THE COMPOSITION OF THE DESOXYRIBONUCLEIC ACID OF SALMON SPERM*

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Several studies from this laboratory have in the past few years dealt with the chemistry of nucleic acids. (For recent summaries, see Chargaff (1, 2).) The development of precise micromethods for the separation and quantitative estimation of the purines and pyrimidines has made possible the investigation of a large number of different nucleic acid preparations. These studies led to the conclusion that the desoxypentose nucleic acids (DNA) exhibited a composition that in many cases differed very considerably (3, 4) from that of calf thymus DNA (5). The present paper provides information on the composition of the highly polymerized DNA from the spermatozoa of the salmon (*Salmo salar*) and compares the results obtained with two different hydrolysis methods employed in this laboratory.

EXPERIMENTAL

Preparation

The spermatozoa serving as the starting material had been collected in Sweden; they formed, when freshly dried in the frozen state in a vacuum, a fine salmon-colored powder.[1] A portion (12.7 gm.) was suspended in 100 cc. of a salt solution representing a modification of the one employed by Pollister and Mirsky (6).[2] After centrifugation of the suspension at 2800 $\times g$, the sediment was distributed in 800 cc. of 10 per cent aqueous sodium chloride by being blended in a high speed mixer for 2 minutes. The mixture was kept for 24 hours and then diluted to a total volume of 5 liters

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[1] We are greatly indebted for this material to Professor J. Runnström and Mr. S. Lindvall.

[2] The components of the solution had the following molarities: sodium chloride 0.123, potassium chloride 0.009, potassium sulfate 0.004, sodium citrate 0.01. All operations were carried out in the cold.

by the addition of 10 per cent NaCl solution. Centrifugation, particularly unpleasant at this step, could be omitted, since the mixture was only slightly turbid and contained little solid material. The addition of 2 volumes of 95 per cent ethanol produced copious fibers which were spooled on a glass hook, drained by squeezing, and washed with 75 and 100 per cent ethanol.

The solution of the threads in 800 cc. of 10 per cent aqueous NaCl was deproteinized (7) by vigorous agitation in a high speed mixer with 150 to 250 cc. of chloroform-octanol (9:1). Six such treatments, each for 3 to 5 minutes, followed by centrifugation, were required. The resulting solution, clarified by centrifugation at 20,000 $\times$ g for 1 hour, was slowly injected below the surface of 2 volumes of ethanol kept at $-10°$. The resulting fibers of DNA were lifted immediately by means of a slowly and uniformly rotating glass hook, well drained, and washed with 75 and 100 per cent ethanol. Following dialysis of their solution in 0.14 M NaCl against running and distilled water for a total of 96 hours, the Na salt of the DNA was recovered by the evaporation of the frozen solution in a vacuum. It formed a white fiber felt, amounting in weight to about one-third of the starting material. In this manner two specimens were isolated.

Properties

Preparation 1 contained N (Dumas) 14.3, P (Pregl-Lieb) 8.9 per cent; Preparation 2, N 14.8, P 8.9 per cent.[3] The colorimetric comparison with the standard preparations of calf thymus DNA previously described (5) and yeast ribonucleic acid (8) made use of the reactions with diphenylamine (9) and orcinol (10) respectively. The results were as follows: Preparation 1, 101, Preparation 2, 108 per cent DNA; Preparation 1, 3.6, Preparation 2, 1.5 per cent ribonucleic acid.

Preparation 1 also was examined spectrophotometrically and viscosimetrically. The ultraviolet absorption spectrum (in M phosphate buffer of pH 7.1) exhibited a maximum at 260 mμ with an ϵ(P) of 6700 and a minimum at 231 mμ with an ϵ(P) of 4600. (For a definition of the expression ϵ(P), see Chargaff and Zamenhof (11).) The specific viscosity η_{sp} in distilled water was determined at 30.3° in an Ostwald-Fenske viscosimeter under gravity (water value, 10.8 seconds); it was found to be 29.6 for a 0.22 per cent solution and 5.6 and 2.0 for 0.11 and 0.055 per cent solutions respectively.

Hydrolysis

Two procedures were compared. In one, which will be referred to here as Procedure 1, the nitrogenous components were liberated and determined

[3] We are indebted to Miss R. Rother for these analyses.

by the methods described previously from this laboratory (5, 8, 12, 13); *i.e.*, the purines were set free by N H_2SO_4 (1 hour, 100°) and chromatographed in aqueous butanol-diethylene glycol (NH_3 atmosphere); the pyrimidines were liberated with concentrated formic acid (2 hours, 175°) after removal of the purines as the hydrochlorides by treatment with methanolic HCl, and chromatographed in aqueous butanol.

