PHOSPHORUS COMPOUNDS IN ANIMAL TISSUES

I. EXTRACTION AND ESTIMATION OF DESOXYPENTOSE NUCLEIC ACID AND OF PENTOSE NUCLEIC ACID*

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The phosphorus compounds of animal tissues can be divided into the following four classes on the basis of their analytical properties, acid-soluble, lipid, nucleic acid, and protein. The separation of the first two groups of compounds from the tissue and from each other is well known and consists simply in extracting the tissue with acid to obtain the acid-soluble compounds and in extracting the tissue residue with fat solvents to obtain the lipids.

It is not sufficient merely to separate the phosphorus compounds into these classes. Methods must also be devised for each compound present in each of the four classes of phosphorus compounds. An elaborate analytical scheme has already been proposed for the estimation of fourteen phosphorus compounds found in the acid extracts of animal tissues (20). Preliminary steps towards the determination of the fatty acids in the lipid fraction have been undertaken (12).

The purpose of the present report is to describe (1) a method for the quantitative extraction of the nucleic acids from animal tissues, (2) the integration of this extraction procedure with previous methods for acid-soluble and lipid phosphorus so that all four groups of phosphorus compounds can be determined on a single tissue sample, and (3) the application of colorimetric methods to the estimation of the nucleic acids in the tissue extracts and the correlation of the results of the measurements with the phosphorus found in the extract. The new extraction method consists simply in heating the tissue with 5 per cent trichloroacetic acid after removal of phospholipids and acid-soluble phosphorus compounds.

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Some of the material reported in this paper was taken from a thesis submitted to the Graduate School of the University of Wisconsin in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

In this paper, “per cent” solutions refer to solutions in which the concentrations are expressed as gm. of material per 100 ml. of solution.
EXPERIMENTAL

Methods and Materials

All of the analytical methods used in this paper were based upon colorimetric reactions and some of the results are expressed as $E$ values ($E = \log \frac{I_0}{I}$ where $I_0/I$ is the ratio of the intensity of the incident light to that of the transmitted light (17)). Measurements of $E$ values were made with the Cenco-Sheard spectrophotometer adapted to the use of selected 13 × 100 mm Pyrex test-tubes. Since the effective thickness of the Pyrex tubes is not known, the $E$ values reported are not true extinction coefficients but are a characteristic of the set of tubes employed and will vary from one set of tubes to another, depending upon the diameter of the selected set of tubes.

Colorimetric Methods—Total phosphorus and nitrogen were measured as described by LePage and Umbreit (20). Desoxypentose nucleic acid (DNA) was measured by the diphenylamine reaction (11) and by the carbazole reaction (11, 15). The orcinol reaction (23) was used to measure pentose nucleic acid (PNA).

Nucleic Acid—Seven samples of DNA, two samples of yeast nucleic acid, desoxyribose, ribose, and the desoxyribosides of DNA were tested in the colorimetric reactions.\(^1\) The DNA samples were prepared from thymus, pancreas, and fish sperm by various methods ((14, 16, 21, 28); also Loring\(^2\)).

Preparation of Tissues—The tissues used for analysis were obtained from animals fed on the regular laboratory ration of mixed grains. In the case of the samples of rat liver, from which the phospholipids were not removed, and the mouse lung, the tissues were obtained from animals under nembutal anesthesia (50 mg. per kilo) and were frozen in liquid air immediately. The other tissues were obtained from animals killed by decapitation. The frozen or fresh tissues were homogenized in the apparatus of Potter and Elvehjem (25).

Results

Color Reactions of Nucleic Acids and Nucleic Acid Derivatives\(^3\)

The nucleic acids used in this study showed considerable variation in their elementary composition but were found to be suitable as standards

\(^1\) The author gratefully acknowledges the following contributions: adenine and thymine desoxyribosides from Dr. T. G. Brady (5); thymine and guanine, thymine, and cytosine desoxyribosides from the collection of the late Dr. P. A. Levene through the courtesy of Dr. A. E. Mirsky; two samples of sodium thymonucleate from Dr. J. P. Greenstein (14); two samples of yeast nucleic acid from Dr. J. A. Bain (2); one sample of sodium desoxyribonucleate (fish sperm) from Dr. H. S. Loring.

