AN IMPROVED COLORIMETRIC METHOD FOR THE QUANTITATIVE ESTIMATION OF THYMINE

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The Hunter (1) procedure was originally developed for the colorimetric detection of minute amounts of thymine. In this procedure thymine is coupled under air with p-diazobenzenesulfonic acid in carbonate solution, and a red color is subsequently produced by the addition of sodium hydroxide and hydroxylamine. Thymine was determined by this means in hydrolysates of deoxyribose nucleic acids (DNA) by Woodhouse (2, 3), by Pircio and Cerecedo (4), and by Day et al. (5). The original method, however, was found to permit only semiquantitative measurements of thymine (6).

The reasons for the analytical limitations of the method were demonstrated by a study (6) of the Hunter reaction. First, it was shown that under air at 20° the coupling of thymine with the diazo reagent required several hours for completion, but that under oxygen at 30° coupling was complete in 20 minutes. In either case, however, a certain amount of coupling of the diazo reagent with both cytosine and uracil occurred during the time required for complete coupling with thymine. Second, it was shown that the red color formed by the addition of alkali and hydroxylamine was transient and that true maximum values of optical density could therefore not be determined. The hydroxylamine was shown to be largely responsible for color instability.

In the improved procedure, consequently, the coupling reaction for thymine is carried out under pure oxygen. The diazo reagent of Koessler and Hanke (7) is employed at twice the concentration used in previous procedures (1-5) to insure the complete coupling of thymine, even in the presence of cytosine and uracil. A mixture of glycerol and sodium hydroxide solution is substituted for hydroxylamine to give an intense and stable red color. The values of optical density of the colors produced with cytosine and uracil are nullified by appropriate blanks when these pyrimidines appear as contaminants in thymine solutions. This modified procedure provides a much more reliable method for the quantitative estimation of thymine.
Materials and Solutions

Oxygen—Tank oxygen, U. S. P., was employed.

Thymine, cytosine monohydrate, and uracil were each recrystallized from water three times. The thymine was further purified by sublimation twice from the lower to the upper plate of a Petri dish. The lower plate was heated slowly and uniformly to 169°, at which temperature thymine began to vaporize, and then very slowly to 200° to insure a more complete recovery. The upper plate was kept in contact with a copper beaker filled with ice water in order to provide a cold surface for condensation.

Thymine Stock Solutions—Three sets of thymine solutions were prepared. Each set ranged in concentration from about 0.005 to 0.120 mg. per ml. All solutions in the first set contained pure thymine. Each solution in the second contained, in addition to thymine, 0.1 mg. of cytosine per ml. Each solution in the third contained, in addition to thymine, 0.1 mg. per ml. each of cytosine and uracil.

Sodium Carbonate—24 gm. were made up to 2000 ml. with distilled water.

Sulfanilic Acid Monohydrate—9.0 gm. were suspended in 90 ml. of concentrated (37.4 per cent) HCl and were made up with distilled water to 1000 ml.

Sodium Nitrite—50 gm. were made up to 1000 ml. and stored under refrigeration.

p-Diazobenzenesuljonic Acid—6.0 ml. and 24 ml. of the stock solution of sodium nitrite were added to 6 ml. of the stock solution of sulfanilic acid, with an interval of 5 to 10 minutes between additions. 5 to 10 minutes later the mixture was made up to 100 ml. with cold distilled water. This diazo reagent was kept in an ice bath and used between 1 and 48 hours after preparation.

Glycerol Alkali—C.p. glycerol was mixed with an equal volume of 6 N sodium hydroxide and used immediately.

Nucleic Acids—The ribose nucleic acid (RNA) was prepared from rat liver by E. L. Grinnan of this Laboratory according to his method (8). DNA-1 and DNA-2 were sodium salts prepared in this Laboratory from calf thymus and rat thymus, respectively, by the Limperos and Mosher (9) modification of the Mirsky and Pollister method (10). DNA-3 was

1 Obtained from the Schwarz Laboratories, Inc., New York, New York.
2 Obtained from the Republic Laboratories, Chicago, Illinois.
3 Any concentration of cytosine and uracil greater than 0.08 mg. per ml. may be used, since the optical densities of colors produced with these pyrimidines remain constant above this concentration.
the sodium salt prepared from rat thymus in Dr. A. Dounce's Laboratory by the method of Kay, Simmons, and Dounce (11) and kindly supplied by them.

Proteins and Amino Acids—The gelatin was U. S. P. grade. The histone was obtained from rat thymus by the Mirsky and Pollister method (10) as a by-product in the preparation of DNA. It was further treated with 10 per cent trichloroacetic acid to remove traces of DNA. The amino acids were histidine monohydrochloride and tyrosine.

Hydrolysis tubes were of the Carius combustion type. Each had a length of 200 mm. and inside and outside diameters of 8 and 10 mm.

Procedure

Colorimetric Estimation of Thymine—A Klett tube was filled with distilled water and sealed with a serum stopper. Oxygen was introduced into the tube through one hypodermic needle and the displaced water was simultaneously removed through a second needle. 6.0 ml. of a solution, containing 2 parts of a thymine solution and 4 parts of the stock sodium carbonate solution, were then added by a calibrated hypodermic syringe, followed by 2.0 ml. of the diazo reagent. The reaction mixture was kept at 30° for 18 to 22 minutes, during which time a deep yellow color appeared. 2.0 ml. of a freshly prepared solution, containing 1 part of glycerol and 1 part of 6 N sodium hydroxide, were then added by syringe to the solution of coupled thymine and mixed by shaking. A red color developed to a maximum intensity in about 1 hour. The optical density was then measured with a Klett-Summerson colorimeter equipped with a No. 52 filter and adjusted to give a zero reading for the blank employed.

