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Studies of Specificity of Deoxyribonuclease from Salmon Testes†

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ABSTRACT: A limited digest of thymus DNA with salmon testes DNase was composed of fragments larger than tetranucleotides. With exhaustive digestion mono-, di-, and trinucleotides were formed. At the (3′) ω terminus G predominated, its frequency ranged from approximately 35 to 72% and increased with decreasing size of fragments. The error caused by the ribose-containing contaminants of DNA is significant, and should not be neglected in the evaluation of nucleoside frequency at the terminal positions of fragments.

A highly potent DNase was isolated from testes of mature salmon, *Oncorhynchus tshawytscha* (Yamamoto, 1971; Yamamoto and Bicknell, 1972). The enzyme was optimally active in the acid pH range, hydrolyzed calf thymus DNA endonucleolytically to yield oligomers bearing 3′-terminal phosphate, did not attack RNA, required no divalent cations, and cleaved native DNA ten times faster than denatured DNA. On the basis of these studies salmon testes DNase was characterized as a DNase II like enzyme (Yamamoto and Bicknell, 1972).

Several laboratories have investigated the specificity of DNase II like enzymes of spleen (Koerner and Sinsheimer, 1957; Doskocil and Sorm, 1961; Vanecko and Laskowski, 1962; Carrara and Bernardi, 1968; Ehrlich *et al.*, 1971; Devillers-Thiery *et al.*, 1973; Thiery *et al.*, 1973; Soave *et al.*, 1973); thymus (Laurila and Laskowski, 1957); tumor cells (Georgatsos, 1967; Ip and Sung, 1968); and brain (Rosen-

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bluth and Sung, 1969). Specificity was evaluated on the basis of frequency of a particular nucleoside in terminal positions of oligomers formed.

As methods for segregation of nucleotide oligomers according to chain length were introduced (Tomlinson and Tener, 1963; Junowicz and Spencer, 1970) it became evident that the frequency of appearance of a given nucleotide at the terminal position often varied with the length of oligomer. This led Laskowski (1967) to formulate a number of restrictions for the interpretation of frequency at terminal positions as evidence for specificity. One of them, the effect of ionic medium on the frequency of a nucleotide at the terminal position, has been experimentally confirmed by Junowicz and Spencer (1973). Another, applicable to enzymes with mixed endo- and exonucleolytic activities, can be partially corrected by comparing the termini of fragments of different lengths, and by the rate of appearance of a particular mononucleotide (Sulkowski and Laskowski, 1962).

A third source of error has been recently found (Tutas 1972, Kedzierski *et al.*, 1973). It is caused by the presence of ribooligonucleotides in many DNA preparations. With nucleases that are nonspecific to sugar, the products released at very early stages of digestion were significantly (up to 50%) contaminated with ribonucleosides. With nucleases that are specific for DNA, like salmon testes DNase, the error from this source appeared at late stages of digestion.

The present paper describes studies of the specificity of DNase from salmon testes on thymus DNA. The specificity is evaluated from the frequency of nucleosides at terminal positions of isostichs isolated from the digest. A slightly modified method of Duch *et al.* (1973) and the method of Duch and Laskowski (1971) were used for analysis of di- and monophosphates, and nucleosides, respectively.

Materials and Methods

Materials. Thymus DNA was prepared according to Kay *et al.* (1952) except that RNA and protein contaminants were removed as described (Kedzierski *et al.*, 1973). After exhaustive digestion with pancreatic and T_1 RNases the level of contaminating RNA in the DNA was about 0.3% (Duch and Laskowski, 1971).

