THE STRUCTURE OF THYMONUCLEIC ACID.

BY P. A. LEVENE AND E. S. LONDON.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

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The earlier work on thymonucleic acid by Levene and Mandel\(^1\) and by Levene and Jacobs\(^2\) led to the isolation of monophosphoric esters of pyrimidine nucleosides and diphosphoric esters of pyrimidine nucleosides. These observations were corroborated by Thannhauser and coworkers.\(^3\)

In a recent publication Levene and London\(^4\) reported the isolation of a guanine nucleoside and the present paper contains a report on the isolation of three additional nucleosides, namely hypoxanthine, thymine, and cytosine nucleosides. The hypoxanthine nucleoside is undoubtedly derived from the adenine nucleoside inasmuch as the yeast nucleic acid digested in the same manner as the thymonucleic acid gave inosine in place of adenosine. The newer findings definitely confirm the formulation for the thymonucleic acid given by Levene, in 1921, which is but a modification of the one given by Levene and Jacobs, in 1912.

In one essential detail the formulation of Levene and Jacobs and of Levene needs revision; namely, the sugar entering in the structure of thymonucleic acid is now found to be not a hexose but a desoxypentose. With this correction, the formulation of the structure of thymonucleic acid becomes as follows:

The reasons for accepting the mode of linking between individual nucleotides as given in this figure have been presented earlier. The nature of the sugar, however, needs to be discussed in some detail.

**Carbohydrate of Thymonucleic Acid.**

In the communication on the guanine nucleoside it was stated that the sugar was obtained in crystalline form and that it analyzed for a desoxypentose. It gave a color test with Kiliani’s reagent for 2-desoxy sugars. It did not form an osazone, but readily formed a benzylphenylhydrazone which analyzed for the hydrazone of a desoxypentose. The three new nucleosides all have the composition of derivatives of a desoxypentose. Thus there can be no doubt as to the composition of the sugar. It remains to reconcile the new findings with the old. From the work of Kossel and his school and from that of Levene, it is known that the sugar of thymonucleic acid on heating with 5 to 10 per cent sulfuric acid is transformed into levulinic acid. This reaction was considered characteristic for hexoses and on this basis it had been assumed

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that the sugar of thymonucleic acid was a hexose. Levene and Mori\textsuperscript{6} found that the reaction is equally characteristic for the synthetic 2-desoxypentoses, as well as for the sugar isolated from the thymonucleic acid. Feulgen\textsuperscript{7} made the important discovery that thymonucleic acid on short hydrolysis with dilute acids gave a positive test with Schiff's reagent and a positive pine stick test. Levene and Mori found that the synthetic desoxypentoses as well as the desoxypentose of nucleic acid reacted in a similar way.

There still remain to be reconciled the analytical data for the crystalline barium salt of the diphosphoric ester of the thymine nucleoside previously described, with the new conception of its structure. In fact, the analytical data of this substance agree better with those required by the newer theory.

\[ C_{11}H_{14}N_{2}P_{2}O_{13}. \text{ Calculated.} \quad C \ 18.37, \ H \ 1.97, \ N \ 3.89, \ P \ 8.62. \]
\[ C_{12}H_{12}N_{2}P_{2}O_{11}. \quad \text{"} \quad 17.84, \ " 1.78, \ " 4.15, \ " 9.21. \]
\[ \text{Found.} \quad 18.11, \ " 1.93, \ " 4.04, \ " 8.97. \]

Thus the substance to which previously the structure of \( d \)-diphosphoric ester of a thymine hexoside had been assigned is in reality the diester of a thymine desoxypentoside.

There is still another point to be mentioned, namely the elementary composition of the thymonucleic acid. The new formulation of \( C_{20}H_{15}N_{15}P_{2}O_{28} \) requires the following elementary composition: \( N \ 16.77 \) and \( P \ 9.89 \). It is noteworthy that the purest sample of the acid prepared by Levene and Mandel in 1908\textsuperscript{8} contained \( N \ 17.70 \) and \( P \ 10.00 \). These high values of the nitrogen and phosphorus content seemed at that time puzzling.