\(^2\) Loring, H. S., unpublished method; personal communication.

\(^3\) Recently Cohen (8) described a new colorimetric method for DNA involving heating the nucleic acid with tryptophane and perchloric acid. The sensitivity of
in the colorimetric reactions provided that the color intensities produced were referred to the phosphorus content of the sample (28).

**Diphenylamino Reaction**—The DNA samples gave an average $E$ value of 0.0224 per microgram of phosphorus. Yeast nucleic acid did not interfere in the reaction. In confirmation of previous observations (8, 24, 31), 1 mole of DNA was found to produce as intense a color as did 2 moles of desoxyribose, desoxyadenosine, or desoxyguanosine. The reaction of the pyrimidine desoxyribosides was slight and incomplete with the diphenylamine reagent (compare (24)).

**Carbazole Reaction**—Both DNA and PNA react with the carbazole reagent. The average $E$ values per microgram of phosphorus were 0.152 and 0.0124 respectively. In measuring DNA in tissue extracts with this reaction, a correction must be applied for any PNA which may be present. Desoxyribose and the desoxyribosides were tested with this reaction but no simple relation existed between the color intensities produced by these compounds and that produced by DNA.

**Orcinol Reaction**—PNA and DNA gave $E$ values per microgram of phosphorus of 0.135 and 0.0166 respectively in the orcinol reaction. The reaction of DNA with this reagent cannot be due to impurities in the DNA samples because all samples of DNA gave the same $E$ values per microgram of phosphorus (see (1)) and because desoxyribose and the desoxyribosides also reacted with the orcinol reagent (1 mole of DNA produced the same color intensity as did 4 moles of desoxyribose, desoxyadenosine, desoxyguanosine, desoxycytidine, or thymidine). In measuring PNA in tissue extracts, a correction must therefore be applied for the reaction of this reaction is enormously increased if the fluorescence of the heated solutions is measured (Coleman electronic photofluorometer No. 12, Filters B-1 and PC-1). Straight line relationships were found to exist between the concentration of DNA (as low as 0.5 to 2.5 $\gamma$) and the intensity of fluorescence. The reaction was carried out as follows: 0.8 ml. of 70 per cent perchloric acid (Eimer and Amend) were mixed with 0.2 ml. of 1 per cent $l$-tryptophane (Hoffmann-La Roche) and 0.6 ml. of water or DNA solution and the mixture was heated for 20 minutes in a boiling water bath. After addition of 6.4 ml. of water, the intensity of the fluorescence was measured. The intensity of fluorescence of both blanks and of DNA solutions is markedly influenced by the time of heating and the perchloric acid concentration. Adenine and thymine desoxyribosides also yield fluorescent materials when heated with tryptophane and perchloric acid. Ribose nucleic acid does not interfere in the reaction. The fluorescent solutions can be extracted with isoamyl alcohol to yield extracts which give linear relationships between fluorescence intensity and DNA concentration. The fluorescence is characterized by a marked sensitivity to ultraviolet light and the fluorescence intensity decreases considerably upon prolonged exposure to ultraviolet light. Although this reaction was not used for any of the analyses herein reported, it seems possible that the method could be developed into an exceedingly useful and sensitive method for DNA if the steadiness of the galvanometer readings could be increased. This might be accomplished by the use of proper organic solvents to extract the fluorescent material.
any DNA present. 1 mole of PNA produced a color as intense as that produced by 2.6 moles of d-ribose.

Separation of Nucleic Acids from Nucleoproteins

In order to measure nucleic acids in animal tissues colorimetrically, it is necessary to be able to remove the nucleic acids quantitatively from other tissue components. The following experiments show that heating the tissue with 5 per cent trichloroacetic acid at 90° for 15 minutes serves this purpose.