Two preparations of salmon testes DNase have been used in this work. Both were prepared according to Yamamoto and Bicknell (1972). Preparation A was lyophilized and stored for 2 months. It was dissolved in 0.01 M sodium acetate (pH 4.7), divided in small portions, stored at −30°, and thawed just before use. Preparation B was lyophilized and stored for many months in the deep-freeze. It was dissolved in 0.02 M sodium acetate, and subjected to the last step of the purification procedure (second chromatography on phosphocellulose, Yamamoto and Bicknell, 1972). The two preparations had the same specific activity. However, some differences in specificity were observed. The available evidence does not allow a definite explanation of this difference. However, a possibility that a barely detectable contamination with ribonuclease was responsible for the observed difference has been investigated and partially supported.

DNase activity was assayed at 30° in 1.2 ml of solution containing 0.3 A_{260} unit[1] of thymus DNA in 0.1 M sodium acetate (pH 4.7). Under these conditions, one unit of DNase activity was defined as that amount of enzyme which caused an increase of 1.0 A_{260}/min. The specific activity was defined as activity per A_{280} unit of enzyme.

Venom exonuclease with a specific activity of about 3.5 U/1 A_{280} unit was prepared by Dr. E. Sulkowski using acetone fractionation according to Williams *et al.* (1961), chromatography according to Richards *et al.* (1967), and denaturation according to Sulkowski and Laskowski (1971). No contaminating 5′-nucleotidase could be detected at the level of 0.1 unit of exonuclease. However, a trace of the nonspecific phosphatase was present.

Standard materials for calibrating columns were either purchased from P-L Biochemicals, Milwaukee (nucleosides and mononucleotides) or prepared (3′,5′-diphosphates) according to Richards and Laskowski (1969b). Prior to use each substance was subject to purification on a Brinkman Model FF electrophoretor.

Methods. Digests of thymus DNA with salmon testes DNase were performed under conditions specified in the legends. The digests were segregated by length according to the procedure of Junowicz and Spencer (1970) on a column of DEAE-Sephadex kept at 65°. LiCl gradient in 7 M urea was used to displace sequential isostichs. Urea was removed according to the procedure of Tomlinson and Tener (1963), and the oligonucleotides were recovered quantitatively. The exceptions were mononucleotides in which recoveries varied from 25 to 90%.

The exonuclease digestion of oligomers bearing 3′-monophosphate was performed at pH 6.2 (Richards and Laskowski, 1969a). It was carried out as described by Duch *et al.* (1973). The reaction mixture contained 0.05 unit of venom exonuclease and 1–2 A_{260} units of an oligomer in 0.1 M ammonium acetate buffer (pH 6.2) containing 1 mM $MgCl_2$ in a total volume of 25 μl. In many cases larger samples were used, with all constituents increased proportionally. The digestion was carried out at 37° overnight (approximately 17–20 hr).

The α terminus[2] of the digested oligonucleotide appeared as a nucleoside, and was quantitatively determined according to Duch and Laskowski (1971). The following coefficients were used to calculate nanomoles of nucleosides from milligrams of the excised paper peak: dT, 0.0020; dG, 0.0019; dA, 0.0022; dC, 0.0066. The method separates deoxyribo- from ribonucleosides, the latter being eluted somewhat earlier. Even though DNA was specially purified to remove RNA, ribonucleosides were observed in the hydrolysates.

Separation of nucleoside mono- and diphosphates was reported by Duch *et al.* (1972). Shortly thereafter, Varian discontinued manufacturing the type of column that had been used, and a change to Reeve Angel columns led to a somewhat inferior separation (Duch *et al.*, 1973). At the time the paper was in press (addendum, Duch *et al.* 1973) better conditions for separation were developed, largely due to the availability of a purer phosphate buffer. Potassium acid phosphate was purified essentially according to Shmukler (1970) except that the first crop of crystals was discarded and the second and third crops were combined

[1] One A_{260} unit refers to the amount of material, which if dissolved in 1 ml will have an absorbance of unity at 260 nm. Protein is expressed in A_{280} units defined in an analogous manner.

