rated. After processing in the usual manner, the product was distilled, b.p. 132–133° (12 mm.), yield 3.2 g. (90%).

(b) *cis–trans* **Ketone.**—By a procedure similar to that above, 0.91 g. (5 mmoles) of *cis–trans*-decahydro-2-naphthoic acid was treated with methyl lithium. The methyl ketone was not distilled but used directly in the perbenzoic acid oxidation.

Perbenzoic Acid Oxidation of Ketones. (a) *cis–cis*-**2-Decalol.**—*cis–cis*-2-Acetyldecalin (2.4 g., 0.013 mole) was allowed to react with 2.6 g. of 85% perbenzoic acid (0.016 mole of peracid) in 15 ml. of chloroform for seven days at room temperature. At the end of this time, the mixture was diluted with ether and washed thoroughly with dilute sodium hydroxide solution to remove all the benzoic and perbenzoic acid. The ethereal solution was concentrated and the residue was treated with Girard Reagent T. The neutral non-ketonic fraction was saponified by refluxing (for two hours) with 20 ml. of 1 *N* aqueous sodium hydroxide in methanol. The decalol was removed by steam distillation. The steam distillate, containing solid decalol, was extracted with ether and the recovered decalol was recrystallized from hexane, m.p. 103–104°, yield 1.35 g. (66%). About 0.3 g. of oil was recovered from the foregoing crystallization liquors and was treated with phthalic anhydride at 130° for two hours. The cooled mixture was diluted with hexane but no solid phthalate ester was formed, indicating the absence of the isomeric *cis–trans*-decalol.

(b) *cis–trans*-**2-Decal**ol.—The crude *cis–trans*-2-acetyldecalin prepared above was treated with perbenzoic acid and the resulting mixture separated into a neutral and an acidic fraction. The neutral fraction, after saponification, was heated for three hours with phthalic anhydride at 135°. The ketone and any unreacted decalol was removed by ether extraction of an alkaline solution of the reaction mixture. Acidification of the alkaline solution yielded a phthalate which rapidly solidified. The solid was extracted with ether, the ether removed and the residue melts 143–149°; yield 714 mg. (45% from acid). Recrystallization from ether–hexane yielded 691 mg. (37% from acid) of *cis–trans*-2-decalyl acid phthalate, m.p. 152–153° (lit.[6] 153°).

cis-**2-Methylcyclohexane Carboxylic Acid.**—*o*-Toluic acid was hydrogenated and purified as described by Macbeth, Mills and Simmonds,[15] b.p. 126° (11 mm.), n^{20}D 1.4635 (lit.[15] b.p. 119° (11 mm.), n^{20}D 1.4644). The *p*-bromophenacyl ester melted at 81–82° (lit.[15] 81–82°).

cis-**2-Methylcyclohexylamine.**—*cis*-2-Methylcyclohexane carboxylic acid (1.42 g., 0.01 mole) was degraded by the Schmidt reaction as described previously and the crude amine was isolated as the benzamide. Recrystallization from both ethanol and ligroin gave pure material; yield 1.45 g. (68.1%), m.p. 110.5–111.5° (lit.[16] 107°).

cis-**2-Methyl-1-acetylcyclohexane.**—*cis*-2-Methylcyclohexane carboxylic acid (9.0 g., 0.064 mole) was allowed to react with 0.16 mole of methyl lithium reagent as described above. A sample of the crude ketone was converted to a semicarbazone, m.p. 182.0–182.5° (lit.[12] 182.0–182.5°). The major portion of the reaction product was distilled through an 18-inch Podbielniak spiral wire column, b.p. 71.6–72.6° (10 mm.), n^{25}D 1.4538, yield 5.5 g. (61.8%). Turner[12] reports b.p. 67–68° (10 mm.), n^{25}D 1.4532.

Hydrogenation of *cis*-2-Decalone Oxime.—The oxime (22.0 g., 0.132 mole) was hydrogenated in 75 ml. of acetic acid, 125 ml. of water and 25 ml. of concentrated hydrochloric acid over 2.0 g. of platinum oxide. The catalyst was filtered, the filtrate made alkaline and the amine isolated by steam distillation; yield 8.0 g. The recovered oxime amounted to 46%. The crude amine was benzoylated in the usual fashion and fractionally crystallized from ethanol to yield 2.6 g. (7.7%) of the amide melting from 200–202° and 3.0 g. (8.8%) of the amide melting from 124.5–126°. Hückel[6] reports 204° and 128°, respectively.

