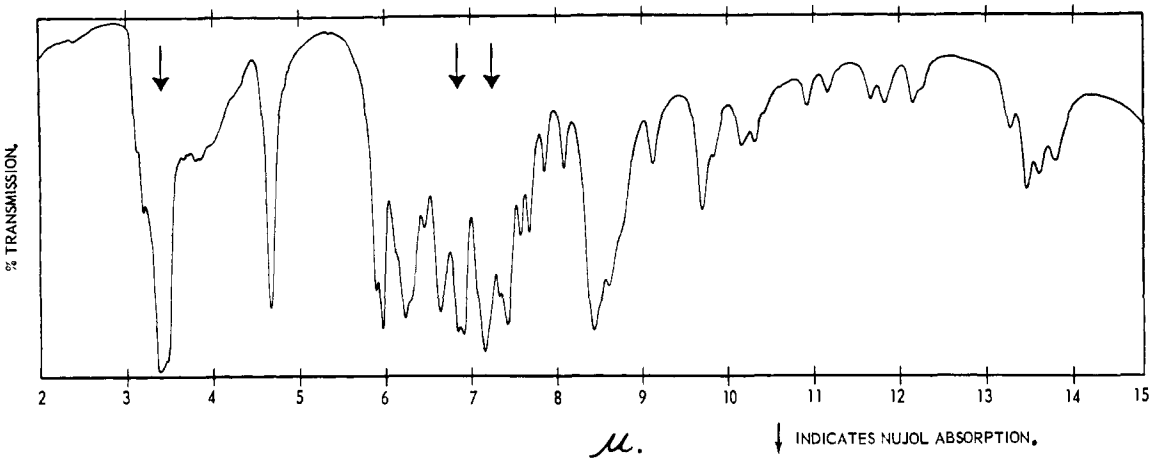

Fig. 1.—Infrared spectrum of azaserine in Nujol mull.

Experimental

Assay.—The inhibitory activity of azaserine toward Crocker mouse sarcoma 180 in mice was employed in the early work on purification, but subsequent studies led to the correlation of this effect with its inhibitory activity against *Kloeckera brevis*.[2] During the course of these studies it was found that both inhibitory activities showed excellent correlation with the ultraviolet and the infrared absorption spectra of partially purified concentrates. Microbiological and absorption assays, because of their simplicity, were thereafter employed and the anti-tumor tests reserved for confirmatory purposes.

A description of the *K. brevis* assay will be published elsewhere.[3] A crude azaserine preparation was employed as a standard in the microbiological assay and in this paper results are expressed as mg. of standard. Crystalline azaserine is 346 times as active as the standard.

When the absorption in the ultraviolet was used for assay purposes, an optical unit was employed. This was arbitrarily defined as that amount of azaserine contained in 1 cc. of solution which exhibits an optical density of 1.0 at λ 250.5 mμ.

Preparations were checked from time to time for the presence of the absorption band at 4.66 μ. It was noted that the intensity of this band increased with an increase in biological potency.

Chromatography on Alumina.—A mixed sample of active stirred-jar culture broths (*p*H *ca.* 7.0) was slurried with 1% Hyflo Super-Cel and filtered to remove the mycelia. The combined filtrate and washings (46 liters) was flash evaporated in a Precision circulatory still at <35° to 0.05 vol. (2.3 liters). To the concentrate was added 9 vols. of 95% No. 3A ethanol with continuous stirring. The inactive precipitate which formed was removed and the ethanol filtrate (23 liters assaying 45.4 mg. standard/cc.) used as the charge in the chromatographic step.

