[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, YALE UNIVERSITY]

RESEARCHES ON PYRIMIDINES. CVII. THE EXAMINATION OF YEAST NUCLEIC ACID FOR 5-METHYLCYTOSINE[1]

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In a paper from this Laboratory on pyrimidines (CV) the authors[3] described the acetol test for thymine and 5-methylcytosine, II, which is applicable in the presence of the pyrimidines, uracil and cytosine, I. A technique was described which permits of the identification of any one of these four pyrimidines in the presence of another. The specific color test (acetol test) recommended for identifying thymine is so delicate that quantities of this pyrimidine as small as one milligram can be detected with ease. The successful application of this acetol test for thymine and methylcytosine adds a new and practical procedure to our present technique for analysis of the pyrimidine content of a nucleic acid molecule.

The program outlined for research in this Laboratory on the "Chemistry of Bacteria" includes a study of the nature of the sugars linked in the nucleic acid of this bacterial cell. This work calls for a large supply of bacteria, but it is finally becoming possible through coöperation with commercial organizations to obtain bacteria, grown on synthetic media, in sufficient quantity to inaugurate an exhaustive study of the respective nucleic acids functioning in different types of bacterial cells. In the present stage of development of nucleic acid chemistry we have practically no knowledge of the composition of nucleic acids of bacterial origin. What accurate data we have regarding nucleic acid has been obtained by a study of a limited number of nucleic acids of animal origin and the nucleic acid of the yeast cell. The recent results of biological experiments organized to include the study of the fundamental organic fractions of bacterial cells, as human, bovine and avian tubercle bacilli, indicate that the nucleic acid units of these cells play a significant and very important part in the life history of these lower organisms.

In the thymus nucleic acid molecule, and also in the nucleic acid of yeast, there is now no doubt as to the nature of the purine groupings which function as primary constituents. With reference to the pyrimidine constituents some doubt has been raised as to whether thymine and uracil are primary products, or whether they are hydrolytic products of methylcytosine, II, and cytosine, I, respectively. Present experimental evidence

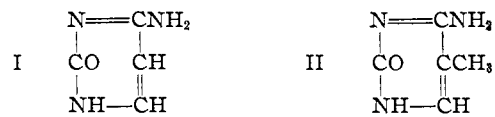
[1] Constructed from a dissertation presented by Henry Harvey Harkins, in June, 1927, to the Faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

[2] Holder of the DuPont Fellowship in Chemistry.

[3] Harkins and Johnson, THIS JOURNAL, **51**, 1237 (1929).

points to the conclusion that both uracil and cytosine function independently in the yeast nucleic acid molecule.

Of the nucleic acids of bacterial origin the one present in human tubercle bacilli—*tuberculinic acid*—has received the most attention. Johnson and Brown[4] made a careful examination of this substance, and they came to the conclusion that it is of the type of animal nucleic acids, that is, it contains a hexose sugar, cytosine and thymine, but no uracil. Later Johnson and Coghill continued the study of this interesting acid and obtained experimental evidence indicating that the cytosine fraction of tuberculinic acid contains a small amount of the pyrimidine 5-methylcytosine,[5] II. This



was the first time that the natural occurrence of this aminopyrimidine has been observed. Johnson and Coghill identified the pyrimidine, II, as its picrate, by means of the characteristic crystallographic properties of this salt. The occurrence of this pyrimidine base in nature suggests again the possibility that the primary group in the thymine series may be 5-methylcytosine, and not thymine, in tuberculinic acid and also thymus nucleic acid. It is also possible that, while thymine is a primary constituent, 5-methylcytosine may be present in small amounts, and actually in an organic combination not to be accounted for by the present, proposed structure of thymus nucleic acid. If this last postulation be true, it might be expected that yeast nucleic acid may also actually contain a small amount of 5-methylcytosine.

In the light of this speculation, it seemed very desirable, therefore, to apply the most delicate tests available for detecting small amounts of 5-methylcytosine, II, in the presence of relatively large amounts of cytosine obtained from natural sources. The test for thymine and 5-methylcytosine which we have described in our previous paper (acetol test) can be applied successfully for detecting 5-methylcytosine under such conditions. Its application has also enabled us to decide in a new way quite conclusively whether 5-methylcytosine occurs mixed with cytosine in the nucleic acid of yeast.