[2] The system of shorthand presentation of the position of oligonucleotides has been described by Richards and Laskowski (1969a). It uses Greek superscript α for the 5′-terminal position whether it has a 5′-phosphoryl or 5′-hydroxyl group. The 3′ terminus in an oligonucleotide of unknown length is called ω.

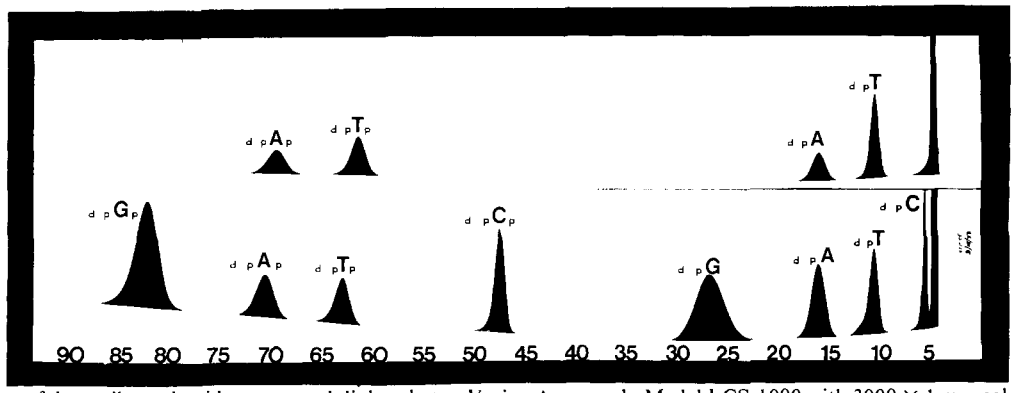

FIGURE 1: Separation of deoxyribonucleoside mono- and diphosphates. Varian Aerograph, Model LCS-1000 with 3000 × 1 mm column filled with Pellionex AS-SAX resin, Reeve Angel. Temperature, 80°; pressure, 700–800 psi, flow rate, 20 ml/hr; full scale deflection, 0.02; linear gradient from 0.0025 M potassium phosphate (pH 3.0) to a mixture of 0.4 M potassium phosphate plus 0.1 M potassium chloride (pH 2.9); initial gradient chamber volume, 50 ml; gradient delay, 15 min. The upper tracing shows the separation of a mixture containing dpT, dpA, dpTp, and dpAp. The lower shows the separation of all four deoxymonophosphates and all four diphosphates. Abscissa time in minutes, chart movement from left to right.

and used. The new conditions for separation of mono- and 3′,5′-diphosphates are described in the legend to Figure 1. A sharp separation of all eight components occurred. The following coefficients were used: dpC, 0.0092; dpT, 0.0041; dpA, 0.0037; dpG, 0.0024; dpCp, 0.0074; dpTp, 0.0053; dpAp, 0.0031; dpGp, 0.0034.

Control experiments were performed to ascertain that the degradation with venom exonuclease was carried to completion. In the system described in the legend to Figure 1 dinucleotides (if present) appear between the 15th and 80th min. Some appear close to the positions of diphosphates. Using dinucleotide isostich, the digestion was performed with variable time intervals. The disappearance of dinucleotides and the appearance of diphosphates (curves are not reproduced) were followed. The experiments showed that under the conditions specified by Duch *et al.* (1973) the reaction was complete in 8 hr. At this time, an amount of nucleosides equivalent to that of diphosphates was observed. For standard digestions, the reaction time was doubled to avoid complication of an accidentally inhibited reaction.

This additional exposure increased the danger of dephosphorylation. Since the accuracy of determining the frequency of nucleosides in terminal positions depends on the absence of dephosphorylation, control experiments were performed to evaluate the magnitude of error caused. Dephosphorylation *via* nonenzymatic origin was evaluated on the isostich of mononucleotides. The material collected from a DEAE-Sephadex column (Junowicz and Spencer, 1970) was diluted 40-fold with water and passed through a DEAE-cellulose column (Tomlinson and Tener, 1963); urea was washed out with water; and mononucleotides were eluted with 2 M ammonium bicarbonate, which was removed by lyophilization. The material was dissolved in water and an aliquot was analyzed for nucleosides. About 10% of the recovered material appeared in the nucleoside fraction.

