THE METABOLISM OF 5-RIBOSYLURACIL

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SUMMARY

A dialyzed homogenate has been prepared from a pseudomonad that has been isolated from creek-mud and shown to contain enzymes which catalyze the following reactions:

5-ribosyluracil —> uridine —> uracil

Treatment of the cell-free extract with ammonium sulfate yields a fraction between 40% to 60% saturation in which only the enzymic transformation of 5-ribosyluracil —> uridine is obtained.

The enzymic reaction requires phosphate and has a broad band type of optimum at pH 6.8 ± 0.5. In contrast to known phosphorylase reactions the activity is inhibited by arsenate. The data so far obtained do not conform to that expected for either of the two pathways known for the degradation of nucleosides. A mechanism of the type catalyzed by a mutase is postulated. In this mechanism the intermediary substance as cofactor is postulated to be 3,5-diribosyluracil, an as yet unknown substance. Certain analogues such as 5-methyluracil and 5-hydroxymethyluracil strongly inhibit the enzymic reaction while others such as 5-bromouracil, 5-amino-uracil, and 5-hydroxydeoxyuridine are inert. 4-methyluracil is also inert. The influence which the carbon to carbon substituted analogues have on the enzymic reaction is strong evidence in favor of the allocation of ribose to position 5 in 5-ribosyluracil, an allocation which has been done on the basis of analogy rather than synthesis.

INTRODUCTION

A new component has been found as a minor constituent of certain ribonucleic acids[1]. This new component has been shown to be an isomer of uridine[2-4] in which the ribose is attached to position 5 of uracil rather than to position 3 and as such is termed 5-ribosyluracil. The ribosidic linkage in this case is unusual in naturally occurring nucleosides in that it is between two carbon atoms rather than between carbon and nitrogen. Such an unusual linkage opens the possibility that the metabolic pathway for the degradation of this nucleoside would be different from that of other known nucleosides. Certain aspects of the following study confirm such expectations.

Abbreviation: Tris, tris(hydroxymethyl)amino methane.

EXPERIMENTAL METHODS

Isolation of the microorganism

Samples of the soil from Strawberry Creek on the campus of the University of California, Berkeley, Calif. (U.S.A.) were suspended in 0.8 % solution of NaCl in the proportion of one part of soil to 9 parts of saline solution. The suspension was permitted to stand and sediment for 1 h at room temperature after which 1 ml of the supernatant suspension was incubated at 36° in 9 ml of the following solution: K_2HPO_4, 0.15 %; KH_2PO_4, 0.05 %; $MgSO_4 \cdot 7\ H_2O$, 0.02 %; 5-ribosyluracil, 0.1 %.

After growth of the organism became visible, aliquots of the suspension were chromatographed on paper according to techniques to be described later. The techniques chosen were such that certain chemical alterations in the structure of 5-ribosyluracil, if present, would be detected. Only one sample of mud from the many which were tried yielded an organism which produced detectable substances from the metabolism of 5-ribosyluracil. Aliquots from this sample were transferred successively in the foregoing medium until the culture was finally plated out in this medium containing 2 % agar.

The purity of the strain of the microorganism was maintained by keeping the parent culture in 0.1 % solution of 5-ribosyluracil. Organisms for seeding the bulk cultures were obtained by inoculating 10 ml of the following medium with a loopful of the suspension of the parent culture.

Difco yeast extract, 5 g; difco peptone, 5 g; glucose, 10 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; $FeSO_4 \cdot 7\ H_2O$, 0.01 g; $MnSO_4 \cdot 7\ H_2O$, 0.01 g; NaCl, 0.01 g; Distilled H_2O, 1 l.

