Hydrazinolysis of Nucleic Acids. I. The Formation of Deoxyribopyrimidinic Acid from Herring Sperm Deoxyribonucleic Acid

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(Received March 23, 1959)

In spite of our recognition of the biological importance of nucleic acid, there are many points on the structure of nucleic acid still left ambiguous, i. e., the nucleotide sequence, the localization in the molecule of certain nucleotides, the structural differences between nucleic acids of different origin, and so on. Some fruitful information on the general structural features of deoxyribonucleic acid has been obtained by Chargaff et al. from chemical degradative studies of a derivative, named apurinic acid, in which deoxyribonucleic acid has been deprived of the purine constituents[1-4]. Many other chemical degradations of deoxyribonucleic acid by which the nucleotide sequence can be studied have been attempted[5-13]. These

1) C. Tamm, M. E. Hodes and E. Chargaff, *J. Biol. Chem.*, **195**, 49 (1952).
2) C. Tamm, H. S. Shapiro, R. Lipshitz and E. Chargaff, ibid., **203**, 673 (1953).
3) C. Tamm and E. Chargaff, ibid., **203**, 689 (1953).
4) M. E. Hodes and E. Chargaff, *Biochim. et Biophys. Acta*, **22**, 348 (1956).
5) A. S. Jones and D. S. Letham, ibid, **14**, 438 (1954).
6) A. S. Jones and D. S. Letham, *J. Chem. Soc.*, **1956**, 2573.
7) A. S. Jones, D. S. Letham. and M. Stacey, ibid., **1956**, 2579.
8) A. S. Jones, D. S. Letham and M. Stacey, ibid., **1956**, 2584.
9) A. S. Jones, M. Stacey and B. E. Watson, ibid., **1957**, 2454.
10) J. A. Lucy and P. W. Kent, *Research*, **6**, 49s (1953).

studies have more or less utilized the particular instability of the purine deoxyribosidic linkages to even a mild acid hydrolysis. Therefore, the information about the nucleotide sequences has been exclusively limited to the pyrimidines in deoxyribonucleic acid. No information was available about the distribution of purines in deoxyribonucleic acid, or of either purines or pyrimidines in ribonucleic acid.

Attention was paid to the reaction of aqueous hydrazine with uracil[14], pyrimidine ribonucleosides[15,16] and ribomononucleotides[16]. Of the latter, uracil and cytosine residues were decomposed to pyrazolone and 3-aminopyrazole, respectively, and simultaneously, ribose 2- and 3-phosphate were obtained from the nucleotide isomers, a and b, respectively. This reaction of hydrazine was successfully applied to both ribo- and deoxyribonucleic acid from which for the first time nondialyzable derivatives almost free from the pyrimidines were obtained and the name ribo- and deoxyribo-apyrimidinic acid were proposed for the products[17,18]. These compounds, especially deoxyribo-apyrimidinic acid, may be useful for structural studies as well as apurinic acid[2,4] and aldehydoapurinic acid di(carboxymethyl)-dithioacetal[7,9].

Anhydrous hydrazine was used for this work, in order to avoid alkaline hydrolysis of the internucleotide linkage which might be caused by the basicity of hydrazine itself even if only a trace of water remained. Since powdered deoxyribonucleic acid of herring sperm took more than 30 min. for its complete dissolution in five parts of anhydrous hydrazine at 60°C, lyophilized preparations were used to prevent a heterogeneous reaction. Experiments using various reaction conditions were carried out and are summarized in Table I. In the course of dissolution in distilled water of the alcohol precipitates after the hydrazinolysis, the viscosity of the solution rapidly decreased. Hydrazine was expected to remain in the precipitates and to cause alkaline hydrolysis. Therefore, in the preparation of DAPYA V, V′ and V″, the alcohol precipitates were dissolved in 0.2 M phosphate buffer of pH 6 and dialyzed. The products thus obtained proved to have longer mean chain lengths and fewer periodate consumptions than others (Table V).