A similar experiment was performed with trinucleotides. They were first digested with venom exonuclease under standard conditions (Table I) and allowed to stand in the deep-freeze (−20°) in the same acetate buffer, pH 6.2. After 6 weeks the sample was thawed, a fresh portion of exonuclease was added, and an aliquot was withdrawn and tested immediately as a zero time sample. The results show that about 7% dephosphorylation occurred during storage.

Dephosphorylation caused by monophosphatases contaminating the preparation of exonuclease was of a similar order of magnitude (7%). This figure is obtained by comparing lines two and three of Table I. An additional experiment with the isostich of dinucleotides was performed (not shown in Table I). An aliquot was digested with venom exonuclease and analyzed on both cation and anion exchange resins. The results showed the presence of mononucleotides (7%). The sum of mononucleotides and 3′,5′-diphosphates was 7% less than the sum of nucleosides. It was concluded that dephosphorylation by a contaminating monophosphatase was less than 10%, since the observed value included nonenzymatic dephosphorylation. The results also suggest that nonenzymatic dephosphorylation decreases with the increasing length of an isostich.

TABLE I: Control Experiment for Dephosphorylation Using Trinucleotide Isostich.[a]

	α Terminus, dN	β Position, dpN	γ Terminus, dpNp
After the first digestion with venom exonuclease	35	32	33
After 6 weeks storage, and immediately upon addition of another portion of venom exonuclease (zero time)	42	28	30
After a second overnight incubation	49	26	25

[a] All values are in mol %.

Results

The first experiments in which preparation A was used were aimed at finding conditions of digestion that produce oligomers of convenient size to be separable by the Junowicz and Spencer (1970) procedure. Figure 2 shows the results obtained with a limited digestion which is insufficient to bring the products into a region of convenient resolution. In Figure 2a less than 10% of the total digest is recovered as fragments shorter than decanucleotides; in Figure 2b these fractions account for about 33%. Interestingly, with limited digestions no small fragments are observed. Under the conditions of digestion of Figure 2a the shortest observed fragment is a tetranucleotide; in Figure 2b, a trinucleotide. The

