[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES AND THE UPJOHN RESEARCH LABORATORIES]

The Chemistry of Amicetin, a New Antibiotic[1,2]

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The physical and chemical properties of amicetin, a new antitubercular antibiotic, have been studied. It has been shown that cytosine, *p*-aminobenzoic acid and *dextro*-α-methylserine are present in the molecule. Degradation of amicetin to *cytosamine* (IV) and to *cytimidine* (VI) is described. A structure is postulated for cytimidine and partial structures are derived for cytosamine and amicetin (III).

Amicetin is an antibiotic produced by *Streptomyces vinaceusdrappus and by Streptomyces fasciculatis*. Fermentation, extraction and purification procedures have been described.[3-5] The purpose of this paper is to discuss the chemistry of amicetin and to present a partial structure of the compound. Amicetin is a colorless, crystalline substance insoluble in water and in most organic solvents but readily soluble in 1-butanol saturated with water. It is amphoteric and dissolves in dilute acids or bases. Two crystalline forms of amicetin have been observed, differing in melting point and solubility but having similar microbiological activity. The lower melting, more soluble form has a poorly defined softening point of 165–169° and is thought to be a hydrate. The higher melting, less soluble form melts sharply at 244–245°. The two forms are interconvertible by recrystallization under appropriate conditions (see Experimental).

Elemental analyses are in agreement with the formula $C_{29}H_{44}N_6O_9$ (formula weight 620.7). Attempts to establish a molecular weight by the Rast method failed because of instability and insolubility in common Rast solvents. The molecular weight was estimated from potentiometric titrations[6] to be about 640. Group analyses indicated two N-methyls, at least three C-methyls, one primary amine and no alkoxyl.

Amicetin absorbs strongly in the ultraviolet, the intensity and position of maximum absorption varying with the *p*H of the solution. Potentiometric titration of amicetin indicated the presenee of three ionizable groups; one was acidic with a pK'_a of 10.4 and two were basic with identical pK'_a values of 7.0.[7] A fourth ionizable group with pK'_a 1.1 was indicated by ultraviolet spectral shifts with changing *p*H.

Amicetin is highly unstable in alkaline solution. Solutions of the compound at *p*H 8 and above were found to lose microbiological activity at a measurable rate. By contrast, solutions in 0.05 *N* hydrochloric acid showed no loss of activity after 24 hours.

When amicetin was allowed to stand at room temperature in dilute sodium hydroxide solution, a crystalline precipitate was deposited. This substance, which we call *cytosamine*, had the composition $C_{18}H_{32}N_4O_6$ according to the elemental analyses and the molecular weight determined by potentiometric titration. Two basic groups were found, having pK'_a values of 3.9 and 7.0. The substance contained one N-methyl group and rapidly decolorized both aqueous bromine and neutral permanganate solutions.

Further hydrolysis of cytosamine by heating with 6 *N* hydrochloric acid led to extensive decomposition. A crystalline hydrochloride was isolated from the mixture and the hydrochloride yielded the crystalline free base on treatment with dilute ammonium hydroxide. This compound, which melted at 310–312°, had the formula $C_4H_5N_3O$. It was identified as cytosine (V). Therefore the cytosamine fragment from amicetin contains cytosine and an unidentified portion, $C_{14}H_{22}NO_5$.

Hydrolysis of amicetin with 6 *N* hydrochloric acid at 75–80° gave another product as the hydrochloride. This salt was quite water soluble but yielded a highly insoluble base when treated with sodium bicarbonate. The base, which we call *cytimidine*, was recrystallized from hot ethanol and melted at 264–266°. The formula $C_{15}H_{17}N_5O_4$ was indicated by analyses. Complete hydrolysis of cytimidine with boiling 6 *N* hydrochloric acid yielded three compounds, two of which were isolated by fractional crystallization from the mixture. The least soluble of these had a melting point of 200° and analysis indicated the formula $C_7H_7NO_2 \cdot HCl$. It was identified as *p*-aminobenzoic acid hydrochloride. The second compound obtained was shown to be cytosine hydrochloride. Evaporation of the mother liquor remaining after removal of these substances gave a sirupy product which dissolved readily in ethanol. Adding aniline to the alcohol solution precipitated a crystalline material having the characteristics of an amino acid. It melted with decomposition at 235–240° and was optically active, $[\alpha]^{25}D$ +6.3°. A positive ninhydrin reaction was obtained with the substance. Analytical values agreed with the formula $C_4H_9NO_3$. Titration gave pK'_a values of 2.3 and 9.4.