Thus the discovery of the nature of the sugar of the thymonucleic acid gives a ready explanation of those peculiarities of the acid which up to date seemed rather puzzling.

\section*{EXPERIMENTAL.}

\subsection*{Introductory.}

The unsuccessful attempts to find the proper conditions for partial hydrolysis of thymonucleic acid to nucleosides have led one of us (Levene) to search for conditions of enzymatic hydrolysis leading

\textsuperscript{7} Feulgen, R., \textit{Z. physiol. Chem.}, \textbf{123}, 197 (1922).
to the desired end. In cooperation with Medigreceanu, he found that there occur in the organs enzymes capable of cleaving nucleic acids to nucleotides, others capable of dephosphorylating nucleotides (nucleotidases), and some capable of hydrolyzing nucleosides (nucleosidases). The nucleotidases were found in several organs and also in the intestinal juice. The latter finding suggested the possibility of accomplishing the desired hydrolysis of nucleic acid by means of intestinal juice. In 1912, through the courtesy of Dr. Carrel, Levene and Jacobs were in possession of a dog with an intestinal fistula which permitted feeding the dog on nucleic acid and collecting the nucleic acid impregnated with enzyme through the fistula. From material obtained in this manner it was then possible to isolate a small quantity of gelatinous material resembling crude guanosine which gave a negative orcinol test and which analyzed as a guanosine hexoside. The material was undoubtedly very impure and apparently contaminated with a carbohydrate which did not belong to the nucleoside inasmuch as on hydrolysis it yielded a hexosazone. In the summer of 1924 Levene performed a number of experiments in Pavlov's laboratory in Leningrad (Petrograd) Russia, attempting to bring about a satisfactory hydrolysis of thymus nucleic acid by means of intestinal juice obtained from dogs with a Thiry-Vella fistula. The experiments were not successful, undoubtedly for the reason that the juice was very poor in enzymes.

The experience of 1912 seemed to suggest the advantage of passing a solution of nucleic acid through a segment of the gastrointestinal tract and collecting it from an intestinal fistula. In order to avoid contamination with remnants of food, it seemed desirable to create a gastric fistula which would permit the establishment of a clean and empty gastrointestinal segment. With this aim in view Professor E. S. London of Leningrad prepared, at the request of Levene, several dogs each with one gastric and one intestinal fistula.

10 I wish to express at this late date my appreciation to Dr. Carrel for his courtesy.—P. A. L.
11 For this courtesy, I wish to express my great appreciation to Professor I. P. Pavlov.—P. A. L.
Mode of Digestion.

The procedure was repeatedly varied in the course of the work, and we are inclined to believe that the optimal conditions for the digestion of the nucleic acid still remain to be established. In the earlier experiments a solution of 50.0 gm. of nucleic acid was allowed to flow through the gastric fistula and the solution collected from the intestinal fistula, the entire procedure lasting from 1 to 2 hours. The volume of the collected fluid varied from 700 cc. to 350 cc. Toluene was added to the solution which was then placed in a thermostat for different intervals, the shortest being 4 days and the longest 7. The degree of digestion varied from experiment to experiment. In the later experiments small portions of gastrointestinal secretions were added daily. Towards the end of the work it was found that hydrolysis of the nucleic acid could be accomplished by adding portions of the secretions daily to the solution of nucleic acid without passing the latter through the segment of the gastrointestinal tract. This fact will permit the working out of more definite optimal conditions. Work in this direction is at present in progress in this laboratory.

The yield of nucleosides differed from experiment to experiment, the maximal being 1.5 gm. of guanine nucleoside from 200.0 gm. of nucleic acid. The other nucleosides were obtained in minimal quantities and many experiments yielded only the guanine nucleoside.