After mixing all ingredients together, the solution was adjusted to pH 6.8 by the use of 0.1 *M* solution of K_2HPO_4. 36 h at 28° produced sufficient growth for the subsequent inoculation of 1 l of the foregoing media in a 2 l flask. By trial, it was found that the optimum time for harvesting the cells was after 36 h of incubation. At this time the cells were in the logarithmic phase of growth and the acidity of the medium had increased such that the pH decreased from 6.8 to 6.6. This was the period during which the cells displayed maximum enzymic activity in the metabolism of 5-ribosyluracil. If the cells were permitted to grow for longer periods of time such that the cells reached a stationary phase with further decrease in the pH of the medium, enzymic activity toward 5-ribosyluracil rapidly decreased.

Cells were harvested by centrifugation for 30 min at 12,000 × *g* in a Lourdes centrifuge, refrigerated at 4°. After centrifugation the cells were washed once with a solution which contained 0.5 % NaCl and 0.5 % KCl and recentrifuged. Approximately 1.5 g of cells (wet wt.) were harvested from each liter of culture.

Preparation of homogenate, supernatant solution, and enzymically active fractions from the supernatant solution

After washing, equal weights of the packed bacterial cells and washed alumina (Alcoa A-301, 325 mesh) were mixed together in a mortar previously chilled in crushed ice. The cells and alumina were ground together[5] vigorously until the thick dry paste became moist. After grinding was completed, 0.02 *M* phosphate buffer at pH 7.0, in the amount of 1 ml/200 mg wet wt. of cells, was gradually added to dilute the thick paste. The mixture was finally centrifuged twice to remove unruptured cells, cell walls,

and alumina. The first centrifugation was conducted at 16,000 × *g* for 10 min and the second at 16,000 × *g* for 20 min.

Part of the supernatant solution was used directly in the measurement of the enzymic activity of the whole cell-free extract, while the remainder was separated into three fractions by the use of ammonium sulfate at 4 to 5°, (a) 40 % saturation, (b) 60 % saturation, and (c) 80 % saturation. The precipitates which were formed on the addition of the ammonium sulfate were removed by centrifugation at 4° and 18,000 × *g* for 10 min.

The precipitates from the ammonium sulfate fractionations were dissolved in 0.002 *M* Tris buffer at pH 7.4. An amount of precipitate as obtained from 500 to 600 mg wet wt. of cells was dissolved in 1 ml of buffer and placed in cellophane dialysis tubing which was then suspended in 5 l of Tris buffer at room temperatures for 0.5 h and finally placed with constant stirring into a refrigerator at 4°. Dialysis was started in buffers at room temperature in order to allow any nucleic acids or polynucleotides which might be present in the precipitate to be fragmented into dialyzable substances and thus reduce the u.v. absorption of the enzyme solution to a minimum. Dialysis for 24 h with several changes of buffer gave satisfactory solutions free from ammonium sulfate and u.v. absorbing substances. The solutions were stored at 4° until used. The greatest enzymic activity was found in the 40–60 % ammonium sulfate fraction.

Preparation of 5-ribosyluracil

There are two published procedures for the preparation of 5-ribosyluracil, that of DAVIS AND ALLEN[1], and that of YU AND ALLEN[2]. The latter procedure takes advantage of the fact that commercial uridine as purchased from the Schwarz Laboratories, Inc., contains about 0.6 % of 5-ribosyluracil as a contaminant. It is less time consuming and hence less expensive to isolate 5-ribosyluracil from the commercial uridine as in the method of YU AND ALLEN than to isolate directly from ribonucleic acids.

Paper chromatography

Chromatograms were developed by the ascending technique on Whatman No. 1 filter paper. In all instances the *n*-butanol–water solvent of MARKAM AND SMITH[6,7] proved effective for the separation of 5-ribosyluracil from other nucleosides. Whatman No. 1 filter paper contains large quantities of u.v. absorbing materials which can be removed by layering the sheets of filter paper in 2 *N* acetic acid in a large flat container and autoclaving for 1 h at 15 lb. Finally the paper is washed several times in 95 % ethyl alcohol and dried. Photographic records of the chromatograms were made by the use of the technique of SMITH AND ALLEN[8], which is essentially that of exposing the chromatograms over Scona reflex paper to an 8 W u.v. lamp with peak emission at 2537 Å.