(1) Presented before the Division of Medicinal Chemistry at the National Meeting of the American Chemical Society at Los Angeles, Cal., March 15–19, 1953.

(2) Inquiries may be addressed to Edwin H. Flynn, The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana.

(3) C. DeBoer, E. L. Caron and J. W. Hinman, THIS JOURNAL, **75**, 499 (1953).

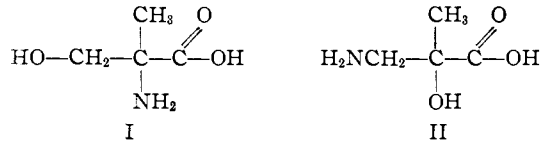
(4) J. W. Hinman, E. L. Caron and C. DeBoer, *ibid*, **75**, 5964 (1953).

(5) M. H. McCormick and M. M. Hoehn, *Antibiotics and Chemotherapy*, **3**, 718 (1953).

(6) The methods employed in estimating molecular weights by the use of titration data were described by T. V. Parke and W. W. Davis at the XIIth International Congress of Pure and Applied Chemistry, New York, N. Y., September 12, 1951.

(7) The acidic or basic natures of the groups were determined by repeating the titration in 66% dimethylformamide–water. We have observed that, in this solvent system, the pK'_a values of acidic groups are generally raised by about 0.5 to 1 *p*H unit while pK'_a values of basic groups remain unchanged from those obtained when the titration is done in water. The titration of amicetin in 66% dimethylformamide showed one group with a pK'_a of 11.1 and two with pK'_a's of 7.0. The presence of two basic groups with identical pK'_a values in the same molecule is a phenomenon we have not observed previously.

The presence of a primary amino group was confirmed by the Van Slyke amino nitrogen determination. Direct comparison with threonine, allothreonine and homoserine showed it to be different from these amino acids. A Kuhn–Roth determination gave 0.5 mole of C-methyl, indicating a branched chain. This was confirmed when the reaction with periodate was studied. One mole was consumed very rapidly and two products were isolated from the reaction mixture, as dinitrophenylhydrazones. They were identified as the derivatives of formaldehyde and pyruvic acid. These facts permit two possible structures for the amino acid, I and II. In the Van Slyke ninhydrin-CO_2