The great resistance of the thymonucleic acid as compared with yeast nucleic acid was very striking. The latter yielded about 5.0 gm. of guanosine from 200.0 gm. of nucleic acid. This fact is in harmony with observations made in 1911 by Levene and Medigreceanu.9

Chemical Procedure for Isolation of Nucleosides.

The first step in the fractionation of the digest consisted in its separation into two fractions, one yielding principally the guanine and hypoxanthine nucleosides, and the other the pyrimidine nucleosides.

The digest of 200.0 gm. of nucleic acid was poured into twice its volume of 95 per cent alcohol and the filtrate was concentrated to about 400 cc. On cooling, this solution generally gelatinized and it could be separated by filtration into the two fractions; the
Thymonucleic Acid
gelatinous fraction serving for the isolation of the purine nucleosides, and the filtrate for the pyrimidine nucleosides. It was found expedient in order to facilitate the filtration to warm the solution of the concentrate on the boiling water bath and to add an equal volume of boiling methyl alcohol. The solution remains liquid while hot, but on cooling a voluminous precipitate is formed which is readily filtered.

**Purine Nucleoside Fraction.**

The above precipitate which contained, in addition to the nucleosides, mineral phosphates and nucleotides was dissolved in hot water and freed from phosphoric acid by means of barium hydroxide. The filtrate from the phosphates was freed from the barium ions and then concentrated to a small volume. On cooling, a semi-gelatinous precipitate formed which served for the isolation of the guanine nucleoside and a filtrate which served for the isolation of the hypoxanthine nucleoside.

**Guanine Nucleoside.**—The precipitate containing the nucleoside is dissolved in water and an excess of 25 per cent solution of basic lead acetate is added. A precipitate is formed which consists principally of nucleotides. To the filtrate an excess of ammonium hydroxide is added which causes the formation of a voluminous precipitate. The mixture is brought to a boil which causes a great part of the precipitate to dissolve. On cooling the filtrate a flocculent precipitate is formed. The mixture is allowed to stand 4 or 5 hours in the cold. The precipitate is then filtered and dissolved in water containing a little acetic acid. Hydrogen sulfide gas is passed through the solution and the filtrate from lead sulfide is concentrated under reduced pressure at a temperature of the water bath not exceeding 30°. The nucleoside then crystallizes in long needles. The substance is practically pure, but for the preparation of the desoxypentose it should be recrystallized twice out of water. The general properties of the substance have already been described, as well as its optical rotation in water. In the present paper the rotation of the other nucleosides will be given in 1.0 N sodium hydroxide and therefore the rotation of this nucleoside also has been measured in this solvent. The result was as follows:

\[
[\alpha]^b_b = \frac{-0.72^\circ \times 100}{1 \times 2} = -36.0^\circ.
\]
As was stated in the earlier publication, the nucleoside is very unstable and in order to determine the optimal conditions for its hydrolysis three experiments were performed. In each case 0.250 gm. of the substance was dissolved in 10 cc. of 0.01 N aqueous hydrogen chloride. The solutions were brought to a boil. This procedure did not consume more than 1 minute. The solution was then kept in a boiling water bath, the first 5, the second 10, and the third 15 minutes. During these intervals a white granular precipitate formed in each tube. At the end of each experiment the solutions were immersed in an ice-alcohol bath and after cooling, the filtrates were brought up to the volume of 25 cc. and the optical rotations of each were measured in a 2 dm. tube. They were as follows:

I \( \alpha_D = -0.49^\circ \).

II \( \alpha_D = -0.46^\circ \).

III \( \alpha_D = -0.48^\circ \).

Inasmuch as the rotation of the desoxypentose in equilibrium is \(-50^\circ\), it follows that each solution contained 0.125 gm. of sugar. Thus it is evident that 5 minutes heating with 0.01 N hydrochloric acid suffices to bring about a complete hydrolysis of the nucleoside.

