HYDROLYSIS PRODUCTS OF NUCLEIC ACIDS LABELED WITH TRITIUM; PREPARATION BY BIOSYNTHESIS*

BY M. L. EIDINOFF, H. C. REILLY, J. E. KNOLL, AND D. H. MARRIANT

(From the Sloan-Kettering Institute for Cancer Research, New York, New York)

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Compounds labeled with C\textsuperscript{14} and N\textsuperscript{15} have proved useful in studies of nucleic acid metabolism. Since tritium, the radioactive hydrogen isotope of atomic weight 3, is now available in relatively high specific activities, it was decided to explore the usefulness of this isotope in such studies. Inasmuch as there are no data concerning the corresponding use of deuterium, it was first considered necessary to know the relative stability of the hydrogen atoms bonded to carbon with respect to isotopic exchange with an aqueous medium at varied pH and temperature. It is known that hydrogen atoms are labile when bonded to oxygen and nitrogen in --OH, --NH\textsubscript{2}, and =NH, and to carbon at a site where enolization can occur (1). The ribose portion of the nucleosides and nucleotides would be expected to retain an isotopic label under moderate reaction conditions, since 6 hydrogen atoms are linked directly to carbon. A measure of stability would be predicted for the hydrogen atoms at the 2 and 8 positions in adenine, the 8 position in guanine, and the 4 and 5 positions in cytosine and uracil, provided that the purine and pyrimidine ring structures did not introduce any unusual lability factors. In order to resolve this point, the four bases were prepared by degradation of sodium nucleate from yeast labeled with tritium. The bases retained appreciable tritium activity after undergoing treatment that would result in isotopic loss at labile atom positions.

The corresponding nucleosides were prepared from the separated nucleotides and their tritium activities measured.

The biosynthetic method used in these studies effected a multifold growth of yeast in a nutrient medium that forced the incorporation of appreciable tritium activity. The method is equally suitable for the incorporation of radiocarbon activity, thus permitting the biosynthesis of

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† Present address, Department of Radiotherapeutics, University of Cambridge, Cambridge, England.

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a large number of labeled compounds of biochemical interest by degrada-
tion of the labeled yeast.

EXPERIMENTAL

_Torulopsis utilis_ SKCC 356\(^1\) was grown in a modification of the medium of Schultz and Atkin (2), consisting of 20 gm. of sodium acetate, 20 gm. of NH\(_4\)H\(_2\)PO\(_4\), 425 mg. of KCl, 125 mg. of CaCl\(_2\)-2H\(_2\)O, 125 mg. of MgSO\(_4\)-7H\(_2\)O, 2.5 mg. of FeCl\(_3\)-6H\(_2\)O, 3.7 mg. of MnSO\(_4\)-4H\(_2\)O, and 1000 ml. of distilled water. 100 ml. portions of the medium were placed in 250 ml. Erlenmeyer flasks and sterilized at 121\(^\circ\) for 15 minutes. For inoculation, a suspension of 24 hour-old slant culture of _T. utilis_ on Sabouraud's agar was prepared in sterile tap water. Direct microscopic cell counts were made on the suspension, buds being counted as half cells. Each flask of medium was inoculated with about 1 \(\times\) 10^7 cells. The cultures were incubated at 25\(^\circ\) \pm 1\(^\circ\) on a rotary shaker for 4 days. At this time the cultures were harvested and were found to contain approximately 3 \(\times\) 10^8 cells per ml., indicating about a 3000-fold increase in cell growth. The principal source of carbon was sodium acetate containing tritium in the methyl group. The activity of the water in the growth medium was 3.5 \(\times\) 10^4 d.p.m. per \(\mu\)M of hydrogen. The activity of the sodium acetate was 1.6 \(\times\) 10^4 d.p.m. per \(\mu\)M. The labeled acetate was prepared by the high temperature equilibration of unlabeled acetic acid and water containing tritium. The latter was obtained from the Isotopes Division, United States Atomic Energy Commission, in the form of hydrogen gas and converted to water by reaction with cupric oxide at 350\(^\circ\).