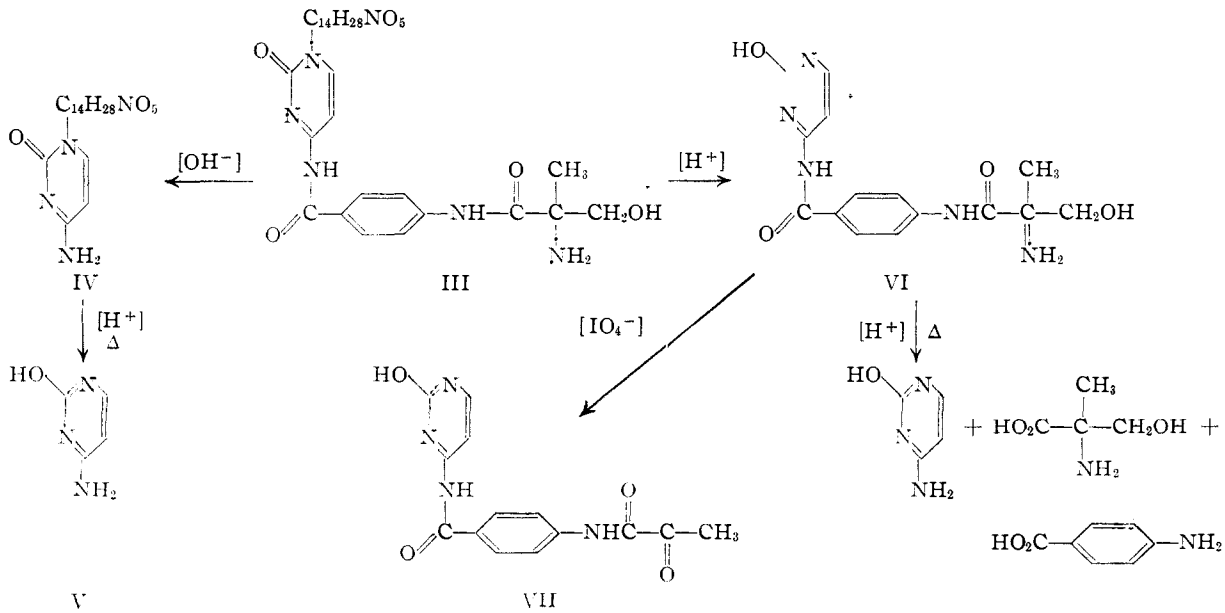


I II

reaction,[8] no CO_2 was formed. This fact favors II as the likely structure. However, it has been reported that α-methyl-α-amino acids behave abnormally in this reaction.[9] To distinguish between the two possibilities, DL-α-methylserine (I) and DL-α-methylisoserine (II) were synthesized. DL-I was prepared by the method of Billman and Parker.[10] DL-II was prepared by a slight modification of the procedure described by Fourneau.[11] These two compounds were compared with the amino acid from amicetin by determination of their dissociation constants and infrared absorption characteristics. Infrared spectra obtained from mineral oil mulls were inadequate to rule out either of the two possibilities, because of the influence of crystal structure on the infrared absorption. However, there was a definitely greater similarity between DL-I and the unknown. Potentiometric titration showed DL-I to have two pK'_a values of 2.3 and 9.4. The pK'_a values for DL-II were 2.7 and 9.15. Since the unknown amino acid gave pK'_a values of 2.3 and 9.46, it is believed that the amino acid in amicetin is *dextro*-α-methylserine (I).

Cytimidine, $C_{15}H_{17}N_5O_4$, therefore consists of three components, cytosine, *p*-aminobenzoic acid and *dextro*-α-methylserine. The linkages in this portion of the amicetin molecule were deduced from the following facts. Potentiometric titration of cytimidine indicated two ionizable groups, one acidic with a pK'_a of 9.8 and the second basic with a pK'_a of 6.9. Since no free carboxyl group was present, it was concluded that the carboxyl groups of *p*-aminobenzoic acid and *dextro*-α-methylserine are involved in amide linkages. Cytimidine gave a negative Bratton–Marshall test[12] showing that the amino group of *p*-aminobenzoic acid was bound. The compound reacted very rapidly with periodate, consuming one mole and forming formaldehyde, ammonia and a crystalline product, $C_{14}H_{12}N_4O_4$ (VII). On the basis of these facts, VI is advanced as the structure for cytimidine.

The known properties of amicetin and the structural features of cytosamine and cytimidine allow the partial structure III for amicetin. Cytosine is the only moiety common to both cytimidine and cytosamine. *p*-Aminobenzoic acid was not present in cytosamine since the Bratton–Marshall test was negative before and after acid hydrolysis. If the elements of cytosine and two hydrogen atoms are subtracted from the sum of the formulas of cytimidine and cytosamine, the formula for



amicetin, $C_{29}H_{44}N_6O_9$, is obtained. It seems apparent, then, that acid hydrolysis cleaved the bond between the cytosine moiety and the uncharacterized portion of the molecule to yield cytimidine.

(8) D. D. Van Slyke, R. T. Dillon, D. A. MacFadyen and P. Hamilton, *J. Biol. Chem.*, **141**, 627 (1941).

(9) H. N. Christensen, T. R. Riggs, H. Fischer and I. M. Palatine, *ibid.*, **198**, 2 (1952).

(10) J. Billman and E. E. Parker, [THIS JOURNAL, **67**, 1069 (1945). Dr. Billman also kindly furnished a sample prepared by him, for comparison purposes.

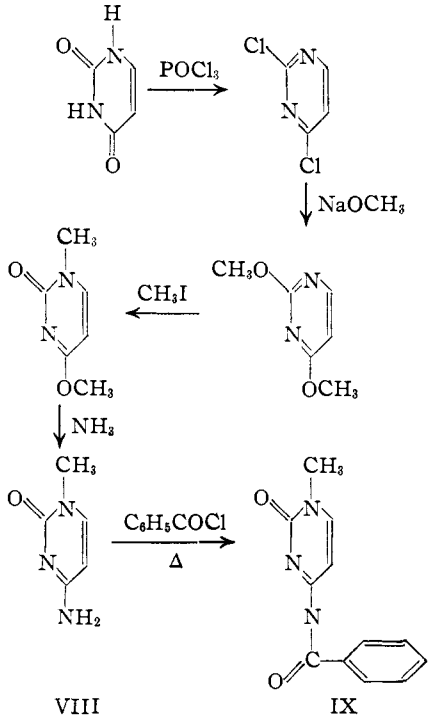
(11) E. Fourneau, *Bull. soc. chim.*, [4] **5**, 229 (1909).