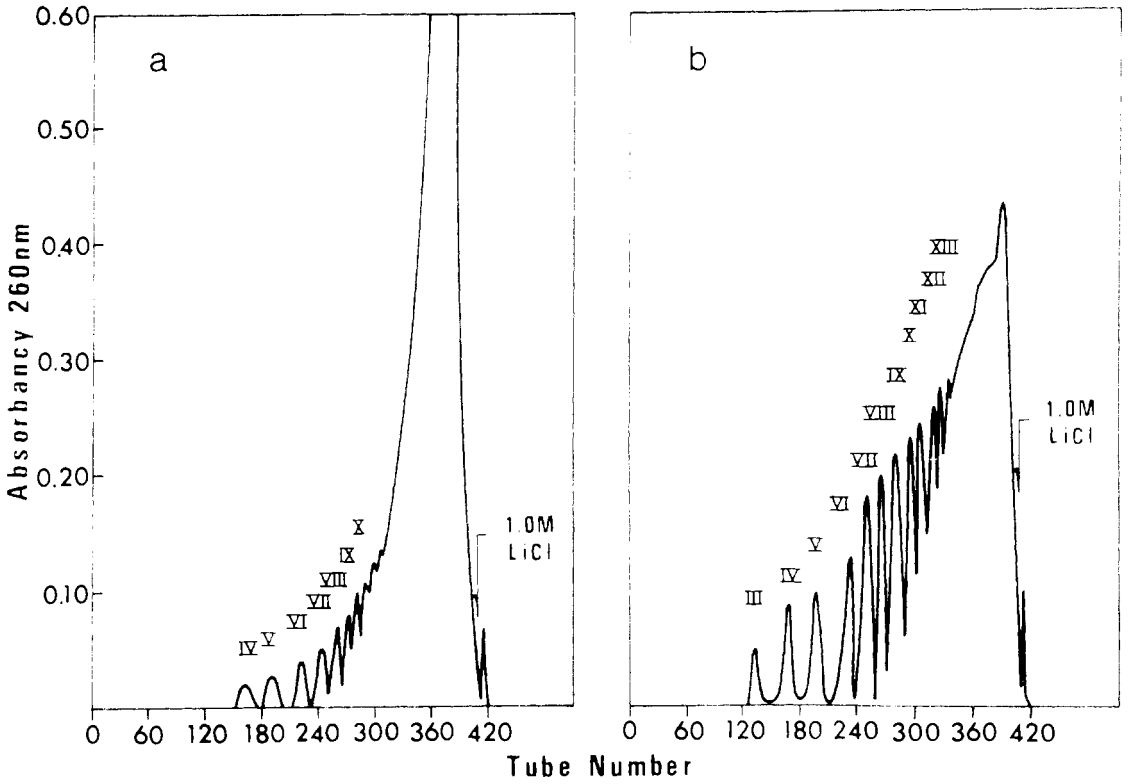

FIGURE 2: Elution profiles of thymus DNA partially digested with preparation A, at 30°. (a) Before digestion, the DNA solution contained 689 A_{260} units. After digestion and before chromatography, the A_{260} units totaled 771 (about 10% hyperchromicity). The Roman numerals located directly above the peaks identify the position and the chain lengths of oligonucleotide isostichs. The A_{260} units recovered were: IV, 6.9; V, 10.3; VI, 9.4; VII, 12.1; VIII, 12.7; IX–XX (chain length estimated), 152; >XXI, 409. The total recovery after chromatography was 614 A_{260} units, or 89%. (b) Before digestion, the DNA solution contained 484 A_{260} units. After digestion, the A_{260} units totaled 623 (about 30% hyperchromicity). The A_{260} units recovered were: III, 12.1; IV, 13.2; V, 17.0; VI, 21.7; VII, 21.6; VIII, 94.5; IX, 24.2; X XV, 152; >XVI, 275.6. Total recovery, 632 A_{260} units, or 101%.

reluctant formation of short fragments resembles the specificity of venom endonuclease (Georgatsos and Laskowski, 1962) and differs from DNase of thymus (Privat de Garilhe and Laskowski, 1955), from micrococcal nuclease (Sulkowski and Laskowski, 1962) and from mung bean nuclease I (Sung and Laskowski, 1962, Wechter *et al.*, 1968). The latter two enzymes show exonucleolytic activity at a rather early stage of the reaction.

The experiment presented in Figure 3 shows that with further hydrolysis the bulk of the digest is represented by fragments smaller than decanucleotides. Mono- and dinucleotides are present. The quantity of mononucleotides is not reported in Figure 3, since the recovery from the Tomlinson and Tener (1963) column was not quantitative.

The termini were analyzed as described under Materials and Methods. Table II reveals that the distribution of nucleosides in terminal positions is similar whether the digestion was limited or exhaustive. The predominant nucleoside at the ω position is G and its content increases with the decreasing size of an oligomer. Another aspect characteristic for preparation A is a low content of T at both terminal positions. Finally, it must be pointed out that some ribonucleosides were observed at the α position. The quantity was never large and they were disregarded in all calculations. Ribonucleosides were most frequently observed with short fragments (di- to tetranucleotides).