BERKELEY 4, CALIFORNIA RECEIVED SEPTEMBER 18, 1950

[CONTRIBUTION FROM THE BIOLOGY DIVISION, OAK RIDGE NATIONAL LABORATORY]

Partial Purification of a Non-phosphorylytic Uridine Nucleosidase from Yeast

BY C. E. CARTER

From yeast plasmolysates an enzyme has been partially purified by ammonium sulfate fractionation which degrades uridine to uracil and ribose in the absence of inorganic phosphate or arsenate, and which does not hydrolyze other ribosides.

The phosphorylytic cleavage of the riboside linkage in inosine and guanosine by an enzyme purified from rat liver,[1] and in several purine and pyrimidine ribo- and desoxyribonucleosides by cell-free extracts of microörganisms[2] suggests a general phosphorylytic mechanism for the enzymatic degradation of ribosides. In this paper an exception to such a generalization is reported involving an enzyme system purified from yeast by ammonium sulfate fractionation which hydrolyzes the pyrimidine riboside uridine in the absence of phosphate, arsenate or activating cations.

Experimental

Materials.—Uridine was purchased from Schwarz and Company and purified by recrystallization from 75% ethanol. The compound chromatographed as a single component and showed no contamination with uracil, cytidine or cytosine.[3]

Preparation of Enzyme.—Three pounds of Fleischmann yeast was plasmolyzed in toluene according to Kunitz.[4] To the clear plasmolysate, solid ammonium sulfate was added to a concentration of 445 g. per liter. After standing 1 hour the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against distilled water. To 350 ml. of this solution, 385 ml. of saturated ammonium sulfate was added, and after standing 1 hour, the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against distilled water with frequent changes for 48 hours. This fraction was then adjusted to *p*H 4.7 and the precipitate, collected by centrifugation, was discarded. The supernatant solution, adjusted to *p*H 7.0 with dilute sodium hydroxide contained about 60% of the original activity and represented about ten- to fifteen-fold purification when assayed as described below. It contained 0–2.5 γ P/ml. and 2.0–2.5 mg. protein/ml. All operations were conducted at 4–10°.

Enzymatic Assay.—Two methods, both based on the differential spectrophotometry of uridine and uracil solutions, were employed. In the first method, employed during fractionation procedures, where a rapid, sensitive test for enzymatic hydrolysis of uridine was required, the great difference in the extinction coefficient at 290 mμ in 0.01 *N* NaOH of uracil (E_M 5.4 × 10^3) and uridine (E_M 30) was applied.[5] In this procedure 0.1 ml. of enzyme solution, 0.025 ml. of a uridine solution containing 20 mg. of uridine/ml. and 0.2 ml. of buffer *p*H 7.0 (phosphate, borate, glycine or veronal) were incubated for 30 minutes at 38°. Four ml. of 0.01 *N* NaOH was then added and the solution read at 290 mμ in the Beckman spectrophotometer. The increment in absorption at this wave length read against a zero time blank is a measure of the uracil formed. The ratio of

(1) H. M. Kalckar, *J. Biol. Chem.*, **167**, 445 (1947).

(2) L. A. Manson and J. O. Lampen, *Fed. Proc.* (Abstract) **9**, 397 (1950).

(3) C. E. Carter, THIS JOURNAL, **72**, 1466 (1950).

(4) M. J. Kunitz, *J. Gen. Physiol.*, **29**, 393 (1947).

(5) J. T. Ploeser and H. F. Loring, *J. Biol. Chem.*, **178**, 431 (1949).

this value to the concentration of protein in the enzyme solution determined in the spectrophotometer, as described by Kalckar,[1] was used to follow the course of purification of the enzyme.