Splitting of Thymus Nucleohistone by Trichloroacetic Acid—A thymonucleohistone solution was prepared from rat thymus by the method of Carter and Hall (6). Aliquots of this solution were heated with an equal volume of 10 per cent trichloroacetic acid for 15 minutes at 90°. Aliquots of the protein-free filtrate were used for the measurement of DNA and total phosphorus. It was possible in this instance to use aliquots of the original thymonucleohistone solution equivalent to the aliquots of the filtrate in the color tests. The results are reported in Experiment 1, Table I. The following conclusions can be drawn: (1) all of the DNA in the thymonucleohistone is removed by the hot trichloroacetic acid treatment, and (2) all of the phosphorus found in the trichloroacetic acid extract is accounted for by the phosphorus in the DNA found.

Further evidence for the splitting of nucleoproteins by trichloroacetic acid has been presented by two groups of workers. Schramm and Dan-

<table>
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<th>Experiment No.</th>
<th>Description</th>
<th>DNA</th>
<th>PNA</th>
<th>P found</th>
<th>P calculated*</th>
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<td>Supernatant from TNH after 15 min. at 90° with 5% TCA</td>
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<td>2†</td>
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<td>Rat protein residue heated 10 min. at 100° with 2% NaOH</td>
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</table>

DNA = desoxypentose nucleic acid; PNA = pentose nucleic acid.

* Calculated from the DNA found, assuming that DNA contains 9.89 per cent phosphorus.

† 1 ml. portions of 20 per cent liver homogenates were extracted twice with cold TCA before being heated with TCA or NaOH.
nenberg (29) have demonstrated that ribose nucleic acid is quantitatively split from tobacco mosaic virus by hot trichloroacetic acid. Kaplan and Greenberg (18) observed splitting of rat liver nucleoproteins by cold trichloroacetic acid after long periods of contact (7 days).

Splitting of Rat Liver Nucleoproteins by Trichloroacetic Acid—The preceding experiment with thymonucleohistone indicated that one extraction with hot trichloroacetic acid was sufficient to liberate all of the nucleic acid from the protein. This was tested further by using rat liver homogenates as sources of proteins containing both DNA and PNA. The intensities of the orcinol and diphenylamine reactions were considered to be valid measures of PNA and DNA respectively. In Experiment 2, Table I, the yields of DNA and of PNA from rat liver upon heating with trichloroacetic acid and NaOH are compared. The yield of PNA was about the same with both reagents, while less DNA was obtained with NaOH. To test the completeness of the extraction of the nucleic acids from rat liver by hot trichloroacetic acid, the tissue residue from the hot acid treatment was either repeatedly heated with trichloroacetic acid or heated with NaOH. Neither treatment increased the yield of nucleic acid above that observed with a single extraction with hot trichloroacetic acid.

Time Required for Splitting of Nucleoproteins—Fig. 1 shows the effect of the time of heating rat tissues with trichloroacetic acid on the intensity of the orcinol and diphenylamine reactions. The experiment was carried out as follows: Acid-soluble and fat-soluble phosphorus compounds were removed from 1.0 ml. portions of a homogenate containing 194 mg. of rat liver and 32 mg. of rat thymus (see the proposed method of analysis for the detailed procedure). The tissue residues were then heated with 5.0 ml. of 5 per cent trichloroacetic acid for varying periods of time. After being cooled and filtered, aliquots of the extract were used for the diphenylamine and orcinol reactions. The resulting $E$ values are plotted in Fig. 1. Maximum amounts of PNA and DNA were obtained with a heating period of 10 minutes or longer.

Concentration of Trichloroacetic Acid Required for Splitting of Nucleoproteins—Acid- and fat-soluble phosphorus compounds were removed from portions of a homogenate containing 194 mg. of rat liver and 32 mg. of thymus as in the preceding experiment. The tissue residues were then heated for 15 minutes at 90° with 5.0 ml. of trichloroacetic acid solutions of varying concentration. After the heating period, 100 per cent trichloroacetic acid was added so that the final concentration of trichloroacetic acid in each mixture was 10 per cent. Aliquots of the filtrates were used in the diphenylamine and orcinol reactions. The results are plotted in Fig. 2.