Four kg. of alumina, previously adjusted to *p*H 5.0 with hydrochloric acid, washed with water and activated by drying at 200° for 4 hours, was slurried with 90% ethanol[4] into a four-inch double wall Pyrex pipe. After the alumina had settled, the bed depth was about 23 inches and the bed volume about 4 liters. The column was capped and a pressure of 10 p.s.i. applied to give a flow rate of 8 liters/hr. When most of the supernatant had drained from the column, the charge was introduced. This in turn was followed by 4 bed volumes of 90% ethanol, 10 bed volumes of 75% ethanol, 3 bed volumes of 50% ethanol and 4 bed volumes of water. The progress of the chromatography was followed easily by checking the ultraviolet absorption at regular intervals during the run. Microbiological assays *vs. K. brevis* were also performed as a final check. The chromatography is summarized in Table II.

This column run is typical of many with respect to the purity of the final products obtained and the over-all recovery. However, in many instances it was found that the 50% ethanol eluates contained considerable biological activity particularly when the 50% ethanol wash was continued through 5 bed volumes. In general, it seemed that the higher *p*H aluminas (*p*H 7–8) would tend to elute earlier giving greater amounts of activity in the 50% ethanol eluates, whereas the lower *p*H aluminas (*p*H 5.0–6.0) tended to elute later giving greater amounts of activity in the water eluates. This varied somewhat from beer to beer, but in general the pattern was as described above for a given starting material. The recovery of activity varied from lot to lot, but in many instances was more than 90% and was seldom less than 65%. It should be pointed out that the ultraviolet absorption of fraction I is rather high, that it decreases in later fractions without being accompanied by antibiotic activity and then begins to increase with the appearance of antibiotic activity. This pattern was consistent in all runs, and it is the increase in ultraviolet absorption following the minimum point which is indicative of the presence of azaserine in the filtrates. It was empirically determined that alumina column fractions which assayed about 20 or more times standard could be chromatographed

TABLE II

FRACTIONATION OF AZASERINE BY ALUMINA CHROMATOGRAPHY[a]

Fraction[b]	Assay, *K. brevis*, Optical units/cc.	Assay, *K. brevis*, mg. std./cc.	Vol., liters	Total mg. std.	% of total	mg. std./mg. prepn.
Control	...	45.4	23	1044200	100	
I	7.12	0	23	0	0	
IA	2.73	0	16	0	0	
IB_1	1.60	0	20	0	0	
IB_2	1.28	0	20	0	0	
IIA	1.50	0	4	0	0	
IIB	1.75	0	4	0	0	
IIC	1.75	0	4	0	0	
IIIA	1.45	0	2	0	0	
IIIB	4.32	8	2	16000	1.5	3
IIIC	35.0	99	2	198000	19.0	42
IIID	49.0	163	2	326000	31.2	94
IIIE	36.0	108	2	216000	20.7	113
IIIF	18.7	44	2	88000	8.4	70
IIIG	8.80	26	2	52000	5.0	49
IIIH	6.20	16	1.4	22400	2.1	36
Total				918400	87.9	

[a] This chromatography can be performed successfully with alumina adjusted from *p*H 5.0–8.0, but *p*H 5.0 alumina is most satisfactory. [b] The numbered fractions I, IA, IB_1 and IB_2, IIA-C and IIIA-H are percolates from the original charge, 90% ethanol, 75% ethanol, 50% ethanol and water, respectively.

(3) D. L. Kohberger, H. C. Reilly, G. L. Coffey, A. B. Hillegan and J. Ehrlich, paper in preparation.

(4) Nine vols. of 95% No. 3A ethanol and 1 vol. water arbitrarily has been called 90% ethanol.

