STUDIES ON THE DETERMINATION OF TOTAL NUCLEIC ACIDS BY ULTRAVIOLET ABSORPTION METHODS

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Ultraviolet absorption methods for the study of nucleic acids have become increasingly useful since the quantitative relationships between nucleic acids and their absorption of ultraviolet light have been shown by Caspersson (1). For example, ultraviolet measurements provide a rapid and simple means of determining the total nucleic acid (TNA) value which may be used to verify the TNA value arrived at by summing the results of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) analyses found by colorimetric methods.

Acid hydrolysis depolymerizes nucleic acids extensively and results in an increased ultraviolet absorption, the value for which is quite reproducible for the same material. The acid hydrolysis also releases purine-bound sugars for their reaction with various chromogenic reagents. Hence the colorimetric assays and determination of TNA by ultraviolet absorption may be performed on the same acid hydrolysate. Trichloroacetic acid (TCA) is commonly employed as the hydrolytic agent because it is also an excellent protein precipitant.

Recent investigations of the application of ultraviolet absorption methods to measurement of TNA in TCA hydrolysates of mammalian tissues have been reported by Tsuboi (2) and Logan et al. (3). As shown by Logan et al., TCA absorbs strongly in the region of nucleic acid absorption (255 to 265 mp) and in a range whereby its absorbancy is changing rapidly with wave length over a small wave length interval. Moreover, during the heating to accomplish the hydrolysis, TCA is partially lost, probably through decomposition.

The studies to be reported here are concerned with further investigations of ultraviolet absorption methods as applied to TCA hydrolysates of biological materials.

EXPERIMENTAL

Materials—

1. Yeast RNA, Nutritional Biochemicals Corporation, Cleveland, Ohio.

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Determination of Total Nucleic Acids

The material was purified by reprecipitation from glacial acetic acid according to the method of Kunitz (4). By analysis the RNA was 14.6 per cent N and 9.0 per cent P.

2. DNA. The material was prepared from calf thymus by the method of Hammarsten (5). By analysis the DNA was 13.7 per cent N and 7.9 per cent P.

3. Rat tissues. The livers, lungs, and spleen from young Sprague-Dawley rats were removed immediately after the animals were killed and were frozen at once on dry ice.

4. Mouse tissues. The livers from National Institutes of Health general purpose Swiss mice were treated in the same manner as the rat tissues.

5. Yeast. Bakers' yeast (Fleischmann), dried in vacuo, was used.

6. Bacteria. 17 hour cultures of Escherichia coli, grown at 37° on horse meat infusion agar, were harvested by washing the nutrient medium with 0.85 per cent saline. The bacteria were recovered by centrifuging the saline wash for 10 minutes at 20,000 × g in a Servall SS-1 centrifuge, the bacteria being washed twice with distilled water, and being dried in vacuo.

The tissues and microorganisms were prepared for these studies by being washed with cold 10 per cent TCA, defatted with alcohol and ether, and finally converted to a dry powder as described by Webb and Levy (6).

Methods

Hydrolysis—In a manner previously described (6), the tissue powders were heated in a boiling water bath for 30 minutes with 5 per cent TCA, and then diluted with an equal volume of 5 per cent TCA (not heated). A blank solution was prepared in a similar manner by heating a like volume of 5 per cent TCA as that used for the powder hydrolysis, followed by dilution with an equal volume of 5 per cent TCA. 1 ml. of the diluted hydrolysates was equivalent to approximately 100 γ of TNA.

Ultraviolet Absorption Measurements—1 ml. aliquots of the diluted hydrolysates and 1 ml. of the blank solution were transferred to 5 ml. volumetric flasks and diluted to volume with distilled water. Optical densities were measured against the blank solution with a Beckman spectrophotometer (model DU) by using 1 cm. quartz cells.

In some experiments, to be described later, the ultraviolet absorbing components were separated from the TCA media before measurement in the ultraviolet region in the following manner.

