THE STRUCTURE OF YEAST NUCLEIC ACID.

IV. AMMONIA HYDROLYSIS.

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The tetranucleotide theory of the structure of yeast nucleic acid was first enunciated by the writer[1] and was subsequently conclusively demonstrated by the experimental evidence furnished by Levene and Jacobs,[2] and by Levene and La Forge.[3] The facts on which the theory was based were: first, the isolation of four nucleosides; second, the isolation of simple pyrimidine nucleotides. These were obtained on partial hydrolysis of yeast nucleic acid. The third fact, important for the development of the theory, was the elucidation of the order of linkage of the components of one simple mononucleotide.[4]

The experimental data obtained until that phase of work permitted no rational formulation of the mode of linkage between individual mononucleotides. Our original graphic representation of the entire molecule of yeast nucleic acid had only an arbitrary schematic sense. In a publication on thymus nucleic acid, Levene and Jacobs made that point clear. Owing to pressure of other work, our own investigations into the problem of the linkage of the mononucleotides was making slow progress, when Thannhauser with his collaborators,[5] and Jones with his collaborators[6] entered the field of nucleic acid study.

[1] Levene, P. A., *Biochem. Z.*, 1909, xvii, 121.

[2] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1909, xlii, 2475, 2703; 1910, xliii, 3151; 1911, xliv, 1027.

[3] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608, 3164.

[4] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1908, xli, 2704; 1909, xlii, 335, 1198.

[5] Thannhauser, S. J., *Z. physiol. Chem.*, 1914, xci, 329. Thannhauser, S. J., and Dorfmüller, G., *ibid.*, 1917, c, 121.

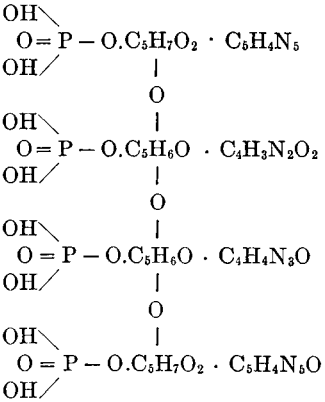
[6] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71. Jones, W., and Germann, H. C., *ibid.*, 1916, xxv, 93. Jones, W., and Read, B. E., *ibid.*, 1917, xxix, 123; xxxi, 39.

By means of either enzyme action or by methods of chemical hydrolysis, they obtained intermediate substances which were regarded by them as di- and trinucleotides.

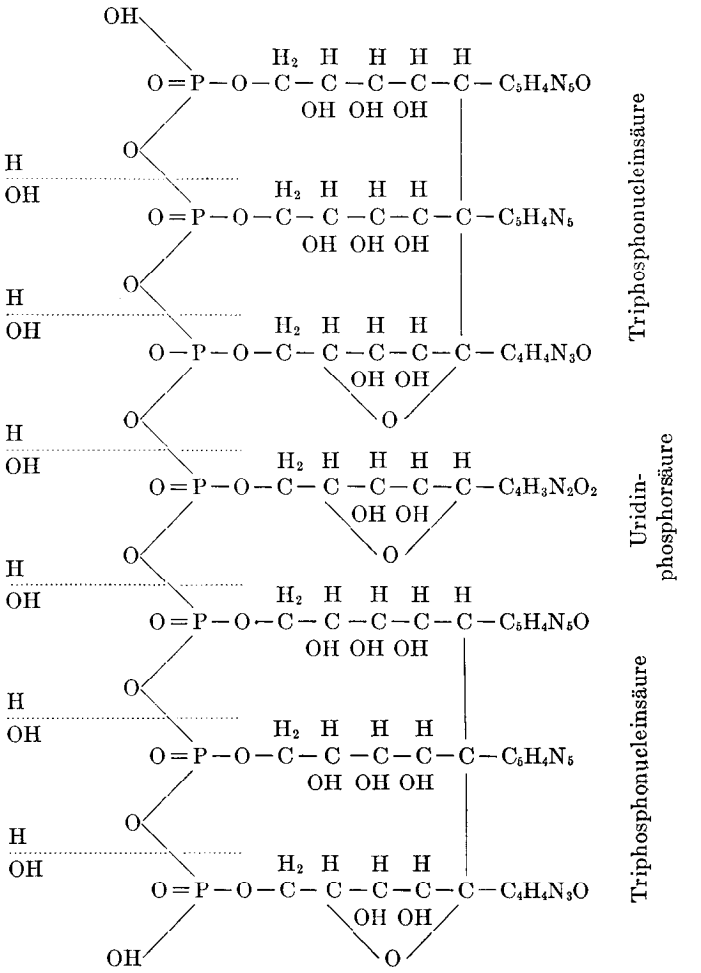
The methods of hydrolysis employed by them in some instances were identical with those employed by us in other instances, slightly modified. The method of separating simple nucleotides as their brucine salts was also introduced by us.