The hydrazinolysis of the deoxyribonucleic acid afforded products the nitrogen content and the N/P ratio of which were lower than those of the parent materials (Table II). Increased removal of the pyrimidines resulted in higher phosphorus contents (Table II). The color intensities by the reaction with diphenylamine were, on the basis of phosphorus content, much more than those given by the parent nucleic acids, although the color intensities were not always parallel to the degree of detachment of the pyrimidines. The estimation of reducing sugar proved that the products had a considerable amount of reducing power, whereas the nucleic acids had only a little. These results, together with the positive qualitative tests for reducing sugar, show that the bulk of the sugar moieties, which in the intact nucleic acid were pyrimidine nucleotides, are in nonglycosidic forms. During the hydrazinolysis and the subsequent treatment, there may be possibilities of side reactions, e. g., formation of hydrazonium salt, hydrazone and sugar alcohol. However, it can be seen from the ratio of N/P that these side reactions occur only slightly if at all.

As shown in Table III the greater part of the pyrimidines was removed from herring sperm deoxyribonucleic acids without impairment of the original interpurine ratios, but small amounts of thymine remained, even in the product obtained through the reaction by which the removal of the pyrimidines was most effective. As for pyrimidine ribomononucleotides, uracil was far more rapidly decomposed by aqueous hydrazine than was cytosine, which was studied by Baron and Brown[16]. Hence it is concluded that thymine is the most resistant to the hydrazinolysis among the main pyrimidine constituents in nucleic acids. The reaction of hydrazine, in the presence of water, gave directly ribose 3-phosphoric acid from pyrimidine ribonucleotides. In the absence of water, however, ureido-deoxyriboside phosphate residues may be formed from the pyrimidine nucleotide residues in the

11) P. W. Kent, J. A. Lucy and P. F. V. Ward, *Biochem. J.*, **61**, 529 (1955).
12) K. Burton and G. B. Peterson, *Biochim et Biophys. Acta*, **26**, 667 (1957).
13) H. S. Shapiro and E. Chargaff, ibid., **23**, 451(1957).
14) H. R. Fosse, J. Hieulle and L. W. Bass, *Compt. rend.*, **178**, 811 (1955).
15) P. A. Levene L. W. Bass, *J. Biol. Chem.*, **71**, 167 (1926).
16) F. Baron and D. M. Brown, *J. Chem. Soc.*, **1955**, 2855.
17) S. Takemura, *J. Biochem.*, **44**, 321 (1957).
18) S. Takemura, *Biochim. et Biophys. Acta*, **29**, 447 (1958).

TABLE I. REACTION OF HERRING SPERM DEOXYRIBONUCLEIC ACID WITH ANHYDROUS HYDRAZINE

Preparation[a]		Amount of DNA	Amount of Anhydrous hydrazine	Reaction temp.	Reaction time	Yield of nondialyzable product	
		mg.	ml.	°C	min.	mg.	%[b]
DAPYA	I	850	3.5	50	30[d]	650	87
DAPYA	II	240	1.0	60	30[d]	170	80
DAPYA	III	120	0.7	60	55	71	72
DAPYA	IV	600	2.8	60	60	389	78
DAPYA	V	248	1.2[c]	37	90	208	95
DAPYA	V′	150	0.7[c]	50	75	113	86
DAPYA	V″	195	1.0[c]	50	150	130	82
APA	II	140	HCl, pH 1.6	37	24 hr.	98	91
APA	IV	250	HCl, pH 1.6	37	26 hr.	183	94
PDRP	ID	150	HCl, pH 1.6	37	26 hr.	39	26
PDRP	IID	70	HCl, pH 1.6	37	26 hr.	17	26
PDRP	IIA	50	0.2	60	60	9.5	17
PDRP	IVD	150	HCl, pH 1.6	37	27 hr.	58	42

a) DAPYA=deoxyribo-apyrimidinic acid; APA=apurinic acid; PDRP=poly-deoxyribose phosphate. DAPYA I and II were obtained from powdered DNA and through acid precipitation; DAPYA III and IV obtained from lyophilized DNA and through acid precipitation; DAPYA V, V′ and V″ obtained from lyophilized DNA and through dissolution in phosphate buffer (See *Experimental*).

b) These are per cent of DNA phosphorus recovered in the product

c) Twice dehydrated hydrazine was used.

d) Reaction times after the complete dissolution of DNA.