RESULTS

Enzymic activity of cell-free extracts

The results which were obtained in one experiment in which 500 μl of cell-free extract in phosphate buffer at pH 7.0 was incubated with 10 μ*M* of 5-ribosyluracil at 31° are shown in Figs. 1 and 2. In order to obtain Fig. 1, aliquots of the incubation mixture were removed at 5, 10, 15, 30, 45, 60, 90 and 120 min and immediately brought to 75° to stop enzymic action. Each aliquot was then applied as a small compact area

on Whatman No. 1 chromatography paper. After all aliquots were applied serially, the chromatograms were developed by submitting the paper to two separate ascending developments in butanol–water solvent. Each development required 18 h. Fig. 1 is a photograph of such a chromatogram taken under u.v. light. The areas in which the enzymic mixture was applied initially do not photograph since all u.v. absorbing material has moved out into three other areas. The substrate, 5-ribosyluracil, and the products of the reaction, uridine and uracil, have moved out in that order. Identification of the substances in the photographing areas was done by comparison of their R_F's in butanol–water and by elution from the paper and measurement of their absorption spectra in u.v. light.

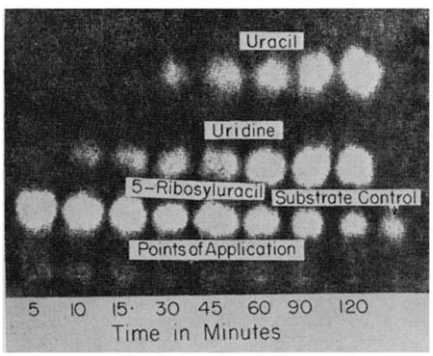

Fig. 1. Chromatogram in butanol–H_2O solvent of a rate study of the enzymic conversion of 5-ribosyluracil ⟶ uridine ⟶ uracil in a whole cell homogenate in phosphate buffer at pH 7.0. Ordinate, time in minutes. Labels which identify the u.v.-absorbing regions are placed over the areas.

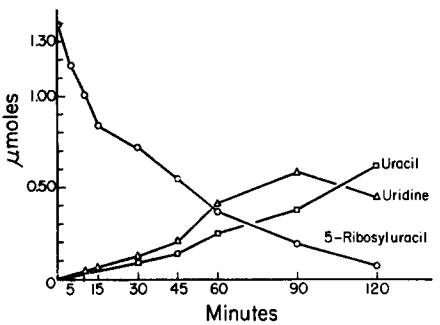

Fig. 2. Time course of the enzymic transformation of 5-ribosyluracil ⟶ uridine ⟶ uracil.

Fig. 2 was prepared from the data which were obtained by elution of the various areas on the chromatogram and measurement of the quantitative amounts of each substance at 2600 Å.

The disappearance of 5-ribosyluracil during the course of the reaction and the appearance and accumulation of uridine and uracil as shown in Figs. 1 and 2 indicate that the enzymic reactions occur in the following steps:

5-ribosyluracil ⟶ uridine ⟶ uracil

The first step in the reaction, 5-ribosyluracil ⟶ uridine has never been reported. The second step, uridine ⟶ uracil is well known. Uracil accumulates since the cofactor[9] necessary for its metabolism has been removed by dialysis.

Enzymic activity of certain fractions obtained by the use of ammonium sulfate

500 μl of each of the three ammonium sulfate fractions in phosphate buffer were incubated with 10 μM of 5-ribosyluracil at 31°. Aliquots of the incubation mixtures were taken as given in the foregoing paragraphs to obtain spectrophotometric data which showed that most of the enzymic activity was present in the fraction obtained at 40 to 60 % saturation and this showed only the first step of the reaction, viz.,

5-ribosyluracil ⟶ uridine

None of the figures that were obtained in this study are reproduced since in the interest of economy of space those figures which are included for the study of the influence of pH serve to document the foregoing statements.

