THE CHEMISTRY OF THE ISOMERIC CYTIDYLIC ACIDS:
ENZYMIC HYDROLYSIS TO CYTIDINE

BY HUBERT S. LORING, MYRTLE L. HAMMELL, LUIS W. LEVY, AND HENRY W. BORTNER

(From the Department of Chemistry and the School of Medicine, Stanford University, Stanford, California)

(Received for publication, January 19, 1952)

It has been shown in previous publications that neither of the cytidylic acid isomers isolated from hydrolysates of yeast ribonucleic acid is oxidized by sodium periodate under conditions in which an unsubstituted \(\alpha,\beta\)-glycol grouping is readily oxidized (1, 2). These results indicate that each compound is phosphorylated in either the \(2'\) or the \(3'\) position and eliminate cytidine-5'-phosphate as a possibility for the structure of either isomer. The remaining possibilities for isomerism involve either a different location of the phosphate group on the same nucleoside unit (Fig. 1, a and b) or, alternately, the presence of an \(\alpha\) or \(\beta\) configuration of the ribose residue phosphorylated in the same or in different positions (Fig. 1, b and c).

In the case of the isomeric adenylic acids obtained from ribonucleic acid hydrolysates, conflicting evidence has been presented as to the reason for the isomerism. Carter obtained evidence on enzymic dephosphorylation that both isomers formed adenosine (3). In a later presentation, however, Doherty (4) found that both compounds were 3'-phosphates and that the isomerism was explained by the presence of an \(\alpha\) configuration (Fig. 1, c) in one isomer and of the \(\beta\) configuration (Fig. 1, b) in the other. It is apparent from the structures shown that dephosphorylation in one case (Fig. 1, a and b) would lead to the formation of the same nucleoside and in the other (Fig. 1, b and c) would yield different nucleosides.

Studies of the utilization of the isomeric cytidylic acids for growth of the pyrimidine-deficient Neurospora were made before and after dephosphorylation to provide evidence regarding the identity of the nucleosides concerned. Contrary to previously published reports which showed utilization of the pyrimidine nucleotides after autoclaving (5), it was now found that the phosphorylated compounds were not utilized by the deficient Neurospora mutant and that the previous results were due to dephosphorylation during autoclaving. After dephosphorylation with prostatic phosphatase, the two cytidylic acid isomers gave a product with the same growth-promoting activity for the pyrimidine-deficient Neurospora mutant 1298, in-

* Aided by grants from the Rockefeller Foundation and the Nutrition Foundation, Inc.
dicative of the formation of the same cytidine. The identity of the nucleoside formed, i.e. cytidine, was established unequivocally by following the dephosphorylation polarimetrically and finding no evidence of mutarotation and by the isolation of the same cytidine sulfate from each hydrolysate. The preparation and properties of the relatively stable lyophilized prostatic phosphatase used in the experiments mentioned above are also described.

EXPERIMENTAL

Preparation and Properties of Lyophilized Prostatic Phosphatase—The source of the acid phosphatase used in these experiments was adenomatous periurethral tissue. The procedure used for the preparation of the enzyme extracts was essentially that described by Kutscher and Worner (6), the extracts being clarified by treatment with Celite before lyophilization. The tissue (30 gm.) was homogenized in a Waring blender for about 5 minutes with 250 ml. of distilled water. The homogenate was covered with toluene and left to autolyze overnight at room temperature. After filtration through cheese-cloth to remove the larger particles, standard Filter-Cel was added and the solution filtered through a bed of Hyflo Super-Cel, giving a pale pink translucent filtrate. The water extract was then lyophilized, the product being a tan-colored powder weighing approximately 2

Fig. 1. (a) β-Ribofuranosidocytosine-2'-phosphate, (b) β-ribofuranosidocytosine-3'-phosphate, and (c) α-ribofuranosidocytosine-3'-phosphate.

1 We should like to express our thanks to Dr. Henry M. Weyrauch, Stanford Lane Hospital, San Francisco, California, who kindly provided us with the samples of tissue.
gm. Heating the dried product at 100° for 10 minutes did not affect enzyme action. The preparation was kept under refrigeration for over a year without a detectable change in activity. Other extracts were made with acetate buffer (0.01 N, pH 5) and magnesium sulfate (0.01 and 0.1 M), but the enzyme activity obtained was similar in all preparations.