TABLE II. COMPOSITION OF THE HYDRAZINOLYSIS PRODUCTS OF HERRING SPERM DEOXYRIBONUCLEIC ACID. PART 1

Preparation[a]		N (%)	P (%)	Atomic N/P	Color reaction with diphenylamine[b]	Reducing deoxyribose residues, mol. per 100 g.-atoms of P[c]
DNA	I	13.4	8.0	3.7	100	5
DNA	II	13.6	8.2	3.7	100	4
DNA	III	13.7	8.2	3.7	100	6
DNA	IV	14.2	8.5	3.7	100	5
DNA	V	13.7	8.2	3.7	100	—
DAPYA	I	12.2	9.1	3.0	135	30
DAPYA	II	12.0	9.6	2.8	156	31
DAPYA	III	12.3	10.0	2.7	133	27
DAPYA	IV	12.2	10.2	2.7	131	36
DAPYA	V	12.3	9.2	3.0	—	—
DAPYA	V′	12.0	9.5	2.8	—	—
DAPYA	V″	11.9	10.1	2.6	—	—
APA	II	6.3	10.6	1.3	116	54
APA	IV	6.5	10.9	1.3	99	51
PDRP	ID	7.4	9.2	1.8	87	51
PDRP	IID	4.7	10.3	1.0	107	63
PDRP	IIA	3.6	9.6	0.85	113	—
PDRP	IVD	3.9	11.0	0.79	110	63

a) See Table I.

b) Compared with each DNA on the basis of P content.

c) Compared with deoxyribose as the standard.

TABLE III. COMPOSITION OF THE HYDRAZINOLYSIS PRODUCTS OF HERRING SPERM DEOXYRIBONUCLEIC ACID. PART 2

Preparation[a]		Mol. of bases/100 g.-atoms of phosphorus[b]						Molar ratios of	
		Adenine	Guanine	Cytosine	Thymine	Total purines	Total pyrimidines	A/G	T/C
DNA	I	29	23	21	27	52	48	1.3	1.3
DNA	II	27	24	22	27	51	49	1.1	1.2
DNA	III	29	23	21	27	52	48	1.3	1.3
DNA	IV	29	23	21	27	52	48	1.3	1.3
DNA	V	28	23	22	27	51	49	1.2	1.2
DAPYA	I	29	23	1.2	19	52	20	1.3	
DAPYA	II	27	24	0.7	8.0	51	8.7	1.1	
DAPYA	III	29	23	0.2	5.4	52	5.6	1.3	
DAPYA	IV	29	23	0.3	4.1	52	4.4	1.3	
DAPYA	V	28	23	1.2	16	51	17	1.2	
DAPYA	V'	28	23	1.0	10	51	11	1.2	
DAPYA	V''	27	24	0.5	5.2	51	5.7	1.1	
APA	II	1.6	2.6	22	27	4.2	49		1.2
APA	IV	0.6	1.0	22	26	1.6	48		1.2
PDRP	IID	2.8	4.3	0.7	6.6	7.1	7.3		
PDRP	IVD	1.8	2.4	0.7	8.3	4.2	9.0		

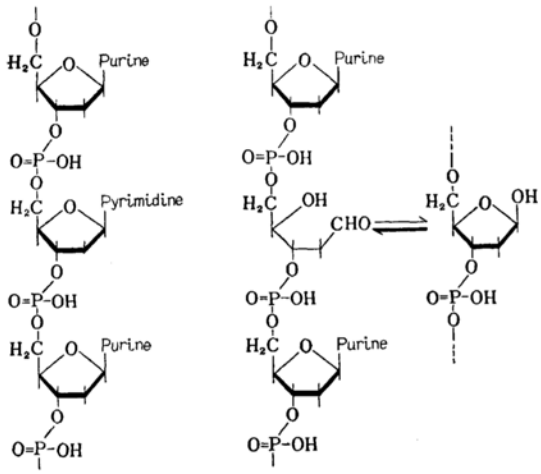
a) See Table I.
b) The values were corrected for a 100% recovery. Actual recoveries of total bases were between 89 and 96%.