(12) A. C. Bratton and E. K. Marshall, *J. Biol. Chem.*, **128**, 537 (1939).

Alkali hydrolyzed the bond between cytosine and *p*-aminobenzoic acid, yielding cytosamine (IV).

These two linkages require further consideration. A titration of the reaction mixture, obtained by mild alkaline hydrolysis of amicetin, showed that the two basic groups (pK'_a values of 7) of amicetin were unaffected but the weakly acidic group with pK'_a 10.4 disappeared. At the same time a new basic group (pK'_a 3.8) and acidic group (pK'_a 3.9) were formed. These were differentiated by titration in 66% dimethylformamide as well as in water. Cytosamine contained two basic groups with pK'_a values of 3.9 and 7.0; therefore, the remainder of the molecule must contain the acidic group and the other basic function. These changes are consistent with a hydrolysis which involves splitting of the cytosine–*p*-aminobenzoic acid linkage. The acidic, pK'_a 10.4 group destroyed in the hydrolysis is apparently the "amide" linkage between cytosine and the *p*-aminobenzoic acid moiety. The degradative changes described above are outlined in the accompanying diagram.

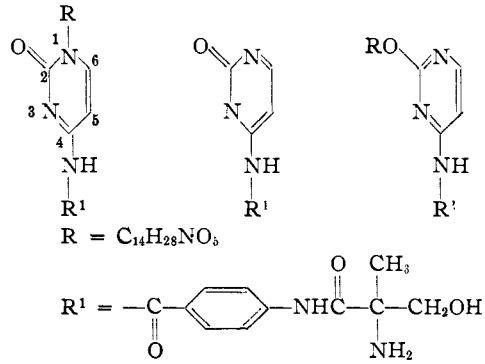
Since the acidity of the NH group of the cytosine–*p*-aminobenzoic acid linkage was unexpected, a suitable model compound was prepared to confirm this observation. 1-Methyl-2-keto-4-benzamido-1,2-dihydropyrimidine was prepared by the following reaction sequence.



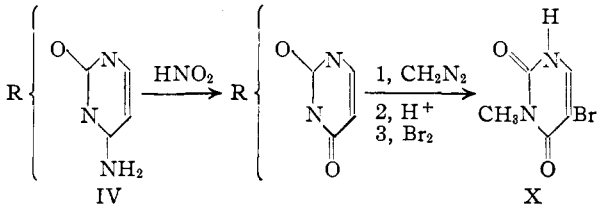
VIII IX

The preparation of 1-methylcytosine (VIII) has been described by Hilbert.[13] The above reaction sequence is simplified and utilizes the observation of Hilbert and Jansen that the 4-alkoxy group will undergo ammonolysis readily.[14] Titration of VIII showed one basic group, pK'_a 4.5. However, IX had one weakly acidic group with a pK'_a of 10.6. When allowed to stand in 0.1 *N* sodium hydroxide for 18 hours the pK'_a 10.6 group disappeared and the basic group of 1-methylcytosine and the carboxyl of benzoic acid were titratable. This behavior parallels that of amicetin.

The acid-labile bond which is hydrolyzed when cytimidine is formed is still to be considered. Because of its acid lability the three likely points of attachment to the cytosine moiety are through the ring nitrogens or the phenolic hydroxyl at 2.



Attachment at position 3 can be excluded by the following information. Cytosamine (IV) was treated with nitrous acid to remove the 4-amino group of the cytosine nucleus. The resulting uracil derivative was then treated with diazomethane, hydrolyzed, and 3-methyluracil was isolated as the 5-bromo derivative X.



IV X

R = $C_{14}H_{28}NO_5$

This degradative procedure was applied in cytidine and uridine structure studies by Levene and Tipson.[15] Attachment at position 2 would seem to be excluded by two facts. First, cytosamine does *not* reduce Fehling solution but acid hydrolysis of the substance gives a solution which does. It has been reported that O-glycosides of 2-hydroxypyrimidines are unstable to base as well as to acid and reduce Fehling solution directly.[16] It is also believed that the oxygen in the 2-position must be fixed in the keto form to provide the acidic NH group present in amicetin and in the model compound IX. Support for this belief comes from the fact that N-benzoylcytosine has only one acidic group which is attributed to the phenolic hydroxyl at position 2. It therefore seems most likely that the $C_{14}H_{28}NO_5$ grouping is attached to the 1-position of cytosine. Analogy with the naturally occurring pyrimidine nucleosides cytidine and uridine also favors this view.