An exhaustive digest was then performed with preparation B. The conditions were similar to those used in the experiment shown in Figure 3, except that the incubation was carried out at 37° instead of 30°. At 37° the rate of the reaction is decreased by 4% which was not considered significant. The isostichs were isolated. Mononucleotides were used as a control for nonenzymatic dephosphorylation, and dinucleotides, for the evaluation of the combined enzymatic and nonenzymatic dephosphorylation, as described under Materials and Methods. The remaining isostichs were analyzed. The results are shown in Table III. The predominance of G at the ω terminus is even more pronounced than shown in Table II. However, A not T was the rarest nucleo-

TABLE II: α and ω Termini of Oligonucleotides Obtained after Hydrolysis of Thymus DNA with Salmon Testes DNase, Preparation A.[a]

Chain Length	α Terminus				ω Terminus			
	T	G	A	C	pCp	pTp	pAp	pGp
Limited Digestion; Experiment Shown in Figure 2a								
IX-XX	9	37	19	35	19	8	28	45
>XX	11	38	18	33	15	7	29	49
Prolonged Digestion; Experiment Shown in Figure 3								
III					22	10	22	46
IV	10	39	22	29	25	8	26	41
V	11	39	22	28	29	9	28	34
VI	12	34	23	31	29	9	32	30
VII	12	31	24	33	23	9	37	31
VIII	13	29	25	33	20	8	39	33
>VIII	16	28	23	33	21	13	40	26

[a] All values in mol %.