Thannhauser first announced the isolation of a trinucleotide from the products of digestion of nucleic acid by enzymes. Later, Thannhauser and Dorfmüller hydrolyzed nucleic acid by means of 25 per cent ammonia and supposedly cleaved the molecule into uridinphosphoric acid and a trinucleotide containing the remaining three nucleotides. On acid hydrolysis with 2 per cent sulfuric acid these authors obtained only uridinphosphoric acid. This publication appeared in Germany in 1917 and did not reach us until 1919. In 1914, Jones and Richards described experiments by which they thought they had cleaved nucleic acid into two dinucleotides; guanin-cytosine, and adenin-uridin dinucleotides. Subsequently, Jones and his coworkers described the same two dinucleotides which they obtained on hydrolysis of yeast nucleic acid by heating the acid in an autoclave in a 2.5 per cent ammoniacal solution for $1\frac{1}{2}$ hours at a temperature of 115°C. On the other hand, on hydrolysis by means of dilute acids; Jones and Read described a cytidin-uracil dinucleotide. On the basis of their respective findings, Jones and Thannhauser presented theories of the mode of linkage between the nucleotides.

According to Jones the structure of yeast nucleic acid is as follows:

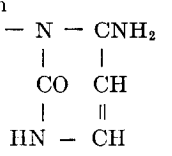


Thannhauser, on the other hand, presents the linkage of the nucleotides in the following way:[7]

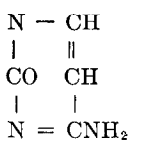


[7] In Thannhauser's representation there is an oversight in regard to the cytidin linking.

It is given



It should be



These theories are based on the following considerations:

That of Jones on the assumption of the reality of the dinucleotides, and on the observations that the so called dinucleotides are tetra-basic. The theory of Thannhauser is based on the assumption of the belief in the reality of the trinucleotide, and second, on the fact that the so called trinucleotide is hexo-basic.

In previous communications we have criticized the conclusions of these writers, on the assumption that their observations were correct.[8] In a later communication,[9] we have shown that the cytidin-uridin dinucleotide was a mixture of uridin and cytidin mononucleotides. Uridinphosphoric acid was obtained as a crystalline barium salt. It may be mentioned here that optical rotation of the crystalline salt air-dry was $[\alpha]_D^{20} = 3.5$, or dry and barium-free $[\alpha]_D^{20} = 5.83$, whereas Thannhauser and Dorfmüller found for their uridinphosphoric acid + 14.4.

On the other hand the barium salt of the cytidinphosphoric acid had the optical rotation of $[\alpha]_D^{20} = +14.0$ or barium-free and dry $[\alpha]_D^{20} = +23.3$ which agrees with the recent finding of Thannhauser for the crystalline cytidinphosphoric acid, which was $[\alpha]_D^{20} = 23$. Thus it is possible that the substance described by Thannhauser as uridinphosphoric acid was of a lesser degree of purity than that of the cytidinphosphoric acid.