**Hypoxanthine Nucleoside.**—The solution containing this fraction was fractionated with lead in exactly the same manner as the guanine nucleoside fraction.

The hypoxanthine nucleoside crystallized in long needles and was recrystallized out of water until the substance was ash-free. The yield of this material was very small, so that all through the work only 1.0 gm. of the substance was accumulated. The air-dry substance contracted at 202° and had no melting point. It analyzed as follows:

3.860 mg. substance: 6.670 mg. CO₂ and 1.655 mg. H₂O.

3.935 “ “ : 0.778 cc. N₂ at 27° and \( p = 759 \) mm.

\( \text{C}_{15}\text{H}_{12}\text{N}_{4}\text{O}_{4} \). Calculated. C 47.60, H 4.79, N 22.21.


The rotation of the substance in water with enough sodium hydroxide to complete solution was the following.

\[ [\alpha]_D = \frac{-0.42^\circ \times 100}{1 \times 2} = -21.0^\circ. \]
0.500 gm. of the substance was taken up in 25 cc. of 0.01 N aqueous hydrogen chloride. The solution was brought to a boil and then allowed to remain in a boiling water bath for 10 minutes. On cooling the solution in an ice-water mixture, the base settled out. To the filtrate silver sulfate was added in excess. The silver salt of the base was removed and from the filtrate the excess of silver was removed by means of hydrogen sulfide. The solution was brought up to the volume of 25 cc. and the solution then showed in a 2 dm. tube the rotation of $-0.54^\circ$, which corresponds to 0.250 gm. of desoxypentose, thus showing that it contained the expected amount of the sugar.

The hydrochloride of the base was redissolved in a slight excess of aqueous hydrogen chloride and the base precipitated by means of ammonia. The base was washed free from chlorides and then had the following composition.

4.475 mg. substance: 1.641 cc. N$_2$ at 30$^\circ$ and p = 751.7 mm.


**Pyrimidine Nucleoside Fraction.**

This fraction in addition to nucleosides contained phosphates, chlorides, and nucleotides. It was concentrated to a small volume and precipitated with 95 per cent alcohol as long as a precipitate formed. The filtrate was freed from phosphoric acid by means of barium hydroxide and the filtrate from the phosphates was freed from the excess of barium ions and then fractionated by means of basic lead acetate in the manner described for the guanine nucleoside.

**Thymine Nucleoside.**—The lead salts soluble on heating were used for the preparation of the nucleoside. From this fraction the lead was removed by hydrogen sulfide and the filtrate from the lead sulfide was cooled to 0$^\circ$ and made acid to Congo red by means of cold dilute sulfuric acid. To this solution, immersed in a cooling mixture, silver carbonate was added until all the chlorine ions were removed. The filtrate from the silver precipitate was neutralized with freshly prepared barium carbonate and through the suspension a stream of hydrogen sulfide gas was passed. The filtrate from silver sulfide and barium sulfate was freed from excess of barium ions and concentrated to a small volume and allowed to
stand in a desiccator under reduced pressure. On standing, a crystalline deposit forms consisting partly of needles and partly of platelets. This deposit consists of thymine nucleoside. For purification it is recrystallized out of water. In the course of the work we came into possession of approximately 4.0 gm. of this substance. About 2.0 gm. were used up in the attempts at hydrogenation in the same manner as the hydrogenation was carried out on uridine. The efforts thus far were not successful, but they will be continued when larger quantities of the material will be available.