When tuberculinic acids are available from new growths of tubercle bacilli of human, bovine and avian types, an exhaustive examination for all pyrimidines occurring in these bacteria will be made in this Laboratory.

The Preparation of Yeast Nucleic Acid.—Although there are many methods described in the literature for the preparation and purification of

[4] Johnson and Brown, *J. Biol. Chem.*, **54**, 721 (1923); **57**, 199 (1923); see also *Am. Rev. Tuberculosis*, **7**, June, 1928.

[5] Johnson and Coghill, THIS JOURNAL, **47**, 2838 (1925).

yeast nucleic acid, it is very difficult to obtain a product which does not give a biuret reaction. Nearly all of these methods are modifications of one proposed by Altmann in 1889.[6] The most important improvements in technique have been recommended by Baumann,[7] Levene and La Forge[8] and also Steudel.[9] The latter investigator examined carefully the action of alkali on yeast nucleic acid and he pointed out the fact that 5% sodium hydroxide solution at room temperature causes nucleic acid to split off guanine-nucleotide. If the temperature is held at 0°, however, no decomposition results. Steudel, therefore, extracted the yeast cell with 5% sodium hydroxide and kept the temperature at or below 0°. In this way he obtained a product which was protein free and which gave good values for nitrogen and phosphorus content. From Steudel's experiments, it may be concluded that a sufficient standard for the purity of a yeast nucleic acid prepared by alkali extraction is a negative biuret test and a theoretical content of guanine. As both of these requirements are easily determined, it becomes a simple matter to decide whether a given nucleic acid is pure.

The method which we used in preparing yeast nucleic acid is closely analogous to Steudel's procedure, although it is not an exact duplicate of his method. After many unsuccessful attempts to prepare a protein-free nucleic acid, the following procedure was adopted and was found to give a biuret-free product in every case.

Three hundred grams of starch-free yeast, containing about 70% of moisture, was suspended in water and cooled to 0° in the ice box. To this cooled suspension was added 322 cc. of sodium hydroxide solution which contained 0.31 g. of sodium hydroxide per cubic centimeter and which also had been cooled to 0°. The suspension was then diluted to a volume of 2 liters. The temperature was kept at 0° for two hours. Acetic acid was then added until the solution was just acid to litmus. (The P_H of the solution was about 6.5.) When the solution had been neutralized its temperature was 15°. The suspension was centrifuged and a slightly turbid liquid resulted. To this was added enough alcohol to produce a solution containing 4% of alcohol. When this solution was filtered a sparkling clear filtrate was obtained. Enough concentrated hydrochloric acid was added to make the filtrate just acid to congo red paper, and finally an equal volume of 95% alcohol. A colorless, flocculent precipitate formed immediately. This was centrifuged, washed twice with 95% alcohol, then with ether three times. The product thus obtained was entirely biuret-free, was perfectly white and contained the theoretical quantity of guanine as determined by the method described by Jones in his monograph on nucleic acids.[10] Several preparations of nucleic acid were made in this manner and a protein-free product was obtained in every case. The weight of the air-dried nucleic acid from 300 g. of moist yeast was usually between 4 and 5 g.

[6] Altmann, *Arch. Anat. Physiol.*, **1889**, 526.

[7] Baumann, *J. Biol. Chem.*, **33**, Proceedings XIV (1917–1918).

[8] Levene and La Forge, *Ber.*, **43**, 3165 (1910).

[9] Steudel, *Z. physiol. Chem.*, **131**, 159 (1923).

[10] Walter Jones, "Nucleic Acids," Monograph on Biochemistry, Longmans, Green and Co., New York City, **1920**, p. 107.

The Separation of Cytosine from Thymine.—If the acetol test for 5-methylcytosine is to be applied to the cytosine fraction of any nucleic acid molecule, it becomes absolutely necessary to know whether cytosine can be separated from thymine and the acetol test made negative on the cytosine so obtained. In case this specific test cannot be made negative in the cytosine unit when this pyrimidine is separated from mixtures known to contain only cytosine and thymine, it is obvious that the acetol test would be of no value in ascertaining the presence of 5-methylcytosine in the basic aminopyrimidine fraction of a nucleic acid, which is known to contain thymine. The following experiment was, therefore, carried out.

