The Determination of the Total Deoxyribose of Deoxyribonucleic Acid*

PETER SCHMID,† CHARLOTTE SCHMID, AND DONALD C. BRODIE

From the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California Medical Center, San Francisco 22, California

(Received for publication, July 23, 1962)

Although several methods (1-5) are used for the determination of deoxyribonucleic acid, none is without some limitations. For example, procedures (1) that depend on the analysis of phosphate in fractionated materials are of limited sensitivity. The use of the absorption at 260 μm as a measure of deoxyribonucleic acid (2) does not provide a procedure giving readily comparable values for different biological materials. The mole fraction, ni, of the most important bases in deoxyribonucleic acid are shown in Table I in which a calculated summated molar absorbancy index aMMD = ΣαMi, based on the molar absorbancy index αMi of each base (10), appears in the last column. A marked difference (7.9 to 9.4 × 10⁶) in the molar absorbancy index of the several materials is observed even though the more recently discovered bases are not considered. Furthermore, it is well known that the absorbancy of deoxyribonucleic acid near 260 μm in the ultraviolet depends to a great extent on the configuration of the molecule.

Analytical procedures based on the indole color reaction have been useful in studying tissue deoxyribonucleic acid. Although this method is of a high order of sensitivity, the results have often been more relative rather than absolute since the kinetics of hydrolysis and color formation have not been understood completely.

Experiences gained in the present investigation of the indole color method as applied to rabbit aorta confirmed the disparity indicated by the results of numerous investigations (11-15) and prompted a kinetic study of the method, the findings of which are being reported. From this study, some of the optimal conditions for the colorimetric determination of deoxyribonucleic acid by the indole color method have been determined which now permit a quantitative determination of total deoxyribose.

I. EXPERIMENTAL PROCEDURE

Reagents and General Methods—Monochloroacetic acid, hydrochloric acid (2.4, 4.6, and 10 M solutions), chloroform, and amyl acetate were reagent grade chemicals.

Stock solutions of deoxyribonucleosides and nucleotides (California Corporation for Biochemical Research) were prepared as follows: deoxyadenosine, 2.11 × 10⁻³ M; deoxyguanosine·H₂O, 1.83 × 10⁻³ M; deoxyctydine·HCl, 1.98 × 10⁻³ M; thymidine, 2.16 × 10⁻³ M; 2-deoxy-d-ribose, 2.30 × 10⁻³ M (pH ~ 2). The concentrations were determined through use of known molar absorbancy indices and ultraviolet absorption measurements. Salmon sperm deoxyribonucleic acid (California Corporation for Biochemical Research) was used as a standard (30 mg per liter adjusted to pH 2.5). Indole reagent (Eastman Kodak) was used as a stock solution (600 mg per liter).

Hydrolysis of nucleosides, DNA, or tissue was performed in 12-ml graduated centrifuge tubes at a designated pH. The tubes, containing 1 ml of deoxyribosyl compound and 1 ml of hydrolyzing agent, were covered with glass balls and heated in a Slaco heater, and the temperature was measured in the reagent blank solution. After an appropriate time interval, the tubes were quickly chilled in ice and 1 ml of indole reagent and 1 ml of HCl added. After development of the color at a specified temperature (see Section III), the tubes were again chilled and extracted three times with 4-ml portions of chloroform, unless otherwise stated. For each extraction, the tubes were shaken 20 times and the organic solvent removed with a capillary pipette. Absorbancies of the color were measured against a water standard. Corrections were made for the absorbancy due to the reagents. Either a Beckman model B or a Cary model 14 spectrophotometer was used (length of optical path, 1.0 cm).

Preliminary Tests—Rabbit aorta was fractionated according to the method of Schneider (16). The nucleoproteins obtained were then heated at 90° in 10% trichloroacetic acid for 15 minutes, followed by heating with indole and HCl according to Ceriotti (11).

The rate studies indicated complex kinetics for the over-all color formation. An initial fast color production was followed by a slower one, which was overlapped by a third reaction that indicated decomposition of the color. Studies with purified DNA gave results similar to those obtained with the tissue. The following reaction processes serve to contribute to the complexities of the over-all color development:

\[\text{Extracted tissue or DNA + acid hydrolysis} \rightarrow \text{degraded DNA, apurinic acid fragments, or both} \]

\[\text{Degraded DNA, apurinic acid fragments, or both } \xrightarrow{\text{indole reaction in strong mineral acid}} \text{colored compounds} \]

In addition to the above, the color is destroyed in acid solution.
Furthermore, an acid-catalyzed reaction of indole leads to the formation of a colored dimer and possibly a trimer (17). These compounds must be, and generally are extracted with solvents such as chloroform, amyl acetate, etc.