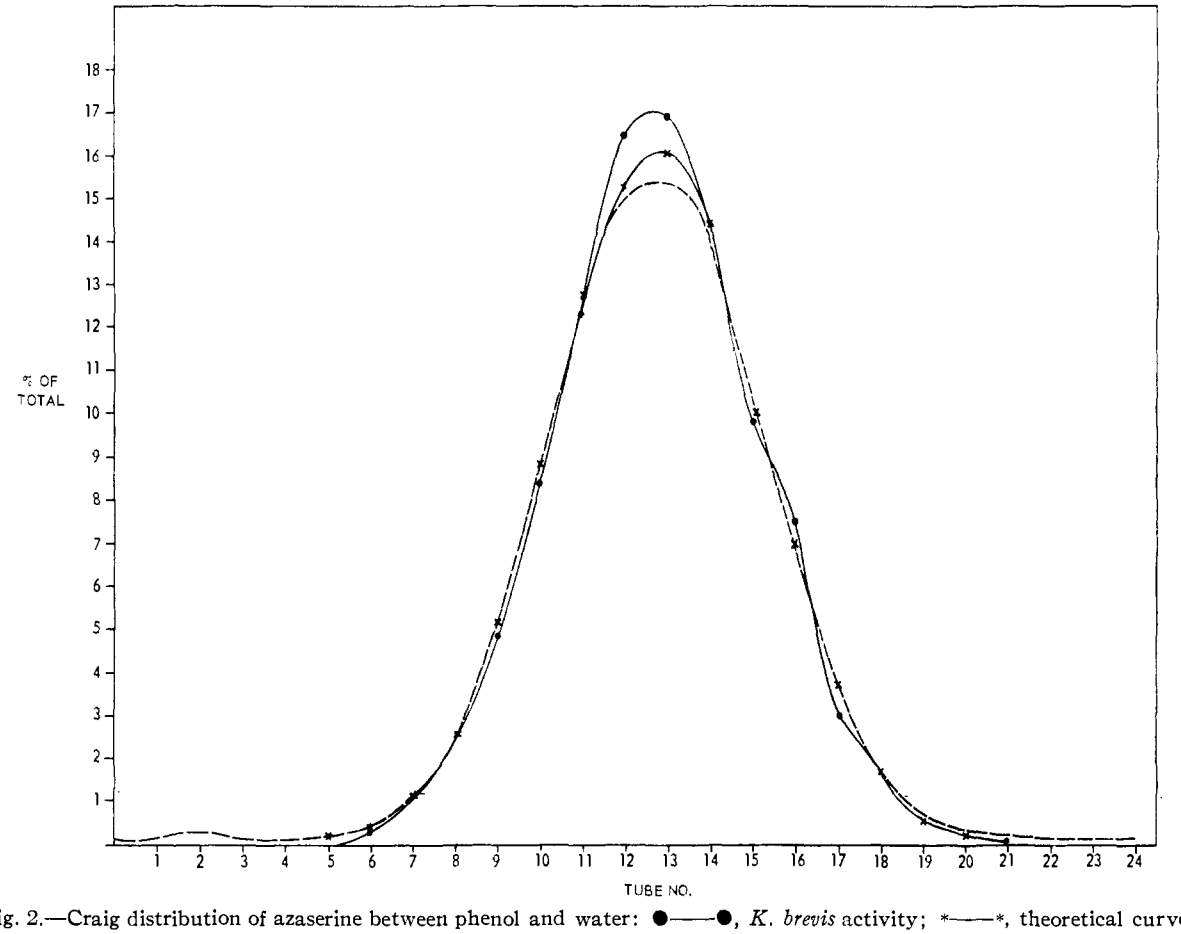

Fig. 2.—Craig distribution of azaserine between phenol and water: ●——●, *K. brevis* activity; *——*, theoretical curve; — —, extinction at λ 250 mμ.

successfully on carbon. It is evident that substantially all the recovered activity is suitable for further fractionation on carbon columns.

Carbon Column Chromatography.—Fractions IIIC–IIIG were combined and concentrated by flash evaporation in a Precision circulatory still at <35° to 110 cc. To this concentrate was added 1.73 cc. of acetone and the volume adjusted to 115 cc. with distilled water. Two cc. of the solution containing 1800 optical units/cc. was removed, and the remainder was used as the charge for the carbon column.

A thin slurry, prepared by stirring 200 g. of Darco G-60 and 200 g. of Celite 545 with water, was poured into a Pyrex glass column 2 inches wide and 5 feet high. The mixture packed to a height of 23 inches with a holdup volume of 1050 cc.