To 0.5 g. of pure cytosine and 2.0 g. of thymine (both of synthetic origin) dissolved in one liter of water, enough concentrated sulfuric acid was added to make the solution 5% in strength (50 g.). A 10% solution of phosphotungstic acid was then added until a precipitate ceased to form. This precipitate was removed by means of the centrifuge and decomposed in the usual manner by trituration with barium hydroxide solution. The barium phosphotungstate was separated by centrifugation and the excess of barium then removed by precipitation with carbon dioxide. The clear and practically colorless solution was diluted to a volume of one liter, the acidity adjusted to 5.0% and the phosphotungstic acid precipitation repeated and carried out as in the first case. The neutral solution containing the cytosine was evaporated to a volume of 50 cc. and tested for thymine in the usual way. A negative result was obtained. In other words, the aminopyrimidine cytosine can be freed from all traces of thymine by means of phosphotungstic acid. Since it is known that 5-methylcytosine and cytosine behave in a similar manner when treated with phosphotungstic acid, it can be concluded from the result of this experiment that it will be possible to apply the acetol test for determination of the presence of 5-methylcytosine in any nucleic acid after preliminary hydrolysis of the nucleic acid molecule.

The Hydrolysis of Yeast Nucleic Acid.—Part of the nucleic acid used in this research was a special product obtained from the firm of Merck and Company. It was found to be protein-free and to contain the theoretical quantity of guanine. The pyrimidine fraction was isolated according to the following procedure.

To 50 g. of nucleic acid was added 300 cc. of water and 100 cc. of concentrated sulfuric acid. The mixture was heated in an oil-bath at 125° for twenty-five hours. The operation was conducted in a one-liter round-bottomed flask connected to a reflux condenser. At the end of this time the mixture was cooled and to it was added 3.6 liters of water. Finely-powdered barium hydroxide was then added until the solution was distinctly alkaline to litmus. The barium sulfate was centrifuged out, the precipitate suspended in two liters of hot water and again centrifuged. The two solutions were combined and concentrated *in vacuo* to a volume of 2 liters. Sulfuric acid was added to this solution until it was distinctly acid, the solution heated to its boiling point and filtered. To the filtrate was added a hot aqueous solution of silver sulfate (36 g.). A voluminous chocolate-colored precipitate came down immediately (the silver salts of the purines). This was filtered off and the solution made alkaline with barium hydroxide to precipitate the silver salts of the pyrimidines. Addition of barium hydroxide was continued until a precipitate no longer formed. The solution was thoroughly cooled and the silver salts of the pyrimidines were centrifuged out. After washing the precipitate with water, it was suspended in 2 liters of water, stirred thor-

oughly to break up any lumps, heated to 60°, made acid with 30 cc. of concentrated sulfuric acid and hydrogen sulfide passed into the solution for several hours. The silver sulfide was filtered off, suspended in hot acid solution and hydrogen sulfide passed in for another two hours to insure complete decomposition of silver salts. The silver sulfide was then removed by means of the centrifuge and the liquid added to the solution obtained from the first centrifugation. The sulfuric acid was removed from this solution with barium hydroxide and the excess of barium removed with carbon dioxide. The clear solution so obtained was concentrated *in vacuo* to a volume of 100 cc. Fifty cc. of this solution was treated with bromine and then made alkaline with barium hydroxide. The purple barium salt of dialuric acid was filtered off and to the filtrate was added 2 g. of barium hydroxide crystals. This mixture was heated under a reflux condenser for fifteen minutes and then distilled to remove acetol. When the distillate so obtained was tested for acetol, a faint but distinct fluorescence could be observed. This would seem to indicate the presence of a small amount of either thymine or 5-methylcytosine. This assumption was not true. The fluorescence was found to be due to acetol arising from a small quantity of nucleosides contained in the pyrimidine fraction. Baudisch has shown that all the simpler carbohydrates, including pentoses, give rise to acetol when distilled with alkali.[11] Hence, an acetol test is not conclusive evidence as to whether either thymine or 5-methylcytosine is present in the pyrimidine fraction until it has been shown that distillation with alkali alone gives a negative acetol reaction.