In a still later publication[10] we reported on the finding that the so called cytidin-uridin dinucleotide was fractionated by us into uridinphosphoric and adenosinphosphoric acids. The former was identified as the crystalline barium salt, the latter as the brucine salt, which at the time of that publication was converted into the barium salt. Since from a large quantity of brucine salt there was obtained only a small quantity of a barium salt analyzing satisfactorily for the salt of the adenosin nucleotide, the publication of the analytical data on that nucleotide was delayed. It was subsequently found that adenosinphosphoric acid is identified most conveniently as the free acid. Crystalline adenosinphosphoric acid was described by Jones and Kennedy;[11] the substance obtained by us differed from that of

[8] Levene, P. A., *J. Biol. Chem.*, 1917, xxxi, 591.

[9] Levene, P. A., *Proc. Soc. Exp. Biol. and Med.*, 1917, xv, 21.

[10] Levene, P. A., *J. Biol. Chem.*, 1918, xxxiii, 425.

[11] Jones, W., and Kennedy, R. P., *J. Pharmacol. and Exp. Therap.*, 1919, xiii, 45.

Jones only in the fact that our material dried in air contained no crystal water, whereas the substance of Jones contained one crystal water. The optical rotation of our substance is $[\alpha]_D^{20} = -38.5$. Whereas the rotation of adenosinphosphoric acid remains constant either in water or in 5 per cent ammonia water, the rotation of guanosinphosphoric acid shows a marked increase in its levorotation in ammonia water. This point may serve for differentiation between the two nucleotides. Thus it is proven that the adenin-uridin dinucleotide is a mixture of two mononucleotides.

The fraction which was originally regarded by Jones as a guanin-cytosine dinucleotide, and from which Read isolated an amorphous guanylic acid, was also found by us to consist principally of guanosinphosphoric acid. In addition a small proportion of uridinphosphoric acid was found in this fraction. The presence of the latter nucleotide might have escaped isolation if not for its recently described property of forming a crystalline lead salt.

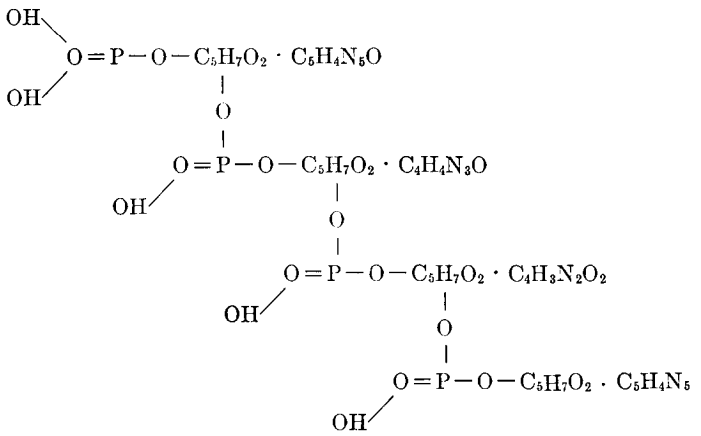
The guanylic acid was isolated in a crystalline form, and constituted the greater part of the fraction.

The method of hydrolysis employed by us consisted in heating the nucleic acid in a 2.5 per cent aqueous ammonia solution for 1 hour at 100°C. Thus the treatment was milder than the one employed by either Jones or Thannhauser. Thus the present findings nullify the experimental evidence in support of the theories of Jones and of Thannhauser. From the theoretical point of view, the theory of Thannhauser is not very tenable for the reason that a carbon to carbon-linking implies a very strong union, whereas the polynucleotide is readily dismembered into mononucleotides. Thannhauser, in fact, accepts it himself with great reserve. As regards the ether-linking accepted by Jones, it must be remarked that, as a rule, an ether-linking represents a very firm union. If one accepts that this rule does not apply to carbohydrates linked in ether form, he should present experimental evidence in support of this view.

However, if the work of Jones and of Thannhauser failed to support their speculations regarding the mode of linkage of the mononucleotides, it has been of great importance in furnishing further proof of the nucleotide structure of yeast nucleic acid;

and also, in making it possible to show that the molecule of nucleic acid is readily decomposed into mononucleotides, and that the linkage between all nucleotides is of the same order.