The charge was added carefully to the column, and a flow rate under gravity of 300–350 cc./hr. was attained. As soon as the charge appeared to be about 2 inches from the carbon surface, fresh 1.5% acetone was added slowly so that a sharp interface formed between the charge and the developing solution. The activity broke through after 2100 cc. (2 holdup vols.) of 1.5% acetone had passed through the column. The emergence of activity is readily recognized by the yellow-green color of the eluate. Fractions were cut as soon as the eluate contained >2 optical units/cc. This experiment is summarized in Table III.

This experiment is typical of many others, some of which have been performed with 10 kg. of carbon and 10 kg. of Celite. The recoveries have usually been 80% or more, and as much as 90% of the recovered material has been 80% pure.

This chromatography can be performed successfully in a number of ways although application of the charge to the column at concentrations of 1667–3334 optical units/cc. (5000–10000 mg. standard/cc.) in 1.0–2.0% acetone solution followed by immediate development with 1.0–2.0% aqueous acetone proved to be one of the easiest and most

TABLE III

Fraction	Optical units/cc.	Vol., cc.	Total optical units	% of starting material	$E^{1\%}_{1\text{ cm.}}$
1	53.5	45	2408	1.2	77
2	178	45	8010	3.9	266
3	353	45	15885	7.8	612
4	490	45	22050	10.8	910
5	665	90	59850	29.4	970
6	365	90	32850	16.2	995
7	204	90	18360	9.0	956
8	66	90	5940	2.9	770
9	28	90	2520	1.2	485
Total recovery			167873	82.4	

efficient ways. The charge can also be applied in much more dilute, completely aqueous solution (333–500 optical units/cc. or 1000–1500 mg. standard/cc.) followed by a slight water wash (0.3–0.5 holdup vol.) and then developed with aqueous acetone (3–5%). This method also was very successful. Both methods were definitely superior to that employing application of the charge in water at 1667–3334 optical units/cc. or 5000–10000 mg. standard/cc., followed by one or more holdup volumes of water wash, followed in turn by elution with 3–5% acetone.

Crystallization of Azaserine.—Fractions 4–7 were combined and dried from the frozen state to a light yellow-green powder weighing 1.295 g. This was dissolved by gradual addition of 250 cc. of boiling 90% ethanol. After filtration and cooling to room temperature, long light yellow-green needles of azaserine separated from solution. These were removed by filtration and dried *in vacuo;* yield 0.765 g., 70.5%; $E^{1\%}_{1\text{ cm.}}$ 1136 at λ250.5 mμ.

An analytical sample of azaserine was prepared by alter-

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.

Acknowledgments.—The authors are indebted to Dr. C. Chester Stock and associates of Sloan–Kettering Institute for Cancer Research, New York, N. Y., for many assays of filtrates and concentrates against Crocker sarcoma 180 in mice, and we wish to express our appreciation for their interest and encouragement in this work. We also wish to express our appreciation to Dr. John Ehrlich and associates for laboratory quantities of culture broths and for microbiological assays; to Dr. John M. Vandenbelt and associates for ultraviolet, infrared and pK'a determinations; to Mr. C. E. Childs and associates for microanalyses; and to Mr. Milton G. Mueller and associates for supplying pilot plant quantities of crude concentrates.

DETROIT 32, MICHIGAN

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

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

BY SALVATORE A. FUSARI, THEODORE H. HASKELL, ROGER P. FROHARDT AND QUENTIN R. BARTZ

RECEIVED JANUARY 29, 1954

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.

(2) S. A. Fusari, R. P. Frohardt, A. Ryder, T. H. Haskell, D. W. Johannessen, C. C. Elder and Q. R. Bartz, THIS JOURNAL, **76**, 2878 (1954).

(3) H. M. Randall, R. G. Fowler, N. Fuson and J. R. Dangl, "Infrared Determination of Organic Structures," D. Van Nostrand Co., Inc., New York, N. Y., 1949, p. 16.

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