The second method, Procedure 2, is considerably simpler and permits the use of even smaller quantities of DNA (2 to 6 mg. for a complete analysis) than was possible heretofore; its use was briefly mentioned in a recent paper (14). It is based on the simultaneous liberation of all purines and pyrimidines by the hydrolysis of the DNA with concentrated formic acid and on their separation, on the same chromatogram, by means of ammoniacal butanol. When it is desired to establish complete balances, *i.e.* to express the molar distribution of individual nitrogenous constituents not only in terms of P present in the hydrolysate, but also in relation to the total N and P of the nucleic acid, about 6 mg. of DNA are used in the analysis. The sample (dried in a high vacuum at 60° for 3 hours) was heated in a sealed small bomb tube (about 220 × 6.5 mm.) with 0.8 cc. of concentrated formic acid (98 to 100 per cent) at 175° for 2 hours. The hydrolysate was transferred quantitatively to a 1 cc. volumetric flask and adjusted to volume by means of small portions of distilled water which served to rinse the hydrolysis vessel. Aliquots, measured with an accurate micro burette in the usual manner (12), were employed for the colorimetric P determination (0.04 cc. of hydrolysate) and for the chromatographic separation (0.01 cc.). When very small quantities of DNA were to be analyzed, 2 mg. of the substance were heated with 0.2 cc. of concentrated formic acid and the hydrolysate was, without volume adjustment, analyzed for P and for individual purines and pyrimidines. In this case, all computations were based on the ratio of moles of nitrogenous constituent to moles of phosphorus present in the hydrolysate (13, 14).

The chromatographic and spectroscopic techniques were, in general, similar to those described previously (8, 12). The paper sheets (16 × 46 cm.), carrying five lanes 3 cm. wide, were left for 20 minutes in an ammonia atmosphere in order to neutralize the acid. The solvent used for the chromatographic separation consisted of a mixture of 6 volumes of *n*-outanol and 1 volume of 0.6 N aqueous ammonia. Two beakers, one containing the solvent mixture, the other 0.6 N ammonia, were placed alongside each paper sheet at the bottom of the jar used for chromatography; the paper sheets were suspended, the solvent trough being left empty, and allowed to equilibrate with the solvent atmosphere for 1 hour in the closed vessel. Then the trough was filled with the solvent mixture and the separation permitted to proceed for 18 to 20 hours, at which time the

solvent front had passed over the lower edge of the paper. After the papers had dried in air, the position of the separated components was marked under a Mineralight lamp or a similar ultraviolet lamp.[4] The components, in the order of increasing distance from the starting point, were aligned as follows (the distance of thymine arbitrarily taken as 100): guanine (26), cytosine (47), adenine (71), thymine.[5] The quantitative determinations were carried out as described before (12).

That the pyrimidines are recovered without loss after heating in concentrated formic acid under the conditions of the hydrolysis has been

TABLE I

*Resistance of Purines to Heating with Concentrated Formic Acid**

Experiment No.	Heating time	Adenine				Guanine			
		Amount in mixture	Amount recovered	Standard error	No. of separation experiments	Amount in mixture	Amount recovered	Standard error	No. of separation experiments
	min.	*γ*	*per cent*	*per cent*		*γ*	*per cent*	*per cent*	
1	0	9.4	95.9	0.7	8	6.6	100.3	1.8	8
2	120	9.4	100.7	1.1	12	6.6	101.1	1.4	12
3	120	9.4	97.8	0.8	9	6.6	102.5	1.2	11
4	0	7.4	99.8	0.8	8	4.4	102.3	2.5	7
5	120	7.4	101.7	0.3	9	4.4	102.8	1.7	12
6	120	7.4	95.7	0.6	12	4.4	93.6	1.6	9

* Solutions in concentrated formic acid of mixtures of adenine and guanine of known concentration were employed. Portions were directly subjected to chromatographic separation and estimation. Other aliquots (0.7 cc.) were heated at 175° for 2 hours in sealed bomb tubes, adjusted with water to a volume of 1 cc., and analyzed.

demonstrated previously (8). Similar data are provided here in Table I for the purines.