All of the data reported in the present paper were obtained without any knowledge of the report of Schramm and Dannenberg (29), which was not concerned with the analytical possibilities of the findings.
and show that a trichloroacetic acid concentration of 3 to 4 per cent is sufficient for maximum yields of nucleic acids. The acidity is the important factor in the splitting of nucleoproteins; if neutralized trichloroacetic acid is used, no splitting occurs. The nucleic acids liberated at zero trichloroacetic acid concentration (Fig. 2) can perhaps be attributed to traces of trichloroacetic acid remaining from the preliminary washing of the tissue residue with cold trichloroacetic acid after lipid removal (see the proposed method).

Proposed Method of Analysis

For purposes of convenience, the analytical method will be described for rat liver. All steps are carried out in 16 mm. Pyrex test-tubes cut to a length of 100 mm. and provided with a pour out lip. Homogenization of the tissue (25) is especially important because it provides an extremely fine tissue suspension which facilitates extraction.

I. Removal of Acid-Soluble Phosphorus Compounds—1 ml. of a 20 per cent rat liver homogenate is mixed with 2.5 ml. of cold 10 per cent trichloroacetic acid and centrifuged. The precipitate is resuspended in 2.5 ml. of cold 10 per cent trichloroacetic acid and centrifuged. The trichloroacetic acid extracts are combined to form the acid-soluble phosphorus fraction. The compounds in this extract can be fractionated as described by LePage and Umbreit (20).
II. Removal of Phospholipids—The tissue residue from (I) is suspended in 1.0 ml. of water, mixed with 4.0 ml. of 95 per cent ethyl alcohol, and centrifuged. The residue is resuspended in 5.0 ml. of alcohol and centrifuged. These steps are used to remove traces of trichloroacetic acid left in the tissue residue from (I). The tissue residue is now boiled three times for 3 minutes each with 5.0 ml. portions of 3:1 alcohol-ether (4). The addition of a few small pieces of pumice-stone greatly facilitates boiling. The alcohol and the alcohol-ether extracts are combined to form the phospholipid fraction.

III. Removal of Nucleic Acids—The tissue residue from (II) is suspended in 1.2 ml. of water, mixed with 1.3 ml. of cold 10 per cent trichloroacetic acid, and centrifuged. The residue is suspended in 5.0 ml. of 5 per cent trichloroacetic acid, heated 15 minutes at 90°, cooled, and centrifuged. The residue is resuspended in 2.5 ml. of 5 per cent trichloroacetic acid and centrifuged. The trichloroacetic acid extracts are combined to form the nucleic acid extract.5

IV. Phosphoprotein—The residue from (III) is mixed with 5.0 ml. of 2 per cent NaOH and dissolved by heating in a boiling water bath for 10 minutes. This fraction is considered to be the phosphoprotein fraction.

Aliquots Used for Analysis—0.5 ml. aliquots of each extract were used for total phosphorus determinations. The following aliquots of extract (III) were used for nucleic acid measurements: diphenylamine reaction, 1.0 ml.; carbazole and orcinol reactions, 0.2 ml. each.

Calculation of Nucleic Acid Results—Since two of the nucleic acid analyses involve corrections, the calculations will be outlined briefly (see the "Results" for the color reaction constants).

\[
A = \text{micrograms DNA P per ml. solution} = \frac{E_{\text{diphenylamine}}}{0.0224}
\]

\[
B = \text{PNA} \quad \text{0.2 ml.} = \frac{E_{\text{orcinol}} - (0.2A \times 0.0166)}{0.135}
\]

\[
C = \text{DNA} \quad \text{0.2} \quad = \frac{E_{\text{carbazole}} - 0.0124B}{0.152}
\]

The results can be converted to micrograms of DNA or PNA by dividing by 0.099 and 0.095 respectively (15).

5 The sensitivity and the accuracy of the nucleic acid analyses would be greatly increased if a means of precipitating the nucleic acids quantitatively from the trichloroacetic acid extract could be devised. Freshly precipitated Zn(OH)₂ was found effective in precipitating DNA (prepared by the method of Hammarsten (16)) in concentrations as low as 20 γ per ml. The Zn(OH)₂ precipitation method failed to precipitate the nucleic acids quantitatively from hot trichloroacetic acid extracts of rat liver, however (28).
Application of Analytical Method

The results of the analysis of rat liver, mouse lung, and rat brain are summarized in Table II. In some cases, the alcohol and the alcohol-ether extractions were omitted.