A 1 ml. aliquot of the diluted tissue hydrolysate was transferred to a 12 ml. centrifuge tube and the contents were neutralized with 2 drops of NH₄OH (28.5 per cent). 1 ml. of the TCA blank solution was treated in a like manner. The tubes were heated for 15 minutes in a boiling water bath to expel excess NH₃ and then cooled in an ice bath to 0-5°. 1 ml. of cold 2 per cent AgNO₃ solution was added to each tube, the contents
were mixed, and the tubes allowed to stand for 15 minutes in an ice bath. The tubes were centrifuged and the supernatant solutions discarded and the residues washed once with cold 1 per cent AgNO₃ and recovered by centrifugation. 4 ml. of 0.1 N HCl were added to each tube and the tubes heated for 10 minutes in a boiling water bath. During the heating period the mouths of the tubes were covered with sealed ampul bulbs to minimize loss by evaporation which, with a 4 ml. volume, did not exceed 0.1 ml. The tubes were cooled, centrifuged, and 2 ml. aliquots of the supernatant solutions transferred to 5 ml. volumetric flasks and diluted to volume with distilled water for measurement.

Results

Ultraviolet Absorption Characteristics of Tissues and Microorganisms; Effect of Time of Hydrolysis—The maximal ultraviolet extinction values for extracts resulting from 15, 30, and 60 minute hydrolyses of powders of several rat tissues and microorganisms were determined. For all the materials examined, there was no significant variation in the extinction values, nor shift in the wave length maxima, for extracts of the same powder which resulted from 15 minutes hydrolysis compared to that resulting from as long as 1 hour hydrolysis. The maxima for all tissues and microorganism extracts, with the exception of yeast, were approximately 265 mμ. The yeast extract (predominantly RNA) showed a maximum at 263 mμ.

In a second experiment in which the addition of extra TCA after 30 minutes hydrolysis of the powders was omitted, the maxima observed were higher, in some instances, ranging from 269 to 272 mμ. This shift in the absorption maxima towards the higher wave lengths was probably due to incomplete precipitation of protein material in the hydrolysates because the TCA concentration is partially lowered under conditions of the hydrolysis (3).

Ultraviolet Absorption Characteristics of RNA and DNA Hydrolyzed with TCA—The wave lengths of maximal absorption and extinction values for purified DNA, purified RNA, and for a 1:1 DNA-RNA mixture hydrolyzed for 30 minutes in TCA were determined. The maximum for DNA was 267 mμ and for RNA approximately 260 mμ, whereas the 1:1 DNA-RNA solution showed a maximum at 265 mμ. Next, the ultraviolet-absorbing characteristics of a synthetic solution, in TCA, of products which would result from the hydrolysis of the 1:1 DNA-RNA mixture, as calculated from the tetrancleotide formula, were studied. The synthetic solution, measured in a final concentration of 1.6 × 10⁻⁵ M for adenine, guanine, and cytidylic acid and of 8 × 10⁻⁶ M for thymidylic and uridylic

1 The materials contained in the solution may be obtained from the California Foundation for Biochemical Research, Los Angeles, California.
acids, showed a maximum from 265 to 268 μm. Measurement was made against a TCA blank solution treated in the same manner as the synthetic solution with respect to heat and dilution. The optical density (1 cm. light path) of a 0.002 per cent solution of the hydrolyzed 1:1 DNA-RNA mixture was 0.520. This solution, in terms of the tetranucleotide formula, was equivalent in ultraviolet-absorbing components to the finally measured synthetic solution. The optical density reading (0.520) was 90 per cent of that found for the synthetic solution at 265 μm, which per cent corresponded well with the per cent purity of the DNA and RNA in the mixture according to phosphorus analysis (88 per cent).

A further experiment confirmed the observations of others (2, 3) that neutralizing the TCA solutions to a pH of 7 with phosphate buffer shifted the absorption maxima of both DNA and RNA to lower wave lengths (approximately 260 and 258 μm, respectively), with no significant change in the maximal ultraviolet extinction values. Routinely, nothing was gained by measurement at 258 or 260 μm, since TCA had a greater absorption at these lower wave lengths.