The effect of pH upon the activity of this phosphatase with various substrates has been studied by earlier workers. In each case the prostatic tissue was homogenized with water, and the water extract of the enzyme used. Kutscher and Worner (6) reported different pH optima when different phosphate esters were hydrolyzed by the enzyme. With α- and β-glycerophosphate, the range was from 5.2 to 6.2, while with phenyl phosphate the values of 4.0 to 5.4 were found. Using yeast adenylic and guanylic acids as substrates, Schmidt, Cubiles, and Thannhauser (7) gave pH 5.3 as the optimum for enzyme action, while Reichard (8) reported 4.3 as the optimum pH value with yeast adenylic acid.

Because of the differences found previously, it seemed desirable to determine the optimum pH conditions for the present lyophilized enzyme preparation with the monoribonucleotides. The substrates studied were disodium uridylate ([α]D = +19°, c 1 per cent) prepared from a recrystallized sample of dibrucine uridylate ([α]D = +59°, c 1 per cent in pyridine) (9), cytidylic acid ([α]D = +50°) (2), crystalline adenylic acid, and guanylic acid, Schwarz and Company, reprecipitated ten times. The hydrolysis experiments were carried out in solutions of 0.1 M sodium acetate buffer at pH 3.5, 4.0, 4.5, 5.0, 5.3, 5.5, 6.0, and 6.5. In volumes of 25 ml., 12.5 mg. of each compound were dissolved independently with sufficient enzyme (0.125 to 0.25 mg.) to cause from 50 to 90 per cent, dephosphorylation in 6 hours at 37°. Samples were taken at 2, 4, and 6 hours and the enzyme inactivated by heating at 70° for 5 minutes. Inorganic phosphate was determined by a modified Fiske and Subbarow procedure (2).

The optimum pH value for enzyme action on all four nucleotides was found to be 5.3 for the three time periods used. Hydrolysis occurred at all pH values tested but differed in rate. Complete dephosphorylation for the substrate concentration mentioned was found at each pH value after 6 hours when the enzyme concentration was increased to 0.04 mg. per ml. The latter enzyme concentration at pH 5.3 was also found sufficient to effect complete dephosphorylation of solutions containing approximately 5 mg. of nucleotide per ml. in 3 hours. A comparison of the rates of hydrolysis on the acid and alkaline sides of the pH optimum showed a more rapid decrease in rate in the more alkaline solutions.

2 This sample was obtained from an alkaline hydrolysate of nucleic acid by elution from Dowex 2 (formate) with 1 N formic acid after separation of the cytidylic acid and adenylic acid a fractions with 0.1 N formic acid.
The absence of cytidine or cytidylic acid deaminase from the lyophilized enzyme preparations was shown by following a hydrolysis of cytidylic acid by optical density measurements over a 48 hour period. The conditions used were similar to those described for measurements of pH optimum. Optical density values at 278 m\(\mu\) remained constant, indicating no deamination to form uridylic acid (or uridine), which has a lower absorption value at this wave-length. Similar experiments with mixtures of cytidylic and uridylic acids led to the same conclusion. The absence of deaminase in certain other prostatic extracts was previously reported by Reichard (8).

**Liberation of Inorganic Phosphate from Isomeric Cytidylic Acids**—The hydrolysis rates of the two cytidylic acids, \([\alpha\] = +50° and +18°, were compared under optimum conditions for a solution containing 0.4 mg. and 0.0024 mg. per ml. of substrate and enzyme respectively. In a representative experiment, aliquots removed after 0.5, 1, 2, and 4 hours gave the following values for percentage hydrolysis for the two compounds in the order mentioned above: 23.6, 37.8, 60.3, 75.9, and 22.6, 35.6, 58.2, and 72.0. While the difference in rates of hydrolysis was slight, it was found in four similar experiments and appeared to be significant.