TABLE IV. ULTRAVIOLET ABSORPTION SPECTRA OF THF HYDRAZINOLYSIS PRODUCTS OF HERRING SPERM DEOXYRIBONUCLEIC ACID[a]

Preparation[b]		Maximum			Minimum		
		mμ.	E_p*	E_{np}**	mμ.	E_p*	E_{np}**
DNA	I	259	7350		230	3100	
DNA	II	259	7200		230	2950	
DNA	III	258	6850		230	2900	
DNA	IV	258	7700		230	3200	
DAPYA	I	257.5	7200	10000	228	2850	3950
DAPYA	II	256.5	6900	11500	227.5	2800	4650
DAPYA	III	255	6450	11100	227.5	2450	4250
DAPYA	IV	255	6650	11900	227	2600	4650
APA	II	268	4450	8400	238	2700	5100
APA	IV	267.5	4450	8900	237	2650	5300
PDRP	ID	260	5100		232	2500	
PDRP	IID	260	3500		235	2350	
PDRP	IVD	256.5	2400		234	1900	

a) All measurements were carried out in 0.1 M phosphate buffer of pH 7.0.
* The extinction coefficient with respect to one gram-atom of phosphorus per liter.
** The extinction coefficient with respect to one gram-atom of nucleotide phosphorus per liter.
b) See Table I.

deoxyribonucleic acid. The subsequent treatment of the intermediate product may hydrolyze the glycosidic linkage between deoxyribose and urea which seems to be unstable in the presence of water. The deoxyribose phosphate residues thus formed may be present in an equilibrium between the furanose (III) and the free aldehydo-form (II) in the aqueous solution of the deoxyribo-apyrimidinic acid, but are certainly present to a considerable extent in the free aldehydo-form[19-21].



(I) (II) (III)

To remove both the purines and the pyrimidines, the apurinic acid from herring sperm deoxyribonucleic acid was treated with anhydrous hydrazine and the deoxyribo-apyrimidinic acid was treated with dilute acid. These products contained bases to the extent of about 10 to 15 mol. % of total phosphorus. The combination of the mild acid hydrolysis with the hydrazinolysis, however, caused greater degradation of the internucleotide linkages than did the hydrazinolysis alone. Moreover, the color intensities with diphenylamine and the estimations of reducing sugar of the products seem to show that a part of the deoxyribose moieties undergo some unknown decompositions.

The mean chain length of apurinic acid (Table V) corresponds to a mean molecular weight of about 5,000. This value is much lower than that obtained by Tamm et al. (15,000; light scattering method)[4] and that by Miura et al. (17,000;

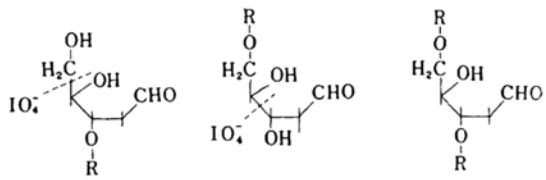
19) S. M. Cantor and Q. P. Peniston, *J. Am. Chem. Soc.*, **62**, 2113 (1940).
20) Chong-Fu Li, W. G. Overend and M. Stacey, *Nature*, **163**, 538 (1949).
21) W. G. Overend, *J. Chem. Soc.*, **1950**, 2769.

TABLE V. MEAN CHAIN LENGTH AND PERIODATE CONSUMPTION OF THE HYDRAZINOLYSIS PRODUCTS OF HERRING SPERM DEOXYRIBONUCLEIC ACID

Preparation*		Mean chain length	Periodate consumption, mol. per 1 mol. of P
DAPYA	I	7.3	0.24
DAPYA	III	8.4	0.20
DAPYA	IV	7.0	0.34
DAPYA	V	15	0.17
DAPYA	V'	10	0.17
DAPYA	V''	10	0.19
APA	IV	18	0.14
PDRP	IVD	4.8	0.57

* See Table I.

surface membrane method)[22]. This discrepancy may be partly due to the quality of the parent deoxyribonucleic acid and also to the presence of many terminal phosphate groups caused by the inclusion in the apurinic acid of a small amount of oligonucleotides. Consequently, it can be assumed that the deoxyriboapyrimidinic acids contain many fragments of much longer chain length than the mean, although fission of the phsophodiester linkages during the hydrazinolysis and the subsequent treatment may have been considerable. Periodate should be consumed only by the nonglycosidic deoxyribose residues which occupy the chain end of nucleotide and do not bear terminal phosphate groups IV and V. If both ends are occupied by nucleotides or bear the terminal phosphate group VI, periodate



(IV) (V) (VI)

can not be consumed. Therefore, it is impossible to predict the amount of the periodate consumption of the hydrazinolysis products from their mean chain lengths. The ultraviolet absorption coefficient of the purines are, on the molar basis, greater than that of the pyrimidines. Hence, in addition to the hyperchromic effects of degradation, the removal of the

22) K. Miura, K. Imahori and I. Watanabe, *J. Chem. Soc. Japan, Pure Chem. Sec.* (*Nippon Kagaku Zasshi*), **78**, 276 (1957).

pyrimidines results necessarily in the higher absorption coefficient with respect to nucleotide phosphorus (E_{np}) than that of the apurinic acid and the deoxyribonucleic acid (Table IV).

