A METHOD FOR THE DETERMINATION OF DESOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID, AND PHOSPHOPROTEINS IN ANIMAL TISSUES*

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The methods used at present for the determination of the higher nucleic acids in organs are based on certain color reactions of their carbohydrate components. The quantitative evaluation of these color tests gives satisfactory results with the free carbohydrates and with the purine nucleosides and nucleotides. The application of the color tests to the higher nucleic acids, however, encounters some serious difficulties which arise mainly from the resistance of the pyrimidine nucleotides against hydrolyzing agents, from the instability of desoxyribose, and from the necessity of separating the nucleic acid from the proteins. An extensive discussion of these problems has recently been given by Davidson and Waymouth (3). It occurred to us that these difficulties might be avoided if the quantitative estimations of desoxyribonucleic acid and of ribonucleic acid could be based on phosphorus determinations rather than on color tests of their carbohydrate components. The prerequisite for such a method would be the possibility of separating quantitatively the phosphorus fraction of ribonucleic acid from that of desoxyribonucleic acid. The separation of both P fractions can be achieved by the selective destruction of ribonucleic acid under the influence of a mild treatment with alkali. Steudel and Peiser (4)

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1 Cori and Cori (1) reported recently that the color produced by yeast nucleic acid in Mejbaum's (2) test corresponded only to 40 per cent of the amount of ribose as calculated on the basis of the tetranucleotide formula. In order to avoid this difficulty, Davidson and Waymouth (3) suggested the use of ribonucleic acid instead of ribose or certain ribose mononucleotides as a standard substance for the colorimetric estimation of ribonucleic acid in tissues. This is not advisable for theoretical and practical reasons. It is very probable that the proportions of purine and pyrimidine nucleotides are different in ribonucleic acids of different biological materials. Furthermore, the only ribonucleic acid which is easily accessible is yeast nucleic acid in the form of its commercial preparations. In our experience, different batches of commercial yeast nucleic acid are not identical in regard to the mutual proportions of their component nucleotides. Even the purification of commercial yeast nucleic acid by repeated precipitations with glacial acetic acid does not lead to preparations of reliable composition.
reported the important observation that ribonucleic acid is quantitatively split into acid-soluble nucleotides when it is incubated for 24 hours at room temperature in an approximately 3 per cent solution of sodium hydroxide. Under these conditions, desoxyribonucleic acid retains its property of being insoluble in mineral acids.

Phosphoproteins differ from both nucleic acids in their behavior towards alkali inasmuch as their phosphorus groups are quantitatively liberated as inorganic phosphate during the incubation with alkali under the conditions just described. Thus, when a mixture of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins is treated with dilute alkali and subsequently precipitated with a strong acid, the total P in the precipitate represents that of the desoxyribonucleic acid, the organic P in the filtrate that of the ribonucleic acid, and the inorganic P in the filtrate that of the phosphoproteins.

EXPERIMENTAL

Procedure for Determination of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins

Removal of Acid-Soluble and Lipid P—The finely minced organ (0.5 to 5 gm.) is weighed analytically and suspended in approximately 20 volumes of ice-cold 7 per cent trichloroacetic acid. The suspension is stirred mechanically for 20 minutes and filtered on a relatively large Büchner funnel over a thin layer of Hyflo filter aid. The filtration proceeds rapidly if a sufficiently large Büchner funnel is used. The filter aid not only expedites the filtration, but in addition greatly facilitates the removal of the extracted material from the filter paper. (The centrifugation of tissue suspensions in trichloroacetic acid is not advisable because the particles do not pack well and have a tendency to float.) The residue is generously washed with an ice-cold, 1 per cent solution of trichloroacetic acid until the filtrate is free of inorganic phosphate. The washings are continued with water until the filtrate is only weakly acid towards litmus, and finally with alcohol and ether.

The residue is suspended in 30 to 40 volumes (of the wet tissue) of a mixture of 75 volumes of alcohol and 25 volumes of ether and boiled for a few minutes. After filtration and washing with ether, the dry residue is finely ground in a mortar and refluxed for 30 minutes with 30 to 40 volumes of a boiling mixture of equal volumes of methanol and chloroform. The residue is filtered on a Büchner funnel and generously washed with ether. Finally, it is dried in an evacuated desiccator.

Treatment of Extracted Tissue Powder with Alkali and Determination of P Fractions—The powder is quantitatively transferred to a test-tube. After addition of an exactly measured amount of N potassium hydroxide
(10 cc. per gm. of fresh tissue), the tube is closed with a rubber stopper and the mixture is incubated for at least 15 hours at 37°. Under these conditions, the tissues (except bone) are completely dissolved. After the incubation, the filter aid is centrifuged off and the total P is determined in a suitable aliquot (1 to 2 cc.) of the solution according to the method of Fiske and Subbarow (5).