Composition

The figures for the purine and pyrimidine composition of the two preparations of salmon sperm DNA, as found in individual hydrolysis experi-

[4] We are very grateful to Dr. A. Marshak of New York University for drawing our attention to the fact that even better definition is obtained when a General Electric mercury lamp (No. G8T5), equipped with Corning glass filter No. 9863, is employed.

[5] When 5-methylcytosine, occasionally encountered as a minor DNA constituent (15), is to be estimated at the same time, a mixture of 6 volumes of *n*-butanol and 1 volume of 0.1 N ammonia has been found preferable as the solvent system (unpublished experiments with Mr. G. Brawerman). The alignment of separated components, again with thymine as the reference point, was as follows: guanine (35) cytosine (51), 5-methylcytosine (65), adenine (80), thymine.

ments performed by the two procedures outlined above, are recorded in Table II. In order not to make Table II unwieldy, the values were reduced to two places and only one set of figures given; *viz.*, the molar proportions of purines and pyrimidines to phosphorus present in the hydrolysate. In the analyses reported here, the values would have remained practically unchanged had they also been recorded in terms of the N and P content of the individual nucleic acid specimen subjected to hydrolysis,

TABLE II

Purine and Pyrimidine Contents of Salmon Sperm DNA

The results are expressed in moles per mole of P in the hydrolysate.

Experiment No.*	Preparation No.	Hydrolysis procedure	Nitrogenous constituent				Recovery of nitrogenous constituents		
			Adenine	Guanine	Cytosine	Thymine	Purines	Pyrimidines	Total
1	1	1	0.27	0.18			0.45		
2		1	0.26	0.19			0.45		
3		1			0.17	0.28		0.45	
4		1			0.18	0.28		0.46	
5		2	0.28	0.20	0.21	0.27	0.48	0.48	0.96
6		2	0.30	0.22	0.20	0.29	0.52	0.49	1.01
7		2	0.27	0.18	0.19	0.25	0.45	0.44	0.89
8		2	0.28	0.21	0.20	0.27	0.49	0.47	0.96
9	2	1	0.25	0.18			0.43		
10		1	0.29	0.20			0.49		
11		2	0.29	0.18	0.20	0.27	0.47	0.47	0.94
12		2	0.28	0.21	0.19	0.26	0.49	0.45	0.94
13		2	0.30	0.21	0.20	0.30	0.51	0.50	1.01

* In each experiment between twelve and twenty-four determinations of individual purines and pyrimidines were performed.

as was done previously (5, 8). Care was taken to ascertain the completeness of hydrolysis in all cases reported.

In Table III, the results yielded by both hydrolysis procedures are compared and examined statistically and average figures for the composition are derived. The molar relationships between the constituents are summarized in Table IV. The significance of the comparison between the value calculated for the average number of gm. atoms of nitrogen per mole of constituent in the hydrolysate and the atomic N:P ratio found in the intact nucleic acid has been discussed previously (5, 8).

It appeared of interest to study the effects of briefer periods of hydrolysis with concentrated formic acid than 2 hours at 175°, as generally used here. When Preparation 2 was heated with formic acid at 175° for 30 minutes, the results (in moles of liberated constituent per mole of P

in solution) were as follows: adenine 0.28, guanine 0.19, cytosine 0.20, thymine 0.07; purines recovered 0.47, pyrimidines 0.27. After heating for 1 hour under the same conditions, the corresponding figures, listed in

TABLE III

Salmon Sperm DNA; Proportions (in Moles of Nitrogenous Constituent per Mole of P in Hydrolysate)

Constituent	Procedure 1			Procedure 2			All analyses	
	No. of hydrolyses*	Mean proportion	Standard error	No. of hydrolyses*	Mean proportion	Standard error	Mean proportion	Standard error
Adenine	4	0.267	0.007	7	0.287	0.005	0.280	0.005
Guanine	4	0.186	0.004	7	0.200	0.006	0.196	0.004
Cytosine	2	0.175	0.001	7	0.197	0.003	0.192	0.006
Thymine	2	0.279	0.002	7	0.273	0.006	0.274	0.005
Total		0.907			0.957		0.942	

* In each hydrolysis between twelve and twenty-four determinations of individual nitrogenous constituent were performed.