In the second procedure, used for kinetic studies of enzyme activity, the differential absorption of uridine and uracil at 280 mμ at *p*H 7.0[5] (E_M uridine 3.5×10^3; E_M uracil 1.4×10^3) was utilized. Incubation was conducted in the spectrophotometer cuvettes at 26° (constant-temperature room) under the following conditions: 0.05–0.200 ml. of enzyme solution, 0.01–0.03 ml. of uridine solution containing up to 500 μg. of uridine and 3 ml. buffer *p*H 7.0. From the extinction coefficients of uridine and uracil it can be seen that the conversion of uridine to uracil results in a 60% decrease in 280 mμ optical density at *p*H 7.0 (see Fig. 2), and by determining optical density at 280 mμ at suitable intervals the time course of the enzymatic hydrolysis of uridine may be precisely followed.

The amount of uracil formed may also be exactly determined at any time during the course of the action by adding sufficient alkali to bring the *p*H of the solution to 11.0–12.0 and measuring the increment of optical density at 290 mμ. Calculations from the data of Fig. 2 illustrate this method. The volume of the enzyme digest mixture was 3.215 ml.; the optical density at 280 mμ at *p*H 7.0, 1.00. Calculating the molarity of uridine from the molecular extinction coefficient (E_M 3.5×10^3) gives 2.88×10^{-4} *M* uridine or 227 μg. of uridine. After the enzymatic reaction had gone to completion the optical density of the solution was determined at 290 mμ following the addition of sufficient concentrated alkali to bring the *p*H of the mixture to 11.0–12.0. Absorption at this wave length in alkali represents uracil formed during enzymatic hydrolysis of uridine. The contribution of uridine to this absorption may be ignored (in theory $D = 0.008$ for a 2.88×10^{-4} *M* solution of uridine). From the experiment illustrated in curve A it was found that, following the addition of alkali to *p*H 11.4 and with the volume of solution 3.315 ml., the optical density of 290 mμ was 1.548. From the extinction coefficient of uracil at 290 mμ at *p*H 11.0–12.0 (E_M 5.4×10^3) this is equivalent to a 2.86×10^{-4} *M* solution of uracil or 106 μg. Under the above conditions the theoretical yield for complete hydrolysis is 103 μg. of uracil.

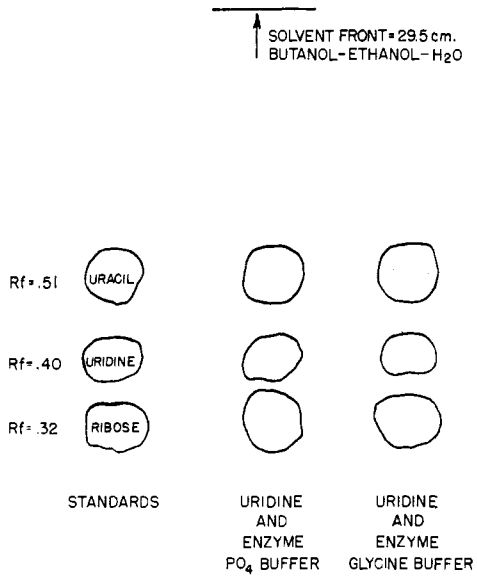

Fig. 1.—Chromatographic separation of the products of the enzymatic degradation of uridine. Solvent system consisted of a mixture of four parts butanol and one part ethanol saturated with water (shaken with an equal volume of water and the aqueous phase discarded). Ribose was detected by spraying with benzidine reagent[6] and uracil and uridine by ultraviolet fluorescence.[3] Incubation mixture consisted of 0.1 ml. enzyme, 0.1 ml. uridine solution containing 200 μg. uridine in 0.1 *M* phosphate or glycine buffer, *p*H 7.0. The mixture was chromatographed at approximately 70% splitting of uridine.

The products of the enzymatic degradation of uridine by the purified yeast enzyme were further identified by paper chromatography.[3] In the butanol–urea system[3] and in the butanol–ethanol–water system it was found, as indicated by differential spectrophotometry, that the enzyme completely hydrolyzed uridine to uracil. In these solvent systems, free ribose migrates to a spot below uridine and uracil and may be detected by any of several conventional reagents for reducing groups of sugars.[6] Employing other solvent systems[7] for sugar chromatography, a component with the distribution characteristics of ribose was found in enzyme digests and the appearance of this component was not influenced by the presence of phosphate or arsenate in the enzyme reaction mixture (Fig. 1).