The following experiment, therefore, was carried out to prove that the acetol test did not come from either thymine or 5-methylcytosine: 50 g. of nucleic acid was hydrolyzed for twenty-five hours with 33% sulfuric acid as described above. The solution containing the pyrimidine was concentrated to a volume of 100 cc., 33 cc. of concentrated sulfuric acid added and the mixture refluxed for twelve hours at 135°. The mixture was then cooled and diluted with 2 liters of water and the sulfuric acid and barium removed in the usual way. The neutral solution was concentrated over a free flame to a volume of 100 cc. To 20 cc. of this solution was added 2 g. of barium hydroxide and the mixture refluxed for fifteen minutes. The distillate from this mixture gave a negative test for acetol, no fluorescence being observed even with the mercury arc light. It may be concluded from this experiment that the pyrimidine fraction tested was completely free of nucleosides. A second experiment was carried out with 20 cc. of the mixture. Bromine was added in excess, the excess removed by boiling and the solution made alkaline with barium hydroxide. The purple precipitate was removed by filtration. Two grams of barium hydroxide was added to the filtrate and the mixture refluxed for fifteen minutes as in the preceding experiment. The distillate from this mixture also gave a negative acetol test. It may be concluded, therefore, that the pyrimidine fraction of yeast nucleic acid, if it contains either thymine or 5-methylcytosine at all, contains these two pyrimidines in a quantity less than 1 mg. per 10 g. of nucleic acid.

Summary

1. A method for preparing a protein-free nucleic acid from yeast has been described. This is based on the original method of Altmann modified as recommended by Steudel.

2. The acetol test for thymine and 5-methylcytosine has been applied to the pyrimidine fraction of yeast nucleic acid, and has been found to be

[11] Deuel and Baudisch, THIS JOURNAL, **44**, 1581 (1924); Baudisch and Deuel, *ibid.*, **44**, 1585 (1924); Baudisch, *Biochem. Z.*, **89**, 279 (1918).

negative. It may be concluded, therefore, that neither of these pyrimidines is present in yeast nucleic acid.

3. By application of the acetol test for 5-methylcytosine to the aminopyrimidine fraction of any nucleic acid, it is now possible to detect this substance when present in very minute quantities.

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RESEARCHES ON HYDANTOINS. XLVIII. SYNTHESIS OF POLYPEPTIDE-HYDANTOINS FROM HYDANTOIN-1-ACETIC ACID[1]

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It is a growing belief that heterocyclic combinations containing active methylene groups (—CH_2—) occur in the structure of normal proteins; therefore, any information which increases the present knowledge of the chemistry of such cyclic groupings is of immediate value in the present period of intense interest in protein chemistry. Any consideration of cyclic amide groupings brings up at once the importance of the ureide structure, and the possible presence of polypeptide-hydantoin combinations in protein molecules. As a result of our chemical study of special bacteria like tubercle bacilli, we have learned that organic combinations are present in these unicellular organisms, some of which may function as inhibitors and some as accelerators of cell growth and development. These facts are important and lead one to ask whether there may not be some truth in the recent query of Prentiss,[3] who writes as follows: "Do chemicals exist in the tissues that are poisonous to infecting organisms and can such be isolated and made in sufficient quantities so that they could be used not only in the treatment of these infections, but possibly also in prevention in special instances? The presence of such substances is only hypothetical but the same may be said of numerous others commonly referred to in medical work as vitamines and whose presence we do not doubt." In fact, H. Kossel[4] studied the action of nucleic acid, a common constituent of cellular tissue, on bacteria and actually found that cholera germs and streptococci are readily killed

[1] This investigation was supported in part by a grant from the Therapeutic Research Committee of the Council on Chemistry and Pharmacy of the American Medical Association.

[2] Constructed from a dissertation presented by Alice Gertrude Renfrew to the Faculty of the Graduate School of Yale University, in June, 1927, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[3] Prentiss, "The Specific Immunity of the Tissues and Its Bearing on Treatment," *Science*, **62**, 91 (1925).

[4] Kossel, *Nature*, **49**, 240 (1894).