TABLE IV

Salmon Sperm DNA; Molar Relationships

Molar ratio*	
Adenine to guanine	1.43
Thymine " cytosine	1.43
Adenine " thymine	1.02
Guanine " cytosine	1.02
Purines " pyrimidines	1.02
P accounted for, % P in hydrolysate†	95.8 (1.6)
Average gm. atoms N per mole constituent	3.7
Atomic N:P ratio in nucleic acid preparations	3.6, 3.7

* The computations of the molar ratio are based on the mean proportions of each nitrogenous constituent found in all analyses (Table III).

† The recovery figure (standard error in parentheses) is based on the average of the total recoveries recorded in all hydrolysis experiments carried out by Procedure 2 (last column, Table II).

the same order, were 0.27, 0.17, 0.19, 0.25; 0.44, 0.44. A comparison of these figures with those listed in Tables II and III will show that the liberation of adenine, guanine, and cytosine was almost complete, even after a heating period as short as 30 minutes, but that thymine lagged far behind. This refractoriness of thymidylic acid is reminiscent of the similar behavior of the ribose nucleotide uridylic acid (16).

Sugar

The sugar released by the purine portion of the DNA molecule was examined chromatographically by the procedures described before (5). The enzymatic degradation to the nucleoside stage was carried out simultaneously with 5 mg. samples of Preparation 1 of salmon sperm DNA and of a specimen of calf thymus DNA. Control experiments in which the nucleic acid was omitted also were performed. The liberation of the desoxy sugar by controlled heating at pH 1.5, the chromatography in three different solvent systems, and the development of the chromatograms with *m*-phenylenediamine (17) followed, in all details, the previous methods (3, 5). The sugar obtained from the salmon sperm DNA occupied the same position on all chromatograms as 2-desoxyribose released from calf thymus DNA.

DISCUSSION

The DNA of salmon sperm analyzed here provides a good instance of a number of regularities stressed in previous publications from this laboratory. It belongs to the "AT type" (4) in which adenine and thymine outweigh guanine and cytosine, in this particular nucleic acid to the extent of about 40 per cent (see Table IV). Not only the ratio of purines to pyrimidines but also that of adenine to thymine and of guanine to cytosine equals 1 (*cf.* (1, 2)). As the number of examples of such regularity increases, the question will become pertinent whether it is merely accidental or whether it is an expression of certain structural principles that are shared by many desoxypentose nucleic acids, despite far reaching differences in their individual composition and the absence of a recognizable periodicity in their nucleotide sequence (14). It is believed that the time has not yet come to attempt an answer.

The present study has offered the opportunity of comparing the results yielded by two different hydrolysis procedures. The methods used heretofore (8) required two separate hydrolyses, one for the determination of purines, another for that of pyrimidines. The simpler procedure, applied in many instances in the present study, in which all nitrogenous constituents are simultaneously liberated by formic acid and separated on one chromatogram, offers many advantages. It requires less material and leads to a somewhat better recovery of the separated constituents. The figures summarized in Tables II and III provide a basis for the comparison of the merits of both hydrolysis methods. It will be seen that the mean ratio of adenine to guanine remained unchanged, *viz.* 1.43 irrespective of the procedure employed. The ratio of thymine to cytosine was 1.59 in Procedure 1, 1.39 in Procedure 2. This seems to be due to a slightly lower recovery of cytosine in the first method of hydrolysis. The ratios listed

in Table IV were based on the mean proportions of each nitrogenous constituent. When the molar relationships found for each hydrolysate were computed individually and compared, as has been done previously (1, 2, 13), the mean ratio of adenine to guanine was 1.44, with a standard error of 0.02; that of thymine to cytosine was 1.42, with a standard error of 0.03.

In the DNA of salmon sperm discussed here the two hydrolysis methods yielded essentially similar results. It should, however, be stressed that it is desirable to use more than one cleavage procedure for the investigation of substances as complicated as the nucleic acids, since eventual differences in the results could provide a useful indication of structural peculiarities.

The identification of the sugar component of salmon sperm DNA as desoxyribose, apparently here attempted for the first time, must remain tentative as long as the chromatographic properties of 2-desoxyxylose, and for that matter of other desoxy sugars, are unknown.

SUMMARY

Highly polymerized preparations of the desoxypentose nucleic acid of salmon sperm were isolated and analyzed for their contents in adenine, guanine, cytosine, and thymine. The results obtained by two different hydrolysis methods were compared. A procedure permitting the complete analysis of as little as 2 mg. of a nucleic acid is described. The sugar component was identified tentatively as 2-desoxyribose.

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