For the determinations of the acid-soluble and inorganic P, another aliquot (5 cc.) is pipetted into a test-tube and precipitated by addition of 0.2 volume (of the aliquot) of 6 N hydrochloric acid and 1 volume (of the aliquot) of 5 per cent trichloroacetic acid. The precipitate contains the total amount of the deoxyribonucleic acid, while the phosphorus-containing hydrolysis products of the ribonucleic acid and the phosphoproteins remain quantitatively in the filtrate. (a) An aliquot of the filtrate serves for the determination of the total P according to Fiske and Subbarow (5). (b) In a second aliquot, the inorganic phosphate is precipitated according to Delory (6). The washed Delory precipitate is dissolved in trichloroacetic acid. The solution is centrifuged if necessary, and the inorganic phosphate is determined according to Fiske and Subbarow (5). (The preliminary isolation of the inorganic phosphate according to Delory is necessary in order to remove protein breakdown products which would interfere with the direct determination of the phosphate according to Fiske and Subbarow.)

Calculation—The difference between the total phosphorus of the alkaline hydrolysate \( T_1 \) and the total phosphorus of the acid supernatant \( T_2 \) represents the phosphorus of deoxyribonucleic acid; the difference between \( T_2 \) and the inorganic phosphorus represents the phosphorus of ribonucleic acid; the inorganic phosphorus represents the phosphorus of the phosphoproteins.

The total volume of the alkaline solution can be calculated from the volume of the \( N \) potassium hydroxide solution by applying a correction for the increment caused by the dissolved tissue powder. It was found experimentally that this increment amounts to 0.2 cc. for 1 gm. of fresh liver tissue.\(^2\) If 10 cc. of \( N \) potassium hydroxide solution are used for the digestion of 1 gm. of wet tissue, the error caused by this change of the total volume is so small that it is sufficiently accurate to add the correction, \( \Delta \) volume = 0.2 cc. per gm. of the fresh tissue, to the volume of the potassium hydroxide solution. In special cases (skin, fat tissue) it will be necessary to

\(^2\) The empirical correction is somewhat larger than the theoretical increment as calculated from the amount of the tissue proteins and the average density of proteins in solutions (1.33). This is due to the fact that the lipid extraction and the drying of the tissue powder at room temperature do not lead to the complete removal of the water.
determine the weight of the extracted tissue in order to calculate the correction.

In order to obtain the amount of nucleic acid, the P figures are multiplied by 10.1 for desoxyribonucleic acid and by 10.6 for ribonucleic acid. It will be preferable, however, to express the results as mg. of nucleic acid P, since it is not certain whether the proportions of the nucleotides in ribonucleic acids of different origin are identical. In addition, it is not possible to give a general conversion factor for the calculation of the amounts of phosphoproteins from the amounts of the phosphoprotein P.

Control Experiments—As a recovery experiment, a mixture of known amounts of desoxyribonucleic acid (prepared according to Hammarsten (7)), yeast nucleic acid, and casein was added to a suspension of minced rat liver. The suspension was analyzed according to the procedure just described. A second sample of the same liver suspension was analyzed without the addition of the mixture. The differences between the corresponding figures in both samples agreed satisfactorily with the amounts of the three compounds added to the liver suspension. See Table I.

If the soluble organic phosphorus after the alkali treatment represents ribonucleic acid, the trichloroacetic acid filtrate must contain ribose and purine bases (either bound or free). In several experiments ribose and total purine determinations were carried out on aliquots of the trichloroacetic acid filtrates. The ribose determinations were carried out according to Mejbaum's (2) modification of Bial's test. For the purine determinations, the aliquots of the acid filtrate were refluxed for 2 hours in the presence of 2 per cent sulfuric acid. The purine bases were precipitated from the hot filtrate by a hot solution of silver sulfate, and the nitrogen of the washed precipitate was determined according to Kjeldahl. Without exception the amounts of purines and of pentose corresponded to approximately one-half of those equivalent to the corresponding values of the organic phosphorus. The amounts of purines found are in agreement with those calculated on the basis of the tetranucleotide formula of yeast nucleic acid. The yield of

### Table I

Recovery of Known Amounts of Desoxyribonucleic Acid, Ribonucleic Acid, and Casein Which Had Been Added to Liver Powder

<table>
<thead>
<tr>
<th>Added Substance</th>
<th>Desoxyribonucleic acid</th>
<th>Ribonucleic acid</th>
<th>Phosphoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, 300 mg. liver powder without addition...</td>
<td>0.190</td>
<td>1.31</td>
<td>0.003</td>
</tr>
<tr>
<td>B, 300 &quot; &quot; &quot; with added mixture.</td>
<td>0.490</td>
<td>1.66</td>
<td>0.141</td>
</tr>
<tr>
<td>B - A.......................</td>
<td>0.29</td>
<td>0.35</td>
<td>0.14</td>
</tr>
<tr>
<td>Added substance....</td>
<td>0.27</td>
<td>0.34</td>
<td>0.15</td>
</tr>
</tbody>
</table>
pentose amounting to approximately 50 per cent of the total present in ribonucleic acid is explained by the fact that the pyrimidine nucleotides yield only negligible amounts of furfural in Bial's test. We found that the color obtained with 50 γ of pure cytidylic acid (8) in Mejbaum’s test corresponded only to 1 per cent of the amount of ribose actually present in the nucleotide.