**Failure of Neurospora Mutants to Utilize Nucleotides**—In previous studies with autoclaved samples of purine and pyrimidine nucleotides, it was found that the purine- and pyrimidine-deficient Neurospora mutants grew in the presence of nucleotides as well as of nucleosides (5, 10). With the realization that appreciable dephosphorylation of pyrimidine nucleotides occurred on heating in slightly acid solution (2), it became desirable to study the utilization of solutions of purine and pyrimidine nucleotides that had not been autoclaved. The latter were sterilized by filtration through sterile Mandler filters. Ultraviolet absorption measurements before and after filtration indicated no change in nucleotide concentration. Aliquots of sterile solutions of the two cytidylic acids and of disodium uridylicate, \([\alpha\] = +19°, c 1 per cent, containing amounts from 0.3 to 6 mg., were added under aseptic conditions to 25 ml. of autoclaved basal medium and inoculated with the Neurospora mutant 1298 as previously described (11). As controls, similar portions of the same solutions were added to basal media and the solutions autoclaved before inoculation. No growth resulted when filtered sterile solutions of either of the two cytidylic acids or of disodium uridylicate were used as contrasted with the autoclaved solutions which gave similar amounts of growth to those found previously (10). If the phosphorylated compounds had been able to enter the conidia used for inoculation, it is likely that nucleosides would have been formed and growth would have resulted. It appears, therefore, that the pyrimidine nucleotides, like many other phosphate esters, do not readily diffuse across cell membranes.

Experiments with the purine nucleotides were carried out in a similar
manner to those with the pyrimidine compounds. The purine-deficient *Neurospora* mutant 28610 was used in the presence of 0.2 to 2.0 mg. quantities of crystalline commercial samples of yeast and muscle adenylic acids and of adenylic acid a and adenylic acid b. The results in these cases were not entirely conclusive in that small amounts of growth were obtained with each of the four compounds studied. After autoclaving, however, amounts of growth comparable to that expected from about 50 per cent of the adenine present were found for the yeast adenylic acid samples; in the case of adenosine-5'-phosphate a slightly larger amount of growth was found, indicating a greater liberation of adenine for this compound.

**Utilization of Dephosphorylated Cytidylic Acids by Neurospora Mutant 1298**—In order to compare the growth-promoting activity of the cytidines produced from the two cytidylic acids, 10 mg. of each compound were dissolved in 25 ml. of water and hydrolyzed with 2 mg. of lyophilized enzyme. Aliquots were removed for phosphate analysis and for assay with mutant 1298. The average amounts of growth found in assays conducted in triplicate with 0.4, 0.6, and 0.8 mg. of cytidylic acid, \([\alpha]_b = +50^\circ\), were 19.5, 24.2, and 29.5 mg., as compared to 18.5, 24.2, and 29.5 mg. with the same quantities of cytidylic acid, \([\alpha]_b = +18^\circ\). From these results it was clear that the growth-promoting activity of the cytidines formed by the action of prostatic phosphatase from the two cytidylic acids was identical.

**Isolation of Cytidine Sulfate after Enzymic Dephosphorylation; Cytidylic Acid, \([\alpha]_b = +50^\circ\)**—A solution containing 1.02 gm. of this isomer in 250 ml. was adjusted to pH 4.0 with ammonia and sulfuric acid and treated at room temperature for 2 hours with 100 mg. of lyophilized enzyme. Analyses for inorganic phosphate showed that hydrolysis was complete. Anions were largely removed from the solution by treating with Dowex 2 (hydroxide) until the pH was about 8. The solution was concentrated to about 10 ml. *in vacuo*, adjusted to pH 2.2 with sulfuric acid, and on further concentration and treatment with alcohol gave 0.779 gm. of crystalline product with \([\alpha]_b^{20.6} = +31.6^\circ\). It was recrystallized from about 30 ml. of water, after filtration of the solution through Hyflo Super-Cel to remove a small amount of insoluble material, by the addition of an equal volume of alcohol; yield, 0.516 gm. A 1 per cent solution in water gave \([\alpha]_b^{20.5} = +35.3^\circ\), in comparison with a commercial sample of cytidine sulfate which gave \([\alpha]_b^{25} = +35.6^\circ\), c 2 per cent in water, or with synthetic β-ribofuranosidocytosine, \([\alpha]_b^{16} = +35^\circ\), c 0.366 in 1 per cent sulfuric acid (12).