Effect of TCA Ultraviolet Absorption on Spectrophotometric Measurements—Although the absorption of TCA in the working range was appreciable, it was possible to correct for it at 265 μm, as shown in the following manner. Various dilutions of a 1:1 DNA-RNA homogenate in water were hydrolyzed in a 5 per cent TCA medium and, after dilution with 5 per cent TCA (see under “Methods”), their optical densities measured against the blank TCA solution. Duplicate determinations were made at each concentration level. The resulting direct proportionality between absorption and nucleic acid concentration over the range 1.25 to 20 γ per ml. (finally measured solution) is shown in Fig. 1.

Some measurements were also made against distilled water. By this procedure it was determined that the average of the optical density readings of the blank solutions was 70 per cent of that found for the lowest concentration (1.25 γ per ml.) of the DNA-RNA solutions.

In order to investigate further the reliability of the ultraviolet measurements when TCA was used as a solvent, dilutions of a hydrolyzed 1:1 DNA-RNA solution were treated with AgNO₃ and the isolated nucleic acid components redissolved with HCl and measured as described under “Methods.” The linear optical density-concentration plot obtained was identical with that for the TCA solutions measured over the same range of concentrations.

To demonstrate the quantitative relationships of the AgNO₃ precipitation procedure and to show that the theoretical ultraviolet-absorbing components were precipitated, the procedure was applied to a synthetic solution (previously described) of the components in TCA. Components
in 1 ml. of TCA solution equivalent to approximately 300 μ of nucleic acids were recovered 98 to 100 per cent (three determinations) according to ultraviolet extinction values in TCA, and in HCl solutions after AgNO₃ precipitation and dissolution of the residue.

![Graph showing the relationship between optical density and TNA concentration.](image)

Fig. 1. Relationship between the optical density and the amount of TNA as obtained by ultraviolet absorption measurements at 265 μ. The abscissa represents amounts of TNA contained in 1 ml. of the finally measured solutions. Readings were made in 1 cm. quartz cells in a Beckman model DU spectrophotometer against the blank TCA solution described in the text. Duplicate determinations were made at each concentration level.

Recovery of Nucleic Acid Ultraviolet-Absorbing Components of Tissues and Microorganisms by AgNO₃ Precipitation—Three portions of mouse liver tissue powder (8.0 mg. each), three portions of rat lung tissue powder (8.0 mg. each), and three portions of the powder of E. coli (4.0 mg. each) were taken for hydrolysis. Aliquots of the diluted hydrolysates were treated with AgNO₃, the residues treated with HCl, and aliquots of the HCl solutions made to volume as described under “Methods.” Optical density readings of these solutions were then compared, respectively, with optical density readings of aliquots of the TCA solutions which represented equi-
valent amounts of the powders. As shown in Table I, average recoveries of the nucleic acid components ranged from 94 per cent in the case of mouse liver to 100 per cent for *E. coli* and rat lung.

**Table I**

Recovery of Nucleic Acid Ultraviolet-Absorbing Components from TCA Hydrolysates of Tissues and Microorganisms

<table>
<thead>
<tr>
<th>Tissue or organism</th>
<th>Hydrolysate No.</th>
<th>Optical density* per mg. extracted powder per 5 ml. solution</th>
<th>Per cent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TCA</td>
<td>HCL</td>
<td></td>
</tr>
<tr>
<td>Mouse liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.420</td>
<td>0.406</td>
<td>96.7</td>
</tr>
<tr>
<td>2</td>
<td>0.420</td>
<td>0.384</td>
<td>91.4</td>
</tr>
<tr>
<td>3</td>
<td>0.414</td>
<td>0.384</td>
<td>92.8</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>93.6</strong></td>
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<tr>
<td>Rat lung</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.330</td>
<td>0.340</td>
<td>103.0</td>
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<tr>
<td>2</td>
<td>0.312</td>
<td>0.320</td>
<td>102.6</td>
</tr>
<tr>
<td>3</td>
<td>0.320</td>
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<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>100.4</strong></td>
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<tr>
<td><em>E. coli</em></td>
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</tr>
<tr>
<td>1</td>
<td>0.748</td>
<td>0.740</td>
<td>98.9</td>
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<td>102.2</td>
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<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td><strong>100.5</strong></td>
</tr>
</tbody>
</table>

* 1 cm. light path at 265 μm.