Distribution of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins in Some Animal Tissues—Table II contains representative figures for the distribution of the three P fractions in some animal tissues. The tissues were minced and fixed in trichloroacetic acid as soon as possible after the death of the animal, but no special precautions were taken to stop the action of the cellular enzymes immediately after the organs were collected. For this reason some of the figures recorded in Table II might be somewhat lower than the actual amounts of the nucleic acids or phosphoproteins present in the intact tissues. This applies especially to the results obtained with pancreas, due to the high nuclease content of the organ.

Table II includes analyses of thymus, pancreas, and egg yolk. The nucleic acids and phosphoproteins in these organs have been thoroughly studied during the earlier period of the biochemistry of the nucleic acids (9, 10). According to these investigations, desoxyribonucleic acid is abundant in the thymus gland and ribonucleic acid in the pancreas, while egg yolk contains a large amount of phosphoproteins but only very small amounts of nucleic acids. It can be seen that the results presented in Table II are in agreement with the conclusions reached by the earlier investigators on the basis of the preparative isolation of the compounds.

It is interesting to note that the amounts of ribonucleic acid in rat liver far exceed those of desoxyribonucleic acid. Davidson and Waymouth (3)
recently reported similar results in an investigation which came to our attention after the completion of our own experiments. The analytical methods used by these authors were entirely different from our procedure.

DISCUSSION

The method presented in this paper is exclusively based on phosphorus determinations. This offers considerable advantage in comparison with procedures in which other components of the nucleic acids, such as the purines or the carbohydrates, are used for the quantitative analysis. The determination of phosphate is more reliable than that of the organic components of the nucleic acids. Furthermore, the necessity of extracting the nucleic acids from the tissue is eliminated.

On the other side, it must be considered that the phosphorus is less specific as a constituent of the nucleic acids than the purines or the carbohydrates. The interpretation of our figures as nucleic acid or phosphoprotein phosphorus rests on the assumption that nucleic acids and phosphoproteins are the only phosphorus-containing substances present in animal tissues after the extraction of the phospholipids and the acid-soluble phosphorus compounds. This assumption has recently been questioned by Davidson and Waymouth (3), who compared in a series of experiments the amounts of total nucleic acid as calculated from total P determinations with those obtained from total purine determinations in tissues after the removal of phospholipids and acid-soluble P compounds. They claimed that in many tissues the nucleic acid values as calculated from the total purine determinations are somewhat lower than those calculated from total P determinations after the extraction of the lipid and acid-soluble P. In lung and brain the discrepancies were considerable.

It appears, however, that the technique used by Davidson and Waymouth (11) for the total purine determinations involves considerable loss of purines due to acid hydrolysis of the nucleic acid. The purine figures obtained with this technique are therefore not suitable for the calculation of the nucleic acid content of the tissues. In the experiments of these authors, the tissue powder was subjected for 6 hours to an extraction with 0.1 N hydrochloric acid in a shaker at room temperature prior to the purine determinations. The acid was changed six times. This treatment is sufficient to cause a considerable loss of purines by hydrolysis of the sensitive linkage between the purines and carbohydrates, especially in thymonucleic acid.³ We investigated this possibility by treating pure thymonucleate

³ The liberation of the purines under these conditions is not accompanied by the formation of acid-soluble P compounds. The P-containing breakdown products are compounds belonging to the group of thymic acids which are insoluble in dilute mineral acids.
(Hammarsten) according to the directions of Davidson and Waymouth (11) and found that the N:P ratio of the nucleic acid had decreased from 1.70 to 1.45 in the course of the treatment. This means a loss of 14.7 per cent of the total N or 22.1 per cent of the purine N. It should be emphasized that the necessary acid extraction of the tissues prior to the nucleic acid determinations should be completed as quickly as possible and with ice-cold solutions. This can be achieved only if the acid extraction is carried out on the fresh tissue. Therefore, the acid extraction must precede the extraction with lipid solvents. If this order is reversed, the complete extraction of the acid-soluble P compounds requires much more time and leads to sizable losses of nucleic acids due to hydrolysis.

SUMMARY

1. A method for the quantitative determination of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in animal tissues has been presented. The method is based on the different behavior of these compounds during a mild treatment with alkali.

2. The amounts of desoxyribonucleic acid, ribonucleic acid, and phosphoproteins in some animal tissues have been reported.

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