Studies on the nature of the remainder of the amicetin molecule will be reported at a later date.

Acknowledgment.—The authors are grateful to Messrs. W. L. Brown, H. L. Hunter, W. J. Schenck, W. A. Struck and associates for microanalyses;

(13) G. E. Hilbert, THIS JOURNAL, **56**, 190 (1934).

(14) G. E. Hilbert and E. F. Jansen, *ibid.*, **58**, 60 (1936). *Cf.* also G. E. Hilbert and T. B. Johnson, *ibid.*, **52**, 4489 (1930).

(15) P. A. Levene and R. S. Tipson, *J. Biol. Chem.*, **104**, 385 (1934).

(16) P. A. Levene and H. Sobotka, *ibid.*, **65**, 469 (1925).

to Messrs. T. V. Parke and J. E. Stafford, Mrs. A. E. Fonkin and Drs. H. Boaz and J. L. Johnson for much of the physical chemical data; and to Dr. R. G. Jones for helpful advice and criticism.

Experimental[17]

The amicetin used in these experiments was produced by fermentation and isolated as described by C. DeBoer, *et al.*[5]

Crystalline Forms of Amicetin.—Amicetin (0.10 g. of the lower melting form) was suspended in 0.5 ml. of water and the suspension was warmed on the steam-bath. The solid dissolved almost immediately and more dense, compact crystals of the higher melting form began to appear. After a few minutes the suspension was filtered while warm, and the crystals dried *in vacuo;* 0.070 g. was obtained. Such preparations had melting points of 243–244° dec.; $[\alpha]^{25}D$ +98° (*c*, 0.8 in 0.05 *N* HCl); +116.5° (*c*, 0.5 in 0.1 *N* HCl). When the high melting form of amicetin was allowed to crystallize from water at temperatures below 35°, long, fine needles were obtained which melted indefinitely at 165–169°. This form could be reconverted to the high melting form by the procedure described above. Analytical data (on pig-dried samples) and microbiological assays were the same for both forms. Typical analytical values for amicetin and two of its salts are given below. *Amicetin.* Calcd. for $C_{29}H_{44}N_6O_9$ (mol. wt. 620.7): C, 56.11; H, 7.15; N, 13.54. Found: C, 56.04, 56.54; H, 6.86, 7.29; N, 14.11, 14.14. The samples were pig-dried for 3 hours at 120° *in vacuo* before analysis.

Amicetin helianthate was prepared by dissolving the free base in water at *p*H 5 with dilute hydrochloric acid, then adding one mole equivalent of methyl orange dissolved in the minimum amount of water. The precipitate of amicetin helianthate was recrystallized by dissolving in boiling methanol and adding water to turbidity. In a capillary the helianthate melted indefinitely at 200–210° with some prior decomposition.

Anal. Calcd. for $C_{29}H_{44}N_6O_9 \cdot 2C_{14}H_{15}N_3O_3S$: C, 55.60; H, 6.06; N, 13.65; S, 5.21. Found: C, 55.35, 55.72; H, 6.38, 6.29; N, 13.93, 13.67; S, 5.02, 5.51.

Amicetin dihydrochloride was prepared by adding two mole equivalents of dilute hydrochloric acid to an aqueous suspension of the free base, then concentrating the solution to dryness *in vacuo*. The salt was recrystallized by dissolving 1.9 g. in 10 ml. of methanol, 8 ml. of water and 2 ml. of 0.05 *N* hydrochloric acid. A total of 100 ml. of acetone was added in portions and crystals separated. When recrystallized in the same manner amicetin dihydrochloride melted at 190–192°.

Anal. Calcd. for $C_{29}H_{44}N_6O_9 \cdot 2HCl$: C, 50.21; H, 6.69; N, 12.12; Cl, 10.22. Found: C, 50.48, 50.40; H, 6.81, 6.51; N, 12.49, 12.65; Cl, 10.26, 9.54.