The yeast was harvested by centrifuging and washed twice with iced-cold 5 per cent trichloroacetic acid solution to remove acid-soluble nucleotides (3), once with water, twice with alcohol, and once with ether. Lipides were removed by standing overnight with a mixture of chloroform-methanol (3:1) followed by gentle refluxing for 1 hour. The yeast was hydrolyzed in \(\eta\) NaOH solution (about 30 ml. per gm.) at 21\(^\circ\) for 20 hours. The solid residue was spun off, washed with a little water, and the combined supernatants brought to pH 4 by addition of ZeoRex cation exchange resin (hydrogen form). The resin together with the flocculent precipitate of protein and deoxyribonucleic acid was spun off (4), the solids washed with a little water acidified to pH 4, and the supernatants neutralized to pH 7 and separated into nucleotides by the method of Cohn (5-7). The separate nucleotides were freed from acid by evaporation to dryness at temperatures not higher than 40\(^\circ\). The nucleosides were prepared by enzymatic dephosphorylation of the corresponding separated nucleotides according

\(^1\)The authors are indebted to The Fleischmann Laboratories of Standard Brands Incorporated for supplying this culture.
to the method described by Schmidt, Cubiles, and Thannhauser (8). The free purine and pyrimidine bases were prepared by the method of Roll and Weliky (9), starting from crude sodium nucleate extracted from the dried yeast by 20 per cent aqueous sodium chloride solution at 100° for two 6 hour periods, followed by alcoholic precipitation. The concentrations of the compounds prepared by the above procedures were determined with a Beckman model DU ultraviolet spectrophotometer. Concentrations were computed with the following molar extinction coefficients at 260 m\(\mu\) and pH 2 (these values were kindly communicated to us by W. E. Cohn): adenine 12,800, adenosine 14,200, adenylic acid 13,900, guanine 8200, guanosine 11,000, guanylic acid 11,800, cytosine 6200, cytidine 6200, cytidylic acid 6800, uracil 8200, uridine 9900, uridylic acid 10,000. The identity of each compound was checked by the following ratios of the absorption at the given wave-lengths (m\(\mu\)) 250:260, 280:260, 290:260, as proposed by Cohn (6).

**Radioactivity Measurements**—Tritium activity was measured by direct counting of solid samples plated out by evaporation from appropriate solvents by the method described by Eidinoff and Knoll (10). To prepare samples for counting, 0.5 ml. of the aqueous solution was pipetted onto an aluminum planchet (7.45 sq. cm. in area) together with 0.5 ml. of methanol and evaporated with stirring under an infra-red light until only a few drops were left. An additional 0.5 ml. of methanol was added and the sample was dried with stirring. Under these conditions, fairly uniform samples can be prepared. The planchets were counted within 1 hour of preparation in a windowless gas flow counter operated in the Geiger-Müller region at 1450 volts. Sufficient counts were taken to obtain a counting precision due to statistical variation of 1 per cent for each sample. Dead time corrections were made and the background and a radioactive standard were checked before and after each series of samples. The solid active layer plated out on the sample pans had thicknesses ranging from 4 to 150 \(\gamma\) per sq. cm. The activities were compared by correcting each observed counting rate to the ideal infinitely thin condition under which no self-absorption would occur. The correction was made by measuring the absorption coefficient with very thin aluminum foil absorbers. It was observed that, up to a thickness of 0.2 mg. per sq. cm., \(I = I^0 e^{-18.7T}\) where \(I\) and \(I^0\) are the counting rates for the aluminum absorber thickness \(T\) (mg. per sq. cm.) and zero respectively. The correction factor, representing the ratio of observed to infinitely thin activities, is \((11) f = (1 - e^{-18.7T})/\sqrt{18.7T}.\)

* The details of this procedure were obtained as a private communication by these authors.
The activity of the four bases and their corresponding nucleosides is given in Table I. The retention of considerable tritium activity in the four bases relative to the specific activity of the nutrient medium demonstrates that hydrogen in the 8 position in guanine and in at least one of the two hydrogen positions linked to carbon in adenine, cytosine, and uracil is resistant to isotopic exchange. In the preparation of the free bases, there were several steps in which labile hydrogen atom positions would isotopically exchange with the medium. These include treatment with (a) 20 per cent NaCl solution at 100° for 12 hours, (b) 70 per cent perchloric acid at 100° for 45 minutes, (c) N HCl at 80° for 15 minutes, and (d) 0.01 N NaOH at room temperature for 2 to 3 days.