**DISCUSSION**

These experiments indicate that in ultraviolet estimations of nucleic acids, when TCA is used as the solvent, adequate correction for the ultraviolet absorption of TCA is possible at wave lengths as low as 265 μm when the TCA concentration is approximately 1 per cent. Because, comparatively, the absorption of TCA is much less, whereas that of the nucleic acids has diminished only slightly, adequate correction for the absorption of TCA is also possible at 268.5 μm, the wave length suggested by Logan *et al.* (3) for measurement of TNA. At this wave length they found E(P) (atomic extinction coefficient per mole of P) for their RNA and DNA standard solutions to be the same, namely 9850.

It is further indicated that the addition of extra TCA after hydrolysis of the biological material is advisable in order to insure an excess of this protein precipitant for complete removal of precipitable material. That the amount of soluble protein breakdown products which remain is probably
small is shown by the fact that there was no increase in the extinction nor shift in the wave length maximum in tissue powder hydrolyzed for as long as 1 hour compared with the same powder hydrolyzed for 15 minutes.

In early experiments, designed to eliminate the effects of TCA and possibly protein absorption, it was observed that the nucleic acid ultraviolet-absorbing components could be quantitatively precipitated in low concentrations with AgNO₃. When the residue was treated with HCl, the precipitated nucleic acid components were dissolved and the absorption of the solvent in the ultraviolet region was negligible. This approach was abandoned as a routine procedure because it was obvious that at the wavelengths used and in the concentration of TCA present, reliable estimation of nucleic acids could be made without the precipitation step. However, the quantitative nature of the precipitation suggests other applications; for example, as a preliminary step in the separation of free bases (purines) from pyrimidine nucleotides. Of further interest, in connection with this precipitation procedure, was the observation that the p-nitrophenylhydrazine test (6) for DNA and the p-bromophenylhydrazine (7) and orcinol (8) tests for RNA (which are essentially tests for sugar components) were all negative when applied to the HCl solution of nucleic acid components. Pyrimidine nucleotides were known to be precipitated under the conditions prescribed. Furthermore, it had been shown by spectrophotometric measurements of the HCl solutions that there was a quantitative recovery of ultraviolet-absorbing material. These facts lend additional evidence to the concept that the sugar components of pyrimidine nucleotides are not released by the mild acid hydrolysis generally used in nucleic acid analysis procedures. For a description of other investigations for the analysis of binary mixtures of purine and pyrimidine components which utilize AgNO₃ precipitation procedures and ultraviolet spectrophotometry, reference may be made to the work of Loring et al. (9).

**SUMMARY**

Estimations of total nucleic acids in trichloroacetic acid hydrolysates of biological material by ultraviolet absorption methods have been studied.

1. When an excess of trichloroacetic acid was assured by the addition of extra amounts after hydrolysis, no significant change in the wave length of maximal absorption or extinction value was observed in tissues hydrolyzed for 15 minutes, compared with the same tissue hydrolyzed as long as 1 hour. Maximum for tissue hydrolysates was approximately 265 μm, which corresponded well with that found for a 1:1 deoxyribonucleic acid-ribonucleic acid solution and that of a synthetic mixture, based on the tetranucleotide formula, of components which would result from the hydrolysis of the nucleic acids.

2. Adequate correction for the ultraviolet absorption of trichloroacetic
acidity at wavelengths as low as 265 m\(\mu\) was possible by treating the blank solution in the same manner as tissue hydrolysates with respect to heating, addition of extra trichloroacetic acid, and dilution.

3. Quantitative separation of the ultraviolet-absorbing components from trichloroacetic acid solution was possible by precipitation with AgNO\(_3\) from an ammoniacal solution at pH 7 and dissolution of the residue with 0.1 N HCl.

BIBLIOGRAPHY

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