The influence of pH upon the enzymic conversion of 5-ribosyluracil to uridine

Fig. 3 is the photograph of a chromatogram and Fig. 4 is the graph of the data which were obtained from the chromatogram in an experiment in which 100 μl of the 40 to 60% fraction in various phosphate buffers were incubated with 0.82 μM of 5-ribosyluracil for 1 h at 31°. The pH-activity curve which is obtained is characteristic with a broad band type of optimum at pH 6.8 $\pm$ 0.5. Inactivation below pH 6.0 or above 7.9 is rapid.

Influence of buffer components on the enzymic reaction

The 40–60% fraction from ammonium sulfate fractionation was dissolved in 0.002 M Tris buffer at pH 7.4 and exhaustively dialyzed against the same buffer.

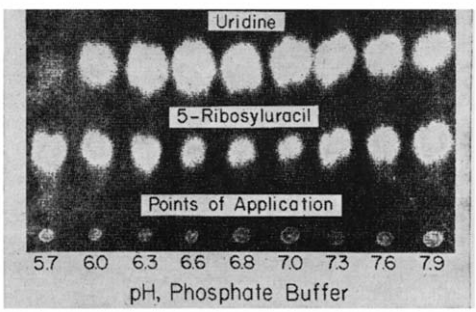

Fig. 3. Chromatogram in butanol–H_2O solvent of a study of the influence of pH on the enzymic conversion of 5-ribosyluracil $\longrightarrow$ uridine. Ordinate, pH. Labels which identify the u.v.-absorbing regions are placed over the areas.

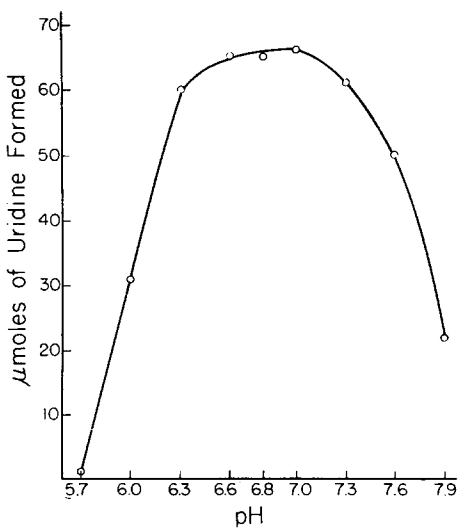

Fig. 4. pH Activity curve of the enzymic transformation of 5-ribosyluracil.

Aliquots of this enzyme-containing fraction were incubated for 2 h with 5-ribosyluracil which was dissolved in (a) Tris buffer at pH 7.4, (b) 10^{-3} *M* arsenate buffer at pH 7.4, (c) 10^{-3} *M* phosphate buffer at pH 7.4, and (d) a mixture of 10^{-3} *M* phosphate and 10^{-3} *M* arsenate buffers at pH 7.4. At the end of the incubation period samples of the mixture were withdrawn and chromatographed in butanol–water solvent. No conversion of 5-ribosyluracil ⟶ uridine could be demonstrated in either Tris buffer alone or arsenate buffer alone. When aliquots of the incubation in 10^{-3} *M* phosphate plus 10^{-3} *M* arsenate were withdrawn at 15, 30, 45, 60, 90 and 120 min intervals and chromatographed such that areas could be eluted and the amounts of 5-ribosyluracil remaining at any given time could be measured spectrophotometrically, the data given in Table I were obtained. These data show that the presence of arsenate ion in phosphate produces a lag in the reaction which results in an 80 % inhibition during the first 15 min of the reaction. After the initial lag no further inhibition in the reaction is observed.

TABLE I

COMPARISON OF ENZYMIC CONVERSION OF 5-RIBOSYLURACIL ⟶ URIDINE

A, phosphate buffer, pH 7.0; B, phosphate buffer + arsenate buffer, pH 7.0.