On the basis of considerations such as these the linkage of the nucleotides could be expressed most simply in the following way:



For the present this form expresses the facts known about the structure of yeast nucleic acid. New facts and new evidence may cause its alteration, but there is no doubt as to the polynucleotide structure of the yeast nucleic acid.

It is unfortunate that, owing to war conditions, the work of Thannhauser was not known to us earlier, also that apparently our work was not known to Thannhauser.

EXPERIMENTAL.

The mode of hydrolysis was practically the same as that described in a previous communication,[10] with a difference in one detail; namely, the temperature of the autoclave was maintained at 100°C.

Treatment of the product of hydrolysis was also the same as described in that communication, and essentially the same as employed by Jones and his collaborators. The fraction precipitated by 98 per cent alcohol will be referred to as guanin fraction, and that remaining in solution as adenin fraction.

Adenin fraction was treated in exactly the same manner as that described in the previous communication. The brucine salt was recrystallized nine times with boiling 35 per cent alcohol.

The crystalline deposit consisted of uridinphosphoric acid previously described. The first three mother liquors, on concentration, gave a brucine salt containing C = 53.00, H = 6.40, and N = 10 per cent. The subsequent six mother liquors, on concentration, gave a brucine salt containing N = 8.5 per cent.

The brucine salt of the first three mother liquors was transferred into the ammonium salt. Originally the ammonium salts were converted into the barium salt. Barium salts, having analytical value sufficiently approaching that required by the theory, were obtained only after many purifications which were associated with much loss. Finally, an attempt was made to transform the ammonium salt into the free nucleotide. This was accomplished without difficulty in the following manner. To the hot solution of the ammonium salt, while the mixture was agitated, a hot solution of neutral lead acetate was added in a slow stream. When the necessary volume of lead acetate (25 per cent solution) was added, the mixture was brought to a boil and filtered. The precipitate was washed in a mortar and filtered; the operation was repeated three times. Finally the precipitate was suspended in water, treated with hydrogen sulfide, and the filtrate from lead sulfide was concentrated under diminished pressure at room temperature. On standing, adenosinphosphoric acid crystallized in long needles resembling the free nucleoside. The substance differed from that described by Jones in that it crystallized without crystal water. The analysis of the air-dry substance was as follows:

0.1010 gm. of the substance gave 0.1268 gm. of CO_2 and 0.0368 gm. of H_2O.

0.1000 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 14.26 cc. of 0.1 N acid.

0.3000 gm. of the substance gave 0.0940 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_7$. *per cent*	Found. *per cent*
C	34.57	34.24
H	4.07	4.08
N	20.17	19.96
P	8.94	8.73

The optical rotation of the substance in aqueous solution was

$$[\alpha]_D^{20} = \frac{-0.77 \times 100}{1 \times 2} = -38.5$$

In a solution of 5 per cent ammonia water the rotation was

$$[\alpha]_D^{20} = \frac{-0.80 \times 100}{1 \times 2} = -40.0$$

Hydrolysis of the Adenosinphosphoric Acid.—2 gm. of the substance in 35 cc. of 1 per cent sulfuric acid were boiled over flame with reflux condenser for 1 hour. The product of hydrolysis was neutralized with sodium hydroxide and to the neutral solution aqueous picric acid was added as long as a precipitate formed. The precipitate was dissolved in hot water and allowed to crystallize.

The analysis of the air-dry substance was as follows:

0.1000 gm. of the substance gave 26.2 cc. of nitrogen gas at $T° = 26°C.$ and $P = 752$ mm.

	Calculated for $C_5H_5N_5.C_6H_2(OH)(NO_2)_3+H_2O$.	Found.
	per cent	*per cent*
N	29.32	29.60

The substance decomposed at 177°C. (uncorrected).