Cytosamine from Amicetin.—Amicetin (5.0 g.) was dissolved in 125 ml. of 0.10 *N* sodium hydroxide and the solution kept at room temperature for 24 hours. During this time crystalline cytosamine had deposited. After cooling at 5° for 15 hours the cytosamine was removed by filtration and washed with two 3-ml. portions of cold water. The product weighed 2.16 g. and softened at 160–165°, then resolidified to a different crystalline form which melted at 260° with decomposition. Cytosamine recrystallized readily from boiling water.

Anal. Calcd. for $C_{18}H_{32}N_4O_6$: C, 54.00; H, 8.05; N, 14.00. Found: C, 54.11, 54.57; H, 7.61, 7.52; N, 13.76, 13.79.

Cytosine from Cytosamine.—Cytosamine (12.5 g.) was dissolved in 250 ml. of 6 *N* hydrochloric acid and the solution was refluxed for four hours. Extensive decomposition occurred, and a black insoluble material was deposited. The solution was decanted from the insoluble residue, and 250 ml. of 1-butanol was added, forming a homogeneous solution. When 100 ml. of water was added, the water phase separated. After three extractions with water in this manner, the aqueous extracts were combined, giving a light brown solution. This was concentrated to a volume of 20 ml. under reduced pressure. A crystalline product separated and was removed after several hours at 5°. The product weighed 2.34 g. and melted indefinitely at 262–265° dec. with some prior sublimation. This hydrochloride was readily converted to the base by dissolving in 50 ml. of water and adding excess ammonium hydroxide. The precipitated base recrystallized readily from hot water and melted at 310–312° dec. It was identified as cytosine by direct comparison of its infrared and ultraviolet absorption spectra with those of authentic cytosine. X-Ray patterns were also identical as were picrate salts prepared from the unknown and cytosine.

Cytimidine from Amicetin.—A solution of 3.0 g. of amicetin in 70 ml. of 6 *N* hydrochloric acid was heated to 70° on the steam-bath. After 5 minutes the solution was removed from the heat and allowed to stand 10 minutes longer. Crystallization of cytimidine hydrochloride occurred, and was completed by keeping the mixture at 0° for 3 hours. The product weighed 1.53 g. and melted at 260–264° dec. This material was converted to the base by dissolving in 50 ml. of water and adjusting the solution to *p*H 8.3. Cytimidine precipitated from the solution. The crystalline product weighed 1.19 g. and decomposed at 255–258°. It was recrystallized from 650 ml. of boiling water and then had a decomposition point of 262–263°. There was no change on further recrystallization.

Anal. Calcd. for $C_{15}H_{17}N_5O_4$: C, 54.37; H, 5.17; N, 21.14. Found: C, 54.46; H, 5.32; N, 21.32.

Acid Hydrolysis of Cytimidine.—A solution of 5.0 g. of cytimidine hydrochloride in 500 ml. of 6 *N* hydrochloric acid was heated under gentle reflux for 15 hours. On cooling, the amber colored solution deposited colorless crystals. Refrigeration caused an additional amount of the same product to separate. A total of 1.88 g. of this material was obtained and identified as *p*-aminobenzoic acid hydrochloride on the basis of titration data (pK'_a's in water: 2.33 and 4.85), ultraviolet and infrared absorption, X-ray diffraction, paper chromatography and elemental analyses.

Anal. Calcd. for $C_7H_7O_2N \cdot HCl$: C, 48.30; H, 4.64; N, 8.07. Found: C, 48.37; H, 4.54; N, 8.15.

The mother liquor from the crystals was concentrated *in vacuo* to about one-half of its original volume. After refrigeration large colorless crystals separated. This material as isolated melted with decomposition at about 255–265°. Papergrams indicated that this product was identical with the crystalline degradation product from cytosamine, namely, cytosine hydrochloride. A total of 1.61 g. of this hydrochloride was isolated. The free base was precipitated from aqueous solution upon treatment of the salt with aqueous ammonia and was identified as cytosine by comparison with an authentic sample.

Anal. Calcd. for $C_4H_5N_3O$: C, 43.24; H, 4.54; N, 37.80. Found: C, 43.78; H, 4.66; N, 37.71.