Several points should be emphasized in connection with Table II. In the first place, extraction of the phospholipids prior to the nucleic acids had little effect on the amount of nucleic acid revealed by the colorimetric PNA or DNA determinations. The higher content of PNA in the lipid-extracted rat liver samples may be due to the smaller number of analyses or to the fact that the analyses were not made on the same samples of tissue. The brain analyses with and without lipid removal were made on the same samples of tissue and here the colorimetrically determined nucleic acids are in excellent agreement. The extraction of the lipids did make a difference in the amount of phosphorus in the trichloroacetic acid extracts accounted for by nucleic acids. The difference was small in

<table>
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<th>Tissue</th>
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<th>PNA (diphenylamine reaction)</th>
<th>DNA (carbazole reaction)</th>
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<th>P calculated*</th>
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DNA = desoxypentose nucleic acid; PNA = pentose nucleic acid.

The average values are given in bold-faced type.

* These calculations were made on the assumption that DNA contained 9.89 per cent phosphorus and 16.76 per cent nitrogen and that PNA contained 9.5 per cent phosphorus and 16.1 per cent nitrogen.

† In these analyses the phospholipids were not extracted prior to the nucleic acid extraction.
the case of rat liver: 92 per cent of the phosphorus was accounted for in the unextracted tissue, while 102 per cent was accounted for in the lipid-extracted livers. The difference was striking in the case of rat brain: 101 per cent of the phosphorus was accounted for by nucleic acids in the lipid-extracted brains, while only 48 per cent was accounted for in the unextracted tissue.

Although all of the phosphorus in the trichloroacetic acid extracts can be accounted for by nucleic acids, this is not the case for nitrogen. The nitrogen found was always higher than that calculated from the nucleic acids found (Table II). This may mean either that the nucleic acids in these tissues do not conform to the accepted tetranucleotide structure of a nucleic acid or that some other nitrogen compound is extracted by the hot acid.

DNA has been measured by two independent methods, the diphenylamine and the carbazole reactions, and the results check very well (see Table II). This finding indicates that the sugars in the DNA split from the protein by the trichloroacetic acid are apparently intact, because Dische has demonstrated that the diphenylamine reaction involves only the purine-bound sugars of DNA, while the carbazole reaction involves only the pyrimidine-bound sugars (11).

Further evidence for the effectiveness of the new method is provided by the fact that the DNA and the nucleic acid phosphorus found in the hot trichloroacetic acid extracts agree well with the values obtained by other workers using different methods (3, 9, 10, 13, 19, 22, 26, 27).

**DISCUSSION**

A method has been described for the extraction and measurement of nucleic acids in animal tissues. A variety of evidence has been presented for the quantitative nature of the extraction. The new method furnishes data not only on nucleic acids, but also data on acid-soluble, lipid, and protein phosphorus. This feature is of especial significance in work with radioactive phosphorus, because in this case it is desirable to be able to separate the four classes of phosphorus compounds in a single tissue sample.

No attempt was made to study the splitting of nucleoproteins by other acids, since it was felt that few if any acids could be found which had all of the desirable properties of trichloroacetic acid. Other acids such as hydrochloric (9, 30) and malonic (7) have been used to split nucleoproteins, but these acids are much poorer protein precipitants than is trichloroacetic acid.

The author is indebted to Dr. V. R. Potter for the suggestion of this problem and for his continued interest in this work.
SUMMARY

1. Several nucleic acid samples, desoxyribose, ribose, and several desoxyribosides were tested and compared in their reactions with the di-
phenylamine, orcinol, and carbazole reagents.

2. It was found that a single extraction with hot trichloroacetic acid would quantitatively remove nucleic acids from thymonucleohistone and
from animal tissues.

3. The effect of the trichloroacetic acid concentration and of the time of
heating with trichloroacetic acid on the amounts of nucleic acids extracted
from liver tissues was studied.

4. The hot trichloroacetic acid extraction was combined with known
methods to produce a scheme for the separation of the phosphorus com-
pounds of animal tissues into four groups: acid-soluble, lipid, nucleic acid,
and protein.

5. Rat liver, rat brain, and mouse lung were analyzed for nucleic acids.

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