The pure thymine nucleoside crystallizes in platelets. Heated in a capillary tube it melts at 185°. The composition of the substance was as follows:

\[
\begin{align*}
5.190 \text{ mg. substance} & : 9.470 \text{ mg. } \text{CO}_2 \text{ and } 2.605 \text{ mg. } \text{H}_2\text{O.} \\
5.100 \text{ " "} & : 0.534 \text{ cc. } \text{N}_2 \text{ at } 26^\circ \text{ and } p = 736 \text{ mm.}
\end{align*}
\]

\[
\text{C}_{10}\text{H}_{11}\text{N}_5\text{O}_4. \text{ Calculated. } \text{C} 49.59, \text{ H} 5.80, \text{ N} 11.56. \\
\text{Found. } \text{" " 49.76, " 5.64, " 11.58.}
\]

The optical rotation of the substance in 1.0 N sodium hydroxide was

\[
[\alpha]_D^\infty = \frac{+0.65^\circ \times 100}{1 \times 2} = +32.5^\circ.
\]

1.5 gm. of the substance were taken up in 1 cc. of 10 per cent sulfuric acid in a sealed tube and heated in a glycerol bath for 4 hours at 130°. On cooling, a crystalline deposit formed. It had all the properties of thymine and for analysis was recrystallized from dilute sulfuric acid. The substance was washed with water, and then with alcohol. It did not combine with the acid. The dry substance analyzed as follows:

\[
\begin{align*}
4.330 \text{ mg. substance} & : 0.856 \text{ cc. } \text{N}_2 \text{ at } 30^\circ \text{ and } p = 751.7 \text{ mm.} \\
\text{C}_3\text{H}_5\text{N}_2\text{O}_2. \text{ Calculated. } \text{N} 22.22. \text{ Found. } \text{N} 22.02.
\end{align*}
\]

The filtrate from thymine was extracted with ether in a continuous extractor for 60 hours. The ethereal extract contained a crystalline deposit of thymine. A similar observation had been made in the extraction of a hydrolysate of thymus nucleic acid. The crystals were removed by filtration and the residue was taken up in 5.0 cc. of water. To the solution were added 0.5 gm. of the
hydrochloride of semicarbazide and 1.0 gm. of sodium acetate dissolved in 5.0 cc. of water. On scratching with a glass rod, the semicarbazone of levulinic acid crystallized immediately. The substance was recrystallized from 98.5 per cent alcohol. The substance melted at 192° (uncorrected). The yield of recrystallized material was 0.225 gm. It had the following composition.

\[ 3.170 \text{ mg. substance: } 0.692 \text{ cc. } N_2 \text{ at } 30° \text{ and } p = 752 \text{ mm.} \]
\[ \text{C}_6\text{H}_{11}\text{NeO}_3 (173). \text{ Calculated. } N 24.25. \text{ Found. } N 24.03. \]

**Cytidine Nucleoside.**—The filtrate from the thymine nucleoside was treated with an excess of a solution of alcoholic picric acid. On standing, an amorphous precipitate formed in which were imbedded microscopic balls of semicrystalline structure. The precipitate was filtered off and washed with ether. The mother liquor on concentration under diminished pressure at room temperature gave another crystalline deposit. The two deposits were combined, recrystallized several times from methyl alcohol, and finally from water. The substance contracted at 190° and did not show any tendency to melt. It had the following composition.

\[ 4.885 \text{ mg. substance: } 0.785 \text{ cc. } N_2 \text{ at } 25° \text{ and } p = 759. \]
\[ 6.370 \text{ " " : } 9.245 \text{ mg. } \text{CO}_2 \text{ and } 1.925 \text{ mg. } \text{H}_2\text{O.} \]
\[ \text{C}_{15}\text{H}_{16}\text{N}_{6}\text{O}_{11}. \text{ Calculated. } \text{C} 39.46, \text{H} 3.53, \text{N} 18.42. \]
\[ \text{Found. } " 39.57, " 3.38, " 18.38. \]

The optical rotation of the substance in water was as follows:

\[ [\alpha]_D = \frac{-0.20\times100}{0.5\times1} = +40°. \]

Thus, in the case of the thyminose nucleosides as in that of the ribose nucleosides, the purine nucleosides rotate to the left, whereas the pyrimidine nucleosides rotate to the right.
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P. A. Levene and E. S. London

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