The mother liquor from the above described products was evaporated to a small volume under reduced pressure. After removing all of the crystalline material (traces of *p*-aminobenzoic acid hydrochloride and cytosine hydrochloride), the resulting oil was dissolved in 10 ml. of 95% ethanol and treated with 30 ml. of 10% aniline in 95% ethanol. The fine, colorless crystals which formed were collected, washed with ethanol and ether and dried. The yield was 1.21 g., m.p. 235–240° with decomposition; $[\alpha]^{22.5}D$ +6.3° (*c*, 1.0 in water). This ninhydrin-positive material possessed the general properties of an amino acid. Elemental analyses indicated it to be isomeric with threonine, but infrared studies showed that it was not threonine, allothreonine or homoserine. Nitrous acid liberated all of the nitrogen but according to the usual Van Slyke ninhydrin determination there was no α-amino nitrogen present. Titration studies detected a basic group of pK'_a 9.4 and an acidic group of pK'_a 2.4. A C-methyl determination indicated the presence of one terminal methyl group. These data, together with degradation and model compound studies as given below indicated this amino acid to be *dextro*-α-methylserine.

Anal. Calcd. for $C_4H_9NO_3$: C, 40.32; H, 7.61; N, 11.76; total NH_2, 11.76; C-CH_3, 12.6. Found: C, 40.40; H, 7.59; N, 11.96; total NH_2, 11.33; C-CH_3, 7.13.

Periodate Oxidation of Cytimidine.—Titration of cytimidine with periodate showed that one mole of the reagent was consumed within 10 minutes. Two milliliters of sodium periodate solution (50 mg./ml.) was added to a solution containing 61 mg. of cytimidine hydrochloride in 10

(17) Melting points were determined with a Köfler micro melting point apparatus unless otherwise indicated.

ml. of water. After 30 minutes the microcrystalline precipitate which formed was collected, washed with water and dried to yield about 45 mg., m.p. >300° with decomposition. This product was virtually insoluble in dilute hydrochloric acid and formed a yellow 2,4-dinitrophenylhydrazone. The elemental analyses were in good agreement for pyruvyl-*p*-amino-aminobenzoylcytosine.

Anal. Calcd. for $C_{14}H_{12}N_4O_4$: C, 56.04; H, 4.03; N, 18.68. Found: C, 55.70; H, 3.95; N, 18.79.

Aqueous dimedon solution was added to the filtrate from the insoluble product and the dimedon derivative of formaldehyde was obtained and identified by melting point.

Structure of the Amino Acid from Cytimidine

A. **Periodate Oxidation.**—The isolated C_4 amino acid (238.3 mg., 2.0 m*M*) from cytimidine was dissolved in 20 ml. of water and 457 mg. (2.0 m*M*) of paraperiodic acid (H_5IO_6) was added. The solution was allowed to stand one-half hour. Previous oxidation experiments had shown the reaction to be complete in about 5 minutes. A solution of 316.5 mg. (1.0 m*M*) of barium hydroxide octahydrate in 15 ml. of water was added and the precipitate removed by filtering. The solution was diluted to 50 ml. and 25 ml. of this was distilled to dryness. The distillate was collected in a cooled receiver, then treated with a solution of 100 mg. of dinitrophenylhydrazine in 100 ml. of 5% hydrochloric acid. After 2 hours the crystalline dinitrophenylhydrazone was removed by filtration. It weighed 25 mg. and melted at 160–163° dec. After recrystallization from 2 ml. of ethanol it was compared with an authentic sample of formaldehyde 2,4-dinitrophenylhydrazone by X-ray diffraction and infrared absorption. The properties of the samples were identical.

The remainder of the oxidation solution (25 ml.) was acidified with 5.0 ml. of 0.2 *N* hydrochloric acid, and then extracted with ether for 5 hours in a liquid–liquid extraction apparatus. The ether extract was added to 100 ml. of the 2,4-dinitrophenylhydrazine reagent, the mixture shaken vigorously, and then the ether was removed by warming. The precipitated 2,4-dinitrophenylhydrazone weighed 110 mg. and melted at 219–220°. A mixed melting point with pyruvic acid 2,4-dinitrophenylhydrazone showed no depression. A comparison of the X-ray pattern and the infrared spectrum of the unknown with those obtained from pyruvic acid 2,4-dinitrophenylhydrazone showed the compounds to be identical.

DL-α-**Methylserine.**—DL-α-Methylserine was prepared by the method of Billman and Parker.[10] It was recrystallized from ethanol–water. The compound sublimed slowly on the Kofler block beginning at 220°. The sublimate melted at 268–272° dec.