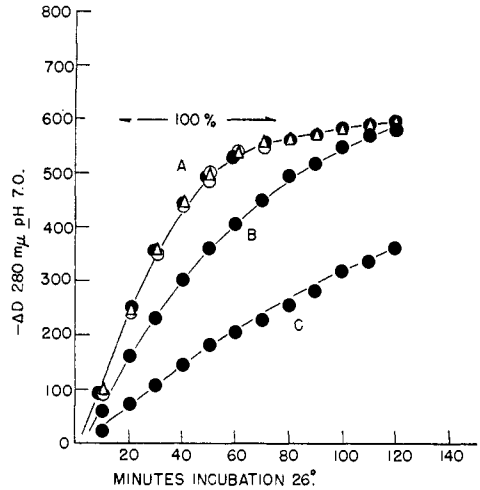

Fig. 2.—The enzymatic hydrolysis of uridine assayed by procedure 2: ●, phosphate; △, glycine; ○, arsenate buffer. Optical density at zero time, 280 mμ *p*H 7.0 1.00; at completion of enzymatic reaction (curve A) optical density 0.400.

Results

Figure 2 illustrates the course of the enzymatic degradation of 227 mg. of uridine in 3 ml. of 0.1 *M* phosphate, arsenate and glycine buffers, *p*H 7.0, plus 0.2 ml. of enzyme solution containing 2 mg. protein per ml. (curve A), 0.1 ml. enzyme (curve B) and 0.05 ml. enzyme (curve C). These data, in contrast to those obtained with the purine nucleosidase of liver described by Kalckar,[1] indicate no requirement for arsenate or phosphate by the yeast uridine nucleosidase. Similar results were obtained with borate and veronal buffers and in distilled water adjusted to neutrality with dilute alkali. The enzyme solution is virtually free of organic or inorganic phosphate (0–2.5 γ total P/ml.), hence the mechanism of the enzymatic cleavage of the riboside linkage in uridine by the yeast enzyme involves a hydrolytic rather than a phosphorolytic reaction.

The *p*H-activity curve for the uridine nucleosidase of yeast is shown in Fig. 3. In this case procedure 1 was employed for assaying enzymatic activity. A well-defined optimum at *p*H 7.0 was found in phosphate, glycine and veronal buffers.

When the data of curve A in Fig. 2 were plotted as $\log D_0/(D_0 - \Delta D/D_0)$ *vs.* time of incubation as shown in Fig. 4 it was found that the reaction fol-

(6) R. H. Horrocks, *Nature*, **164**, 444 (1949).
(7) E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 1659 (1949).

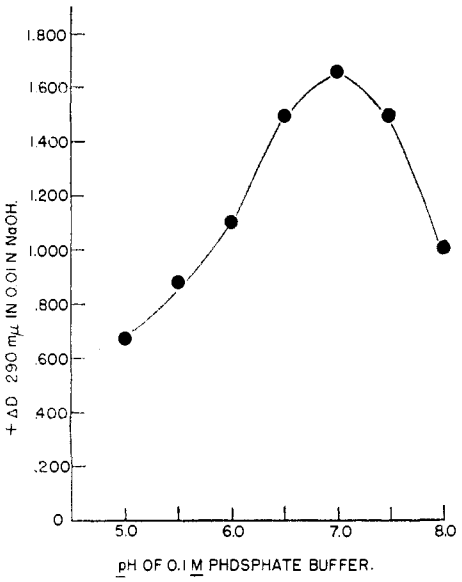

Fig. 3.—The *p*H-activity curve of yeast uridine nucleosidase in phosphate buffer.