Time in minutes	*Control uridine μM A*	*Uridine μM B*	*% of lag*
15	0.067	0.014	79
30	0.115	0.039	66
45	0.155	0.093	41
60	0.177	0.127	28
90	0.214	0.155	27
120	0.242	0.168	30

Influence of certain 4- and 5-substituted pyrimidines on the enzymic reaction

The course of the reaction was studied in the presence of the following readily available analogues, (a) 4-methyluracil, (b) 5-bromouracil, (c) 5-hydroxymethyluracil, (d) 5-aminouridine, (e) 5-hydroxydeoxyuridine, and (f) 5-methyluracil (thymine). Prior to use in the enzymic system each of the substances was purified by chromatography in the butanol–water system. Each of the substances was mixed with 5-ribosyluracil and the active enzyme fraction in phosphate buffer at pH 7.0 and incubated for 2 h at 31°. At the end of the incubation period, aliquots were chromatographed, eluted and measured spectrophotometrically in the usual manner to obtain the data that are given in Table II. Strong inhibition of the enzymic reaction is displayed by those substances which contain a carbon to carbon linkage at position 5. Within the limits of the substances available, position 5 substitution which is not a carbon to carbon linkage such as –Br, –OH, or $-NH_2$ does not inhibit the reaction. Also substitution of a carbon to carbon linkage at position 4 as in 4-methyluracil does not inhibit the reaction. It should be noted at this point that the structure assigned to 5-ribosyluracil[2–4] is on the basis of analogy rather than synthesis. The inhibition which is observed to occur with 5-hydroxymethyluracil and 5-methyluracil is strong supporting evidence for the allocation of the ribose to position 5 in 5-ribosyluracil.

TABLE II

INFLUENCE OF CERTAIN PYRIMIDINES ON THE REACTION 5-RIBOSYLURACIL → URIDINE

	5-ribosyluracil μM	*Uridine recovered μM*	*% of inhibition*
4-methyluracil	0.450	0.442	—
5-bromouracil	0.450	0.440	—
5-hydroxymethyluracil	0.450	0.324	28
5-methyluracil	0.450	0.05	90
5-aminouridine	0.450	0.425	—
5-hydroxydeoxyuridine	0.450	0.439	—

Use of [2-^{14}C]uracil in an attempt to establish a mechanism for the enzymic reaction, 5-ribosyluracil → uridine

The foregoing data already point to the possibility that an unusual type of enzymic reaction has been found; however, it was felt necessary to conduct an experiment to decide whether or not the reaction were catalyzed by a nucleoside phosphorylase. If such were the case the transient formation of uracil could be expected to be followed by the transfer of the ribose to position 3. If radioactive uracil were to be added to the enzymatic reaction the incorporation of a small but definite percentage of the uracil into uridine could be expected.

[2-^{14}C]Uracil was purchased from Isotope Specialties Co., Burbank, California, and purified by chromatography in butanol–water solvent. After purification [2-^{14}C]-uracil in an amount containing $8 \cdot 10^5$ counts/min was mixed with 5-ribosyluracil and active enzyme fraction in phosphate buffer at pH 7.0 and incubated for 2 h at 31°. At the end of the incubation period the mixture was chromatographed in butanol–water solvent. In order to eliminate possible absorption of radioactivity the areas on the first chromatograms were eluted and rechromatographed in the same solvents. The area which contained the uridine was scanned and found to contain less than 150 counts/min. Further chromatographic purification gave no detectable radioactivity hence it can be inferred that free uracil is not an intermediate in the enzymic pathway.

DISCUSSION

At the present time the catabolism of pyrimidine nucleosides is known to occur by way of either one of two possible types of reaction, (a) an hydrolase type of reaction characterized by the addition of H_2O to the glycosidic linkage[10], or (b) a phosphorylase type of reaction characterized by the addition of phosphate to the glycosidic linkage[11].

If the enzymic hydrolysis of 5-ribosyluracil were to take place in an hydrolase type of reaction, uracil should be a direct product in the reaction. In the actual study of the reaction sequence in the breakdown of 5-ribosyluracil, uracil is formed only as a result of nucleoside phosphorylase reaction on uridine and not directly from the 5-irbosyluracil. The absolute requirement for phosphate ions also rules out known types of hydrolase reactions.