II. INFLUENCE OF HYDROGEN ION CONCENTRATION AND TIME ON HYDROLYSIS OF DNA

The time-dependent influence of hydrogen ion concentration on the rate of hydrolysis was determined as in "Experimental Procedure." To 1 ml of DNA stock solution was added 1 ml of either 10% monochloroacetic acid, 10% trichloroacetic acid, or 2.4 M HCl to yield, respectively, the final hydrogen ion concentrations listed in the legend of Fig. 1. Tubes were then heated at 90° for a period up to 120 minutes, removed at times indicated in Fig. 1, chilled, and treated to yield solutions with final concentrations of 1.2, 0.6, and 0.0 M HCl, respectively, and 0.015% indole. These solutions were then heated for 25 minutes at 100°. After two extractions with amyl acetate, the absorbancy was measured at 490 mp. Fig. 1 illustrates the influence of heating at 90° at several hydrogen ion concentrations on color production. The results indicate that with preliminary hydrolysis at a pH below 2.0, a substantial amount of the intermediate(s), which gives rise to the color, is destroyed. Under less acidic conditions (pH above 2.0) which give rise to apurinic acids and free purines, no such change can be observed. However, a significantly higher concentration of color is produced initially with 1.2 M HCl.

The above results were confirmed in experiments in which deoxyribonucleosides were heated for 80 minutes with bisulfate solution (10%, pH 2.5). The ultraviolet spectra before and after heating with bisulfate showed that deoxyguanosine and deoxyadenosine were hydrolyzed quantitatively. Under the same conditions, deoxyctydine and thymidine were hydrolyzed only to the extent of approximately 30%.

III. INFLUENCE OF HYDROGEN ION CONCENTRATION AND TEMPERATURE ON COLOR DEVELOPMENT

The influence of hydrogen ion concentration on the development of color was investigated at various temperatures. DNA stock solution, 1 ml, and 1 ml of monochloroacetic acid (final pH 2.5) were first heated for a period of 80 minutes at 90°. Subsequently, the tubes were heated with indole reagent in 0.6, 1.2, and 2.5 M HCl, respectively, at each of several temperatures as indicated in part in Fig. 2. From this figure, it can be seen that the temperature strongly influences the stability of the color produced by indole in 2.5 M HCl.

The rate of color production at 85° is not markedly lower than at 100°, although in other experiments conducted at 60°, the rate of color formation decreased considerably, and at 40°, very little color was produced within 30 minutes. With 0.6 M HCl at 85°, the color is not stable, and the color intensity is only 85% of that produced in 2.5 M HCl.

The influence of hydrogen ion concentration is therefore 2-fold. First, the glycosidic bond of the pyrimidine nucleotides that is not hydrolyzed with monochloroacetic acid must be cleaved to permit color development. The second effect is related to the stability of the color formed with indole. The experiments indicated that optimal conditions for the color development are reached at 85° and a final concentration of 2.5 M HCl.

<table>
<thead>
<tr>
<th>Source of DNA</th>
<th>Reference</th>
<th>Mole fractions</th>
<th>Average molar absorbancy index $A_{max} \times 10^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>6</td>
<td>0.202</td>
<td>0.213</td>
</tr>
<tr>
<td>Guanine</td>
<td>6</td>
<td>0.202</td>
<td>0.213</td>
</tr>
<tr>
<td>Cytosine</td>
<td>6</td>
<td>0.232</td>
<td>0.218</td>
</tr>
<tr>
<td>Thymine</td>
<td>6</td>
<td>0.235</td>
<td>0.235</td>
</tr>
</tbody>
</table>

IV. SPECTRA OF COLOR PRODUCED BY INDOLE WITH VARIOUS SUGARS

Spectra of the color produced by various sugars in the indole reaction are given in Fig. 3. Sugars were heated first with monochloroacetic acid for 80 minutes at 90°, after which time indole and HCl were added, and the solution was heated for 120 minutes at 85°. Spectra of the unextracted solutions were recorded and corrected for reagent blank. Deoxyribose, ribose, and arabinose showed the same absorption maximum at 490 mp, the spectra being qualitatively identical with those produced by DNA or RNA. However, the molar absorbancy index for the color produced by either ribose or RNA is only 2.6% of that produced by deoxyribose (Section V). In Fig. 4, the spectrum of the reagent blank is seen to have an absorption maximum at 470 mp. Three extractions with chloroform removed at least 87% of this interfering color, and three extractions of the "deoxy color" were both necessary and sufficient. If a sample was extracted up to nine times, no change in the absorbancy...
The stability of deoxyribose was tested in experiments in which the sugar was added to monochloroacetic acid (above pH 2). One set of tubes was stored at 0°, while a second set was heated up to 80 minutes at 90°. After the color had been developed with indole in 2.5 M HCl, no difference in absorbancy was observed. This was found to be true also for deoxyadenosine and deoxyguanosine. This is further evidence that all the deoxyribose is used for production of color. Fig. 5 shows the rate of color production for DNA, deoxyribosyl compounds, and deoxyribose. The data have been normalized to compare rates of formation of the color. The rate of color formation is identi-
Acid hydrolysis with monochloroacetic acid, pH 2.5, for 80 minutes with the exception of the pyrimidine derivatives for which 140 minutes of heating was necessary. Absorbancy was measured at the time of completion of color development (see Fig. 5).