The blank was prepared with water in place of the thymine solution when the solution to be analyzed contained thymine alone, but with cytosine or cytosine and uracil when these pyrimidines appeared as contaminants in the solution to be analyzed. The cytosine and uracil solutions were each employed in concentrations of 0.1 mg. per ml.

Three separate colorimetric determinations were made for each thymine stock solution. The average optical density for each group of three determinations was plotted against the thymine concentration of the corresponding standard solution. The resulting curves for pure thymine, thymine with cytosine, and thymine with cytosine and uracil were each resolved into two straight lines intersecting at 0.0555 mg. of thymine per

4 Department of Biochemistry, School of Medicine and Dentistry, The University of Rochester, Rochester, New York.

6 Obtained from the Nutritional Biochemicals Corporation, Cleveland, Ohio.

6 Obtained from Merck and Company, Inc., Rahway, New Jersey.
ml. From these lines equations were derived which related optical density and concentration (Fig. 1).

Estimation of Thymine in Nucleic Acids—A dry, weighed sample of nucleic acid or other material was transferred to a Carius combustion tube together with 4 ml. of 9 N sulfuric acid. The tube was then sealed in a flame and the sample was hydrolyzed at 125-130° for 22 to 26 hours. After the tube was cooled and opened, the hydrolysate was transferred quantitatively to a 50 ml. centrifuge tube with 16 ml. of distilled water in 1 ml. portions.

An excess of solid silver sulfate was added to the hydrolysate in order to permit complete precipitation of silver purines. After refrigeration overnight the material was centrifuged and the supernatant fluid decanted. The precipitate was washed twice by centrifugation with a cold saturated silver sulfate solution which was 1.8 N with respect to sulfuric acid. The washings from this process, together with the original supernatant fluid, were transferred to another 50 ml. centrifuge tube. This solution, containing the pyrimidine fraction of the nucleic acid hydrolysate, was treated twice with hydrogen sulfide in order to remove the excess silver ions. After the silver sulfide precipitate had been centrifuged, the combined supernatant fluid and washings were transferred quantitatively to a 100 ml. beaker and boiled to remove the excess hydrogen sulfide.

The solution was cooled and neutralized with solid sodium carbonate.
It was then shaken twice with ether in order to remove the aldehydic breakdown products of desoxyribose. The aqueous layer was then adjusted to the desired volume, usually 10, 25, or 50 ml., and finally mixed with twice its volume of the stock sodium carbonate solution. Three 6.0 ml. aliquot portions were then taken for colorimetric analysis. The concentration of thymine in the DNA hydrolysate was calculated by means of the appropriate standard equation (Fig. 1) from the average of the three optical densities.

### Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample weight</th>
<th>Thymine added</th>
<th>Total thymine found</th>
<th>Thymine content of sample (total thymine minus added thymine) per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymine</td>
<td>0.888</td>
<td>0.891</td>
<td>100.3 ± 0.6*</td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>21.32</td>
<td>0.882</td>
<td>99.9 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>10.14</td>
<td>0.888</td>
<td>0 ± 2</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>10.41</td>
<td>1.33</td>
<td>0 ± 2</td>
<td></td>
</tr>
<tr>
<td>DNA-1</td>
<td>7.93</td>
<td>1.51</td>
<td>7.82 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>DNA-2</td>
<td>41.04</td>
<td>3.18</td>
<td>7.75 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>DNA-3</td>
<td>16.05</td>
<td>2.03</td>
<td>7.10 ± 0.06</td>
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</tr>
<tr>
<td>DNA-4</td>
<td>10.74</td>
<td>0.788</td>
<td>7.15 ± 0.06</td>
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</tr>
<tr>
<td>DNA-5</td>
<td>10.97</td>
<td>0.782</td>
<td>7.13 ± 0.06</td>
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</tr>
<tr>
<td>DNA-6</td>
<td>14.63</td>
<td>1.91</td>
<td>6.97 ± 0.06</td>
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</tr>
<tr>
<td>DNA-7</td>
<td>22.95</td>
<td>1.60</td>
<td>6.97 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>DNA-8</td>
<td>33.41</td>
<td>2.33</td>
<td>6.97 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

* These deviations include the standard deviation from the standard equation (Fig. 1) and the volume errors inherent in relating mg. of thymine per ml. to mg. of thymine per total volume of sample solution.

### RESULTS AND DISCUSSION

The improved procedure was found to be valid for the isolation of the pyrimidine fraction from DNA and for the colorimetric estimation of thymine in this fraction. The validity of this procedure was proved by the complete recovery of thymine from samples of pure thymine and from samples to which thymine had been added (Table I). Thymine could not be determined in samples contaminated with tyrosine or tyrosine-containing histone, since tyrosine, which remained as a contaminant in the pyrimidine fraction, couples with the diazo reagent (12). Thymine could be determined, however, in samples contaminated with histidine or histidine-containing gelatin, since histidine, which would also have coupled with the diazo reagent to produce color (7), had been removed as the insoluble silver salt in the extraction process.
ESTIMATION OF THYMINE

The thymine contents of representative samples of DNA (Table I) were estimated by this improved method.

SUMMARY

An improved colorimetric method has been developed for the quantitative estimation of thymine in hydrolysates of desoxypentose nucleic acids.

BIBLIOGRAPHY

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