Some fission of the internucleotide linkages occurred during the hydrazinolysis and the subsequent treatment, and small amounts of intact thymine were left in the preparations. However, there will be possibilities to improve the preparative method. Since about 80% of the material was nondialyzable and the interpurine ratios were not distorted during the reaction, the deoxyribo-apyrimidinic acid must be useful for the structural studies of deoxyribonucleic acid. The apyrimidinic acids were used for the study of the specificity of pancreatic deoxyribonuclease which are reported in the subsequent paper.

Experimental

Nitrogen was determined by micro-Kjeldahl method and phosphorus by Allen's method[23]. The color reaction of the hydrazinolysis products with diphenylamine[24] was carried out and compared with each parent deoxyribonucleic acid as the standard. The nonglycosidic deoxysugars which were initially bound to the pyrimidines also participate in the color reaction. Reducing deoxysugar was determined by means of Fujita-Iwatake's method[25]. These results are summarized in Table II. The base composition was determined chromatographically after hydrolyzing samples in a scaled tube with 70% perchloric acid at 100°C for 1 hr.[26] (propan-2-ol—hydrochloric acid[27], Toyo Roshi Co. 51a paper, descending). Each value listed in Table III represents the average of at least three individual determinations. The absorption spectra were determined in 0.1 M phosphate buffer of pH 7.0 (Fig. 1 and Table IV). The mean chain length was determined by the ratio of the total phosphorus to the terminal phosphate which was liberated as inorganic phosphorus by prostatic phosphomonoesterase[28] (Table V). The periodate consumption was carried out at pH 4 with sodium metaperiodate, in the dark at 10°C or below, for 60 min.; excess periodate was reduced with sodium arsenite at pH 8 and the excess arsenite was back titrated with standard iodine solution[29] (Table V).

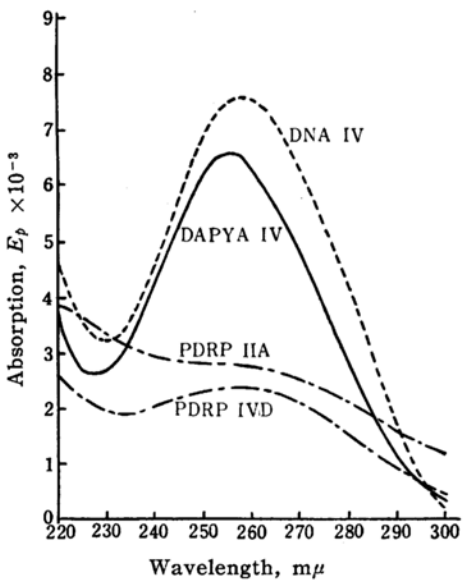


Fig. 1. Ultraviolet absorption curves of the hydrazinolysis products of herring sperm deoxyribonucleic acid in phosphate buffer, pH 7.
E_p=extinction per g.-atom of phosphorus.

Dehydration of Hydrazine.—Eighty per cent hydrazine hydrate was dehydrated with sodium hydroxide pellets at 113°C for 2 hr. and distilled[30]. For more strict dehydration, the distillate was again refluxed with sodium hydroxide pellets at 180°C for 3 hr. and distilled[31].

Deoxyribonucleic Acids.—Five preparations of the sodium deoxyribonucleate of herring sperm, DNA I to V, were used. DNA III was a highly polymerized specimen (MW. ca. 6×10^6)[32]. Before treatment with anhydrous hydrazine, the nucleic acids were dried over concentrated sulfuric acid in vacuo at 60°C for 5 hr. or at 100°C for 3 hr. The compositions of the specimens are summarized in Tables II and III.