Brucine Salt of Adenosinphosphoric Acid.—2 gm. of the nucleotide were dissolved in hot water and the solution was neutralized with a solution of brucine in methyl alcohol. On cooling, the solution nearly solidified. The crystals of the brucine salt of the nucleotide were filtered off with suction, and the substance was recrystallized three times out of 35 per cent alcohol.

The air-dry substance on heating in a sealed capillary tube melted as follows: At 177°C. it began slightly to contract; at 195°, the substance effervesced, remaining perfectly colorless; at 225° a second point of effervescence was observed, the substance turning dark. The substance analyzed as follows:

0.1020 gm. of the substance gave 0.1978 gm. of CO_2 and 0.0572 gm. of H_2O.

0.2000 gm. of the substance gave 17.6 cc. of nitrogen gas at $T° = 24°C.$, $P = 769$ mm.

0.3000 gm. of the substance gave 0.0244 gm. of $Mg_2P_2O_7$.

	Calculated for $C_{10}H_{14}N_5PO_7.(C_{23}H_{26}O_4N_2)_2 \cdot 7H_2O$.	Found.
	per cent	*per cent*
C	53.28	52.88
H	6.40	6.28
N	10.00	10.23
P	2.47	2.27

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-0.74 \times 100}{1 \times 2} = -37\ 0$$

The guanin fraction consisted principally of guanosinphosphoric acid (guanylic acid). It was treated in a general way in the manner indicated by Read. The lead salts were converted into the brucine salts, and these were fractionated in the same manner as the salts of the adenin fraction. However, the substance obtained from all the nine mother liquors contained over 10 per cent of nitrogen and only the fraction constituting the ultimate crystalline deposit contained on analysis about 8.75 per cent of nitrogen. Surprisingly also this fraction consisted in the main of guanylic acid. The brucine salts were converted into the ammonium salts. These were dissolved in boiling water and to the hot solution a hot solution of lead acetate was added in a slow stream. The mixture was then brought to a boil and filtered hot. The lead precipitate was freed from lead and concentrated under diminished pressure at room temperature. Generally an amorphous, somewhat gelatinous precipitate settles out. In some instances the solution turns into a semiliquid jelly. To bring about final crystallization, no general rule can be given. At times repeated precipitation with lead acetate will lead to a filtrate which, on concentration, solidifies into a crystalline mass. Often it is advisable to precipitate the nucleotide by means of lead acetate fractionally. The later fractions as a rule crystallize with less difficulty.

The properties and analysis of the crystalline guanylic acid were described in a previous communication.[12]

When the brucine salts with 8.75 per cent of nitrogen were converted into ammonium salts, and when these were taken up

[12] Levene, P. A., *J. Biol. Chem.*, 1919, xl, 171.

in hot water, part of the substance remained insoluble. This residue consisted of brucine salt which escaped, being converted into ammonium salt. The brucine salt on analysis showed a nitrogen content of N = 7.8 per cent. This brucine salt was then converted into the ammonium salt. The latter was dissolved in boiling water, and a hot solution of neutral lead acetate was added. The mixture was brought to a boil and filtered hot. The filtrate was seeded with a few crystals of the lead salt of uridinphosphoric acid, and allowed to stand near a hot water bath. It was found that when the cooling of the filtrate proceeded rapidly a gelatinous lead salt settled out. If, however, the cooling was progressing slowly the lead salt of uridinphosphoric acid settled out in crystalline form. For analysis the substance was dried to constant weight under diminished pressure at the temperature of xylene vapor. It analyzed as follows:

0.1118 gm. of the substance gave 0.0856 gm. of CO_2 and 0.0218 gm. of H_2O.

0.1848 gm. of the substance employed for Kjeldahl nitrogen estimation required for neutralization 7.7 cc. of 0.1 N acid.

0.2772 gm. of the substance gave 0.0574 gm. of $Mg_2P_2O_7$.

	Calculated for $C_9H_{11}N_2PO_9Pb$. *per cent*	Found. *per cent*
C	20.40	20.73
H	2.10	2.18
N	5.29	5.84
P	5.86	5.78