<table>
<thead>
<tr>
<th>Compound</th>
<th>C.p.m. per µM X 10^-3 (corrected)</th>
<th>Thickness of sample mg. per sq. cm.</th>
<th>Self-absorption factor</th>
<th>No. of samples</th>
<th>Average deviation per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>4.6</td>
<td>0.022</td>
<td>0.82</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Adenosine</td>
<td>13</td>
<td>0.125</td>
<td>0.38</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Guanine</td>
<td>2.1</td>
<td>0.050</td>
<td>0.62</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>Guanosine</td>
<td>5.9</td>
<td>0.027</td>
<td>0.78</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Cytosine</td>
<td>0.55</td>
<td>0.028</td>
<td>0.78</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Cytidine</td>
<td>8.4</td>
<td>0.013</td>
<td>0.90</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Uracil</td>
<td>0.54</td>
<td>0.0081</td>
<td>0.93</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Uridine</td>
<td>9.4</td>
<td>0.152</td>
<td>0.33</td>
<td>2</td>
<td>11</td>
</tr>
</tbody>
</table>

The relative specific activities listed in Table I are not in agreement with those calculated on the basis that the specific activity of each hydrogen linked to carbon is the same, regardless of position in the purine and pyrimidine rings and the ribose group. This discrepancy could arise from (a) differences in utilization of tritium from the water and the organic precursors derived from acetate, (b) differences in relative reaction rates involving the hydrogen isotopes of masses 1 and 3, and (c) differences in partial loss or washout of tritium during the degradation of the yeast nucleic acid. The approximately 2-fold greater activity of adenine relative to guanine is in accord with the assumption that the 2 and 8 positions in the former are each equal in activity to the 8 position in the latter compound. The activities of the nucleosides are greater than those of the corresponding bases by 8.4, 7.9, and 8.9 x 10^4 c.p.m. per µM in the case of adenine, cytosine, and uracil respectively. These figures are in accord with the reasonable assumption that the ribose groups in all the nucleosides
have an equal activity arising from a single synthetic route. On this basis, however, the specific activity of the guanosine is unaccountably low.

Although the principal objective of these studies was to demonstrate the retention of the hydrogen isotopic label in the purine and pyrimidine rings, the biosynthetic method adopted has a more general usefulness. This investigation has also demonstrated that nucleosides and nucleotides can be prepared with a tritium specific activity equal to, or 1 order of magnitude less than, the tritium activity of the nutrient medium.

If acetate containing radiocarbon had been used as the sole source of carbon in the nutrient medium, the manifold growth of yeast would yield labeled compounds whose specific activity is equal to that of the acetate. Since acetate labeled with radiocarbon in the methyl group is easily prepared in high activity from BaC\textsubscript{14}O\textsubscript{3} (12), the biosynthetic procedure should be very satisfactory for the preparation of randomly labeled compounds of equally high activity from yeast.

**SUMMARY**

1. Nucleic acid hydrolysis products were prepared from yeast grown in a nutrient medium containing tritium. Adenine, guanine, cytosine, and uracil, all containing this isotope, were prepared from the yeast sodium nucleate. Since one step in this procedure involves the treatment of the nucleate with 70 per cent perchloric acid at 100°, it is evident that hydrogen atoms in the 8 position in guanine, the 2 or 8 position (or both) in adenine, and the 4 or 5 position (or both) in cytosine and uracil have a substantial resistance to isotopic exchange in aqueous media.

2. The principal source of carbon in the nutrient medium was sodium acetate. A variety of highly radioactive compounds can be prepared by this biosynthetic method, since the growth is manifold and since acetate labeled with radiocarbon or tritium in the methyl group is readily prepared.

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