Designation of the Reaction Products.—The hydrazinolysis gave products in which the nucleic acid was more or less deprived of the pyrimidines. In order to avoid confusion and to simplify descriprions, the name deoxyribo-apyrimidinic acid (DAPYA) is given to all the hydrazinolysis products and the name polydeoxyribose phosphate (PDRP) to the products obtained either by the hydrazinolysis of the apurinic acid (APA) or by the mild acid hydrolysis, as used for the preparation of apurinic acid, of the hydrazinolysis product. The preparation numbers of the reaction products are the same as those given to the parent deoxyribonucleic acids, i. e., DAPYA II and APA II were obtained from DNA II, PDRP IIA from APA II, and PDRP IID from DAPYA II.

23) F. W. Allen, *Biochem. J.*, **34**, 858 (1940).
24) Z. Dische, *Mikrochem.*, 8, 4 (1930); F. B. Seibert, *J. Biol. Chem.*, **133**, 593 (1940).
25) A. Fujita and D. Iwatake, *Biochem. Z.*, **242**, 43 (1932).
26) A. Marshak and H. J. Vogel, *J. Biol. Chem.* **189**, 597 (1951).
27) G. R. Wyatt, *Biochem. J.*, **48**, 584 (1951).
28) P. R. Whitfeld and R. Markham, *Nature*, **171**, 1151 (1953).
29) J. R. Dyer, "Methods of Biochem. Anal.", Vol. III, Interscience Publishers, Inc., New York, (1955). p. 111.
30) I. M. Kolthoff, *J. Am. Chem. Soc.*, **46**, 2009 (1924).
31) D. H. R. Barton, D. A. J. Ives and B. R. Thomas, *J. Chem. Soc.*, **1955**, 2056.
32) K. Iso and I. Watanabe, *J. Chem. Soc., Japan, Pure Chem. Sec. (Nippon Kagaku Zasshi)*, **78**, 1268 (1957).

Preparation of the Deoxyribo-apyrimidinic Acids.—The mixture of lyophilized herring sperm deoxyribonucleic acid and anhydrous hydrazine was kept free from moisture under the conditions described in Table I. Within a few minutes, the nucleic acid dissolved in the hydrazine, excepting deoxyribonucleic acid III which took about 10 min. for its complete dissolution. After cooling, absolute alcohol (5 vol.) was added to the highly viscous solution, the fibrous precipitate was made into a powder by scratching, centrifuged, washed with absolute alcohol, with ether, and dried. The dry solid was dissolved with cooling in cold distilled water and the solution dialyzed against running tap water for 20 hr., against frequent changes of distilled water at 4°C for 40 hr. and the inside fluid was freeze-dried. Light yelllow colored products were obtained. The composition of the products thus obtained is listed in Tables II and III.

When the alcohol precipitate was dissolved in 0.2 M phosphate buffer of pH 6 instead of distilled water, the solution was dialyzed against the same buffer at 4°C for 16 hr. against running tap water and finally against distilled water. Faint yellow products were obtained and are described as DAPYA V, V′ and V″ in the tables.

Preparation of Poly-deoxyribose Phosphate.—Apurinic acid was prepared from herring sperm deoxyribonucleic acid in the same way as it was prepared by Tamm et al.[1] Apurinic acid II was dried at 60°C for 5 hr. and then treated with anhydrous hydrazine as above. Deoxyribo-apyrimidinic acids I, II and IV were also treated by the same mild acid hydrolysis as used for the preparation of apurinic acid. The nondialyzable products of yellowish brown color were obtained from each treatment and were incompletely soluble in water, although these gave clear solutions before freeze-drying. The composition of these products is given in Tables II and III.

Qualitative Tests for Nonglycosidic Deoxysugar.—In contrast to the deoxyribonucleic acid, the hydrazinolysis products and the apurinic acid reacted with ammoniacal silver solution yielding brown precipitates, reduced Fehling's solution on warming, reacted with benzidine giving a brown color, reduced mercuric chloride solution yielding a white precipitate, and gave pink color with reduced fuchsin solution, but the reaction of the hydrazinolysis products was much slower than that of the apurinic acid. The hydrazinolysis products spotted on filter paper gave a reddish purple color with the aniline hydrogen phthalate reagent used for the development of chromatogram of reducing sugars[33].

The author thanks Professor F. Egami for his close interest and Professor I. Watanabe, the University of Tokyo, for a supply of herring sperm deoxyribonucleic acids. The expense of this study was defrayed in part by a grant from the Ministry of Education.

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33) S. M. Partridge, *Nature*, **164**, 443 (1949).