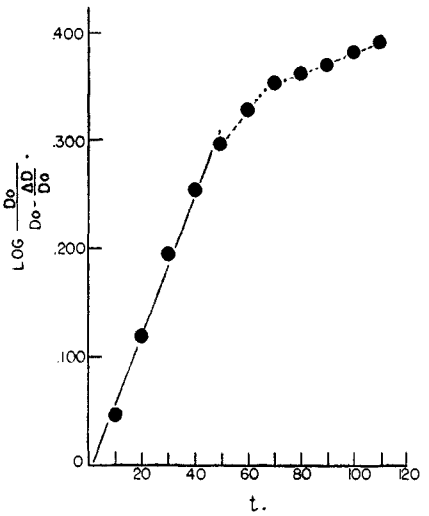

Fig. 4.—Kinetics of the enzymatic hydrolysis of uridine by yeast uridine nucleosidase. Data of curve A Fig. 2 plotted as log $D_0/(D_0 - \Delta D/D_0)$ *vs.* time.

lowed first order kinetics up to 83% hydrolysis of the substrate. The effect of products of the reaction on the rate of enzymatic hydrolysis of uridine was studied and it was found that the addition of 200 μg. of uracil to the reaction mixture produced a 27% inhibition of uridine nucleosidase activity whereas the addition of 2000 μg. of ribose produced only a 30% inhibition.

The purified yeast enzyme possesses only uridine nucleosidase activity; adenosine, inosine, guanosine, cytidine and thymidine are not degraded. As in the case of purine nucleosidase, the homologous nucleotide (uridylic acid) is not a substrate for yeast uridine nucleosidase. Uridylic acid does not act as an inhibitor of uridine nucleosidase.

Discussion

The finding of a hydrolytic pyrimidine nucleosidase in plasmolyzed yeast extracts suggests that a mechanism of synthesis and degradation of ribosides alternative to the previously described phosphorylitic nucleosidases exists in nature. The species distribution of the hydrolytic nucleosidases remains to be determined. We have been unable, however, to establish hydrolytic pyrimidine nucleosidase activity in mammalian tissues where all pyrimidine nucleosides have been found to be relatively resistant to enzymatic degradation.

The hydrolytic uridine nucleosidase of yeast may represent an instance of an isolated or anomalous pathway of riboside metabolism.

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[CONTRIBUTION FROM THE BALLISTIC RESEARCH LABORATORIES]

Catalytic Reduction with Hydrazine

BY LESTER P. KUHN

The following reactions have been found to take place in the presence of a palladium or platinum catalyst: $2RONO_2 + 2N_2H_4 \rightarrow 2ROH + N_2O + 3H_2O + 2N_2$, $2RONO + N_2H_4 \rightarrow 2ROH + N_2O + H_2O + N_2$, $2R'NO_2 + 3N_2H_4 \rightarrow 2R'NH_2 + 3N_2 + 4H_2O$, where R is an alkyl group and R′ is aryl. When methylhydrazine is used instead of hydrazine methane and ethane are formed in addition to the products shown above.

Hydrazine is a strong reducing agent and reacts rapidly with many inorganic oxidizing agents such as ceric, ferric, cupric and iodate ions. In fact the determination of hydrazine is generally effected by titration with an oxidizing agent.[1] Toward organic oxidizing agents on the other hand the effectiveness of hydrazine as a reducing agent is variable depending upon the compound to be reduced. Certain quinones,[2] tetranitromethane,[3] halogenated polynitromethanes,[3] N-chloro and N-bromo compounds[3] are readily reduced by hydrazine at room temperature. The following compounds have also been reported to be reduced by hydrazine: nitrate esters at 150–200°,[4] *m*-dinitrobenzene to *m*-nitroaniline at room temperature,[5] nitrobenzene[6] slowly at room temperature and more rapidly at elevated temperature, and various substituted nitrobenzenes[5,7] at 80–140°. In the present work no perceptible reaction was observed between nitrobenzene or dinitrobenzene and hydrazine in alcohol solution at room temperature in 18 hours.

(1) Penneman and Audrieth, *Anal. Chem.*, **20**, 1058 (1948).
(2) Dutt and Sen, *J. Chem. Soc.*, **123**, 3420 (1923); **127**, 297 (1925).
(3) Macbeth and co-workers, *ibid.*, **117**, 880 (1920); **119**, 1356 (1921); **121**, 904, 116, 2169 (1922).
(4) Walther, *J. prakt. Chem.*, **53**, 436 (1896).
(5) Bollenbach, *ibid.*, **76**, 233 (1907).
(6) Rothenburg, *Ber.*, **26**, 2060 (1883).
(7) Muller and Zimmerman, *J. prakt. Chem.*, **111**, 277 (1925).