DL-α-**Methylisoserine.**—DL-α-Hydroxy-β-chloroisobutyric acid,[11] 5.0 g., was dissolved in 100 ml. of concentrated ammonium hydroxide and the solution was heated in a pressure bottle on the steam-bath for 18 hours. After evaporation to dryness *in vacuo*, the residue was treated with 25 ml. of concentrated hydrochloric acid, then excess hydrochloric acid was removed under reduced pressure. The residue was extracted with three 25-ml. portions of ethanol and the insoluble portion was discarded. Aniline (5 ml.) was added to the ethanol solution to precipitate DL-α-methylisoserine. This was recrystallized from 80 ml. of water, giving 1.4 g., m.p. 280–285° dec. Two additional recrystallizations from the water–alcohol mixture gave DL-α-methylisoserine melting at 282–285° dec.

Anal. Calcd. for $C_4H_9NO_3$: N, 11.76. Found: N, 11.31.

1-Methyl-2-keto-4-amino-1,2-dihydropyrimidine.—1-Methyl-2-keto-4-methoxy-1,2-dihydropyrimidine[18] (3.0 g.) was placed in a pressure bottle and 50 ml. of methanol saturated with ammonia was added. The mixture was heated in a steam-bath for 24 hours, then cooled to 0°. The crystalline product weighed 1.65 g. and melted at 300–302° dec., unchanged by recrystallization from hot water. Hilbert[13] has reported a melting point of 303° for this compound.

1-Methyl-2-keto-4-benzamido-1,2-dihydropyrimidine.—To 5 ml. of pyridine was added 0.50 g. of 1-methyl-2-keto 4-amino-1,2-dihydropyrimidine. Benzoyl chloride (0.4 ml.) was added, with no apparent reaction. The mixture was heated on the steam-bath for 2 hours but all of the solid did not dissolve. An additional 2.0 ml. of pyridine and 0.2 ml. of benzoyl chloride were added and the mixture was brought to boiling with an open flame. The solid dissolved. After heating for one hour on the steam-bath the mixture was cooled to 0°. Crystallization occurred. The reaction mixture was poured onto 20 g. of cracked ice. The crystalline product was removed and washed with water, giving 0.37 g. of solid, m.p. 210–215°. Recrystallization from ethanol raised the melting point to 221–222°.

Anal. Calcd. for $C_{12}H_{11}N_3O_2$: C, 62.87; H, 4.84; N, 19.33. Found: C, 62.79; H, 4.79; N, 18.19.

Reaction of Cytosamine with Nitrous Acid.—Cytosamine, 10.0 g., was dissolved in 200 ml. of water by adding glacial acetic acid to *p*H 5. Ten grams of sodium nitrite was added to the solution, followed by 12.5 ml. of glacial acetic acid added dropwise, with stirring. The solution was kept at room temperature for 4 hours, and then evaporated to dryness under reduced pressure. The residue was extracted by adding 50 ml. of ethanol, warming to 60°, and then cooling to room temperature. The insoluble fraction was discarded. The ethanol extract was concentrated to dryness under reduced pressure, and the amorphous, cream colored solid was used for reaction with diazomethane as described below.

3-Methyl-5-bromouracil from Deaminated Cytosamine.—The product obtained from the nitrous acid treatment described above was dissolved in 150 ml. of methanol and a solution of diazomethane in methylene chloride was added in portions until a slight excess remained. The diazomethane was prepared from 10.0 g. of nitrosomethylurea in the usual manner. Vigorous reaction occurred as the diazomethane was added to the methanol solution. After one hour at room temperature the solvents were removed under reduced pressure. The solid was dissolved in 250 ml. of 4 *N* hydrochloric acid and the solution was refluxed for 4 hours. The dark solution was taken to dryness under reduced pressure and the residue was triturated with three 50-ml. portions of hot benzene. The benzene extract was evaporated to dryness and the product dissolved in 2 ml. of glacial acetic acid. Bromine was added dropwise until the color persisted, then the acetic acid was removed under reduced pressure. After three recrystallizations from water a constant melting point of 228–230° dec. was obtained.

Anal. Calcd. for $C_5H_5N_2O_2Br$: Br, 38.95. Found: Br, 38.70.

These values are in agreement for the expected product, 3-methyl-5-bromouracil. Johnson and Heyl[19] have reported a melting point of 228–230° for this compound.

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