<table>
<thead>
<tr>
<th></th>
<th>( A_{\text{Max}} \times 10^{-5} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxyribose</td>
<td>13.7</td>
</tr>
<tr>
<td>Deoxyadenosine</td>
<td>13.1</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>12.5</td>
</tr>
<tr>
<td>Deoxyctydine</td>
<td>12.6</td>
</tr>
<tr>
<td>Thymidine</td>
<td>12.5</td>
</tr>
<tr>
<td>Ribose</td>
<td>9.8</td>
</tr>
<tr>
<td>Arabinose</td>
<td>1.26</td>
</tr>
<tr>
<td>Adenine</td>
<td>0.39</td>
</tr>
<tr>
<td>Deoxycytidylic acid</td>
<td>0.30</td>
</tr>
</tbody>
</table>

The validity of Beer’s law was also tested. No deviation was found with concentrations up to 10 \( \mu \)g per ml of deoxyadenosine, corresponding to an absorbancy of 0.5.

Since deoxyribose is responsible for the production of the color, the molar absorbancy index for the deoxyribonucleosides and deoxyribose should be identical. Table II indicates that this is true within experimental error. The molar absorbancy index of ribose is only 2.6% of that of deoxyribose. Therefore, small amounts of RNA will not affect DNA determinations.

VI. DISCUSSION

The analysis of the reaction of indole with deoxyribose shows that the extensively modified indole method as described in this paper affords a sensitive basis for quantitative measurements of DNA as well as of deoxyribonucleotides of both purines and pyrimidines. Through the use of monochloroacetic acid at a pH above 2, a stable color is developed on the addition of indole reagent and HCl. It is well known that indole undergoes electrophilic substitution reactions with great facility and that the reaction of indole with aldehydes or ketones yields dimethylmethanes or 3-alkylideneindoles. Indeed, electrophilic substitution of indole with deoxyribose, ribose, and arabinose in acid solution yields in all three cases a spectroscopically identical chromophor system. However, there are differences in their physical properties. The chromophor system produced with deoxyribose does not partition into an organic phase such as chloroform. This is in contrast to the chromophor system produced with ribose or arabinose, which is extractable to a large extent. Furthermore, the chromophor system produced by DNA does not differ from that of deoxyribose. This may indicate that any deoxyribose 3-phosphate is hydrolyzed under the conditions used here. On the other hand, if the phosphate ester is stable, it indicates that the phosphate group does not affect the chromophor system.

Table II and Fig. 5 establish that the total deoxyribosyl content of a DNA preparation as well as of a mixture of deoxyribonucleotides and deoxyribonucleosides can be determined quantitatively. Within experimental error, the molar absorbancy index of the color produced with deoxyribose and the four deoxyribonucleosides is found to be \( 1.3 \times 10^4 \).

In a procedure similar to that described by Ceriotti (3), Park and Ullrich (19) found that their method is not generally suitable for determining total deoxyribosyl content of a mixture of deoxyribonucleotides. Webb and Levy (20) recommend a reasonably pure DNA preparation as a satisfactory reference material. Such a procedure would be recommendable assuming that deoxyribonucleotides either do not react at all or react quantitatively. Under conditions used by Park and Ullrich (19), these assumptions are not valid. Fig. 5 indicates that for relatively short periods of heating (20 minutes) where 85% of deoxyadenosine has reacted, a substantial amount of the sugar component of the pyrimidine components reacts but not quantitatively. With longer heating periods, however, deoxyribonucleotides as well as deoxyribofuranosides react quantitatively. Therefore, it is no longer necessary to isolate and purify DNA for the use as a reference material. This study has shown that commercially available deoxyadenosine is a reliable primary standard.

SUMMARY

A kinetic analysis of the reactions which led to the quantitative formation of color from deoxyribosyl compounds and indole has permitted the establishment of optimal conditions for the colorimetric determination of deoxyribonucleic acid, including the influence of pH, temperature, and the time of the rates of hydrolysis and color production.

Molar absorbancy indices for the color produced with the purine and pyrimidine nucleosides are shown to be identical. A primary standard, such as deoxyadenosine, is proposed for deoxyribonucleic acid determinations. This eliminates uncertainties due to compositional variations found in isolated deoxyribonucleic acids.

Acknowledgment—We wish to express our thanks to Professor J. J. Eiler for his interest and advice throughout the development of this work.

REFERENCES

The Determination of the Total Deoxyribose of Deoxyribonucleic Acid
Peter Schmid, Charlotte Schmid and Donald C. Brodie


Access the most updated version of this article at
http://www.jbc.org/content/238/3/1068.citation

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

Click here 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/238/3/1068.citation.full.html#ref-list-1