If the catabolism of 5-ribosyluracil were to follow the pathway of a phosphorylase type of reaction then both ribose-1-phosphate and uracil should be direct products of the reaction. Also arsenate could be substituted for phosphate with the expectation of the formation of ribose-1-arsenate. The enzymic transformation of 5-ribosyluracil

$\longrightarrow$ uridine is shown to have an absolute requirement for phosphate but to be inhibited by arsenate. One of the possible explanations of this apparent contradiction may be that the reaction, 5-ribosyluracil $\longrightarrow$ uridine may take place in a minimum ot two steps and that the ribose-1-phosphate which may be formed in the first step may be necessary for the second step. In the presence of arsenate, ribose-1-arsenate is formed but immediately undergoes spontaneous hydrolysis[12] to form ribose which is inactive. All attempts to separate the reaction into steps failed.

Since all of the experimental evidence fails to give unequivocal support that the enzymic action is either that of a hydrolase or a nucleoside phosphorylase an alternative may be that of a mutase type of reaction. Phosphoglucomutase is a well known mutase. The reaction catalyzed by phosphoglucomutase is given in the following equation with glucose-1,6-diphosphate as cofactor:

Glucose-1-phosphate + glucose-1,6-diphosphate $\rightleftharpoons$ glucose-1,6-diphosphate + glucose-6-phosphate

In analogy with the foregoing reaction the transformation of 5-ribosyluracil to uridine can be written tentatively in the following manner with 3,5-diribosyluracil as cofactor:

5-ribosyluracil + 3,5-diribosyluracil $\longrightarrow$ 3,5-diribosyluracil + 3-ribosyluracil

Since the enzymic reaction takes place by the use of exhaustively dialyzed extracts the presence of 3,5-diribosyluracil in the extracts can only be assumed in the event that it forms a non-dialyzable complex with the enzyme protein. The transformation as studied was irreversible.

If the reaction as studied should have the characteristics of a mutase as provisionally formulated then the reaction, 5-ribosyluracil $\longrightarrow$ uridine could be classed also as an anabolic reaction accounting for the synthesis of both uridine and 3,5-diribosyluracil which are then later incorporated into ribonucleic acids. Uridine, of course, is a well known component of ribonucleic acids but 3,5-diribosyluracil has not yet been detected as a component of nucleic acids. The u.v. absorption characteristics of 3,5-diribosyluracil should be the same as those which are observed for uridine hence means other than spectrophotometric would have to be devised for its detection. The possible occurrence of 3,5-diribosyluracil as a component of ribonucleic acids becomes of paramount importance in an understanding of the structure inasmuch as this would provide the first unambiguous data on the possibility of branching in the ribonucleic acid structure.

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NON-ENZYMIC FORMATION OF DIAZOTIZABLE AMINE FROM RIBOSYLADENINE

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SUMMARY

Adenosine is cleaved non-enzymically in the presence of ketopentose, Cu^{++}, and pyrophosphate at 70° to yield small amounts of 5-amino-1-ribosyl imidazole. A similar diazotizable amine may be produced non-enzymically from ATP under conditions found in biological experiments. The preparation and characterization of the imidazole derived from adenosine are described. Two additional adenosine derivatives which are produced during the reaction have been partially characterized as 6-amino substituted compounds.

INTRODUCTION

ATP in the presence of pentose phosphate undergoes a non-enzymic degradation which leads to the formation of small amounts of diazotizable amine. This reaction may occur as an artefact in biological experimentation and illustrates some conditions which labilize the pyrimidine moiety of purine ring systems. The production of diazotizable amine from ATP is attributable to the ketopentose content of pentose phosphate preparations and requires the presence of cupric ion. While ketopentose phosphate appeared to be the most reactive carbohydrate component, other compounds of a ketose character, such as fructose and glycol aldehyde, promote formation

Abbreviations: ATP, adenosine triphosphate; Tris tris(hydroxymethyl)aminomethane.