**Cytidylic Acid, \([\alpha]_b = +18^\circ\)**—A solution containing 0.774 gm. in a volume of 430 ml. at pH 5.6 was treated with 10 mg. of enzyme and, after standing

---

3 These samples were kindly provided by Dr. C. E. Carter.

4 The original sample was kindly provided by Dr. L. Laufer of the Schwarz Laboratories, Inc.
at room temperature for 12.5 hours, was treated with Dowex 2 (hydroxide) and sulfuric acid as described above. A yield of 0.516 gm. of crystalline material with $[\alpha]_b = +30.6^\circ$ was obtained, which on recrystallization gave 0.322 gm. of product with $[\alpha]_b^{24} = +35.4^\circ$, c 1 per cent in water. A comparison of the decomposition and mixed decomposition points of the cytidine sulfates from the two cytidylic acids with the twice recrystallized commercial sample showed identical behavior in each case. When placed in the bath at 228°, all the samples decomposed at 230-231° with evolution of gas.

In the experiments just described, polarimeter readings were taken during the conversion of the cytidylic acids to cytidine. The results showed that the optical activity of the two solutions, which were different initially, converged and finally gave the same value. Because an appreciable amount of time was required for hydrolysis, particularly in the case of the isomer with $[\alpha]_b = +18^\circ$, and a relatively low cytidylic acid concentration had been used, the experiments were repeated with higher enzyme and cytidylic acid concentrations, rotations being read at approximately 2 minute intervals. The solutions used contained 0.4 per cent cytidylic acid and 0.04 per cent enzyme at pH 5.8. The rotations of the two solutions with time, as plotted in Fig. 2, showed that hydrolysis proceeded smoothly with the formation of the same cytidine, as the phosphate group was removed from either nucleotide. While the results do not preclude an instantaneous isomerization of an unstable cytidine to the form whose rotation was measured, such a possibility would appear unlikely. It seems more reasonable that the same nucleoside is present in both of the cytidylic acid isomers and that the reason for isomerism is a location of the phosphate on the 2'-carbon of the ribose in one case and on the 3'-carbon in the other.

**DISCUSSION**

The fact that both cytidylic acids isolated from hydrolysates of yeast nucleic acid give the same cytidine proves that the isomerism must involve
the location of the phosphate group on the ribose side chain rather than the configuration of the 1'-carbon of the ribose. Because neither compound reduces periodate, it is also logical to conclude that in one case it is the 2' position which is esterified while in the other the phosphorylation must involve the 3'-carbon. While it is not possible at present to decide definitely which isomer is the 2'- and which the 3'-phosphate ester, a comparison of their physical properties leads to a tentative conclusion regarding their chemical structures. It can be argued a priori that the compound having the amino and phosphoric acid groups in closest proximity to each other should show the greater tendency toward zwitter ion formation with concomitant decrease in solubility, acidity, and ultraviolet absorption. Examination of Fisher, Hirschfelder, and Taylor models of the 2'- and 3'- phosphates of β-ribofuranosidocytosine reveals that the amino and phosphate groups are in closest proximity in the 2' compound. As the cytidylic acid with [α]_20° = +18° is also the least soluble, the least acidic, and shows the lowest ultraviolet absorption maximum, it seems reasonable to assign it tentatively the 2'-phosphate structure. The final conclusion, however, must await more rigorous structural proof.

SUMMARY

The preparation of relatively stable lyophilized prostatic phosphatase with a pH optimum of 5.3 for uridylic, cytidylic, adenylic, and guanylic acids, free from cytidine or cytidylic acid deaminase, is described.

The pyrimidine and purine nucleotides, in contrast to the nucleosides, are not utilized for growth of the deficient Neurospora mutants 1298 and 28610, respectively.

Several lines of evidence are presented which show that dephosphorylation of the two cytidylic acids leads to the formation of the same cytidine. The results indicate that the isomerism must involve the location of the phosphate group in the 2' position in one case and in the 3' position in the other.

BIBLIOGRAPHY

THE CHEMISTRY OF THE ISOMERIC CYTIDYLIC ACIDS: ENZYMIC HYDROLYSIS TO CYTIDINE
Hubert S. Loring, Myrtle L. Hammell, Luis W. Levy and Henry W. Bortner


Access the most updated version of this article at [http://www.jbc.org/content/196/2/821.citation](http://www.jbc.org/content/196/2/821.citation)

Alerts:
- When this article is cited
- When a correction for this article is posted

[Click here](http://www.jbc.org/content/196/2/821.citation.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/196/2/821.citation.full.html#ref-list-1](http://www.jbc.org/content/196/2/821.citation.full.html#ref-list-1)