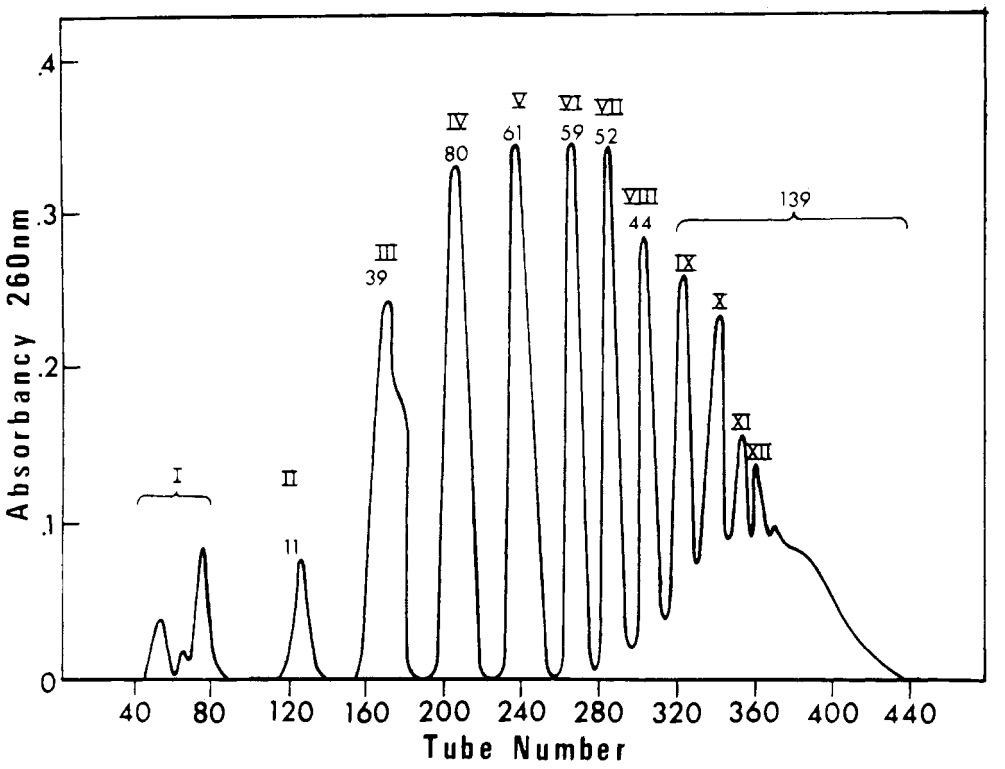

FIGURE 3: Elution profile of hydrolysates of DNA extensively digested with salmon testes DNase, preparation A. Thymus DNA (344 A_{260} units) was incubated with testes DNase in 0.1 M sodium acetate (pH 4.8) for 8 hr at 30°. DNase (2 units) was added at 0 hr and an additional 2 units added at 4 hr. At termination of digestion, the A_{260} of the hydrolysate totaled 500 units (45% hyperchromicity). Chromatography of the digest was performed according to Junowicz and Spencer (1970). The Roman numerals positioned directly above each peak indicate the respective chain length, and the corresponding arabic numbers indicate the total A_{260} units of each fraction. The amount of mononucleotides recovered after removal of urea was 4 A_{260} units; presumably 75% of the material has been lost in the process. Assuming 16 A_{260} units for the sum of mononucleotides, about 5 are represented by pyrimidine mononucleotides, 1 by dpA, and 10 by dpG. Tubes 317–420 were pooled. The total recovery was 489 A_{260} units, or 98%.

TABLE III: Composition of Isostichs Obtained after Prolonged Digestion of Thymus DNA with Salmon Testes DNase, Preparation B.[a]

Chain Length	α Terminus, dN				Internal, dpN				ω Terminus, dpNp			
	T	G	A	C	pC	pT	pA	pG	pCp	pTp	pAp	pGp
III	7	32	36	25	26	61	2	11	7	18	3	72
IV	14	37	23	26	9	55	22	14	9	21	3	66
V	19	35	22	27	16	47	22	15	13	26	4	57
VI	24	35	23	18	18	45	23	13	16	26	3	54
VII	21	28	23	28	20	44	21	15	16	26	3	54

[a] The isostichs of mono- and dinucleotides were used for control expriments as described with Materials and Methods. All values in mol %.

TABLE IV: The Same Experiment as Shown in Table III Except That the Values are Given in Nanomoles.[a]

Chain Length	α Terminus, N	Internal, pN	ω Terminus, pNp
III	2.0 (35)	1.8 (32)	1.9 (33)
IV	1.8 (25)	3.7 (51)	1.9 (25)
V	1.5 (20)	4.5 (60)	1.5 (20)
VI	0.4 (17)	1.5 (65)	0.4 (17)
VII	2.4 (19)	8.2 (66)	1.9 (15)

[a] The figures in parentheses are in per cent.

side at the ω terminus. T was very high at the internal positions, particularly in tri- and tetranucleotides, suggesting that it occupies a penultimate position (either β or ψ).

The same experiment has been recalculated to show the quantity of products obtained (Table IV). The results show that with overnight incubation an average error caused by dephosphorylation is even smaller than calculated from the control experiments. The chain length of an isostich (except hepta) is easily calculated from either pN/N + 2 or pN/pNp + 2. A small excess of N over pNp is a measure of dephosphorylation. However, in no case (except heptanucleotides, which were heavily contaminated with heptaribonucleotide, see below) is there an error large enough to produce doubt as to the chain length of an isostich.

TABLE V: Composition of Isostichs Obtained after Prolonged Digestion of Thymus DNA with Salmon Testes DNAse, Preparation B.[a]

	α Terminus, dN				Internal, pN				ω Terminus			
	T	G	A	C	pC	pT	pA	pG	pCp	pTp	pAp	pGp
I[b]					21	32	7	40				
II	11	21	62	6					3	27	2	68
III	8	34	27	31	12	54	20	14	9	28	3	60
IV	10	31	28	31	17	45	24	14	17	23	8	52
V	20	28	28	24	19	38	30	13	18	29	7	46
VI	25	26	24	25	24	34	32	10	20	31	10	39
VII	32	22	22	24	26	33	32	9	20	33	11	36

[a] All values in mole %. [b] The isostich of mononucleotides is composed of 3′-mononucleotides (Np's), all other isostichs are composed of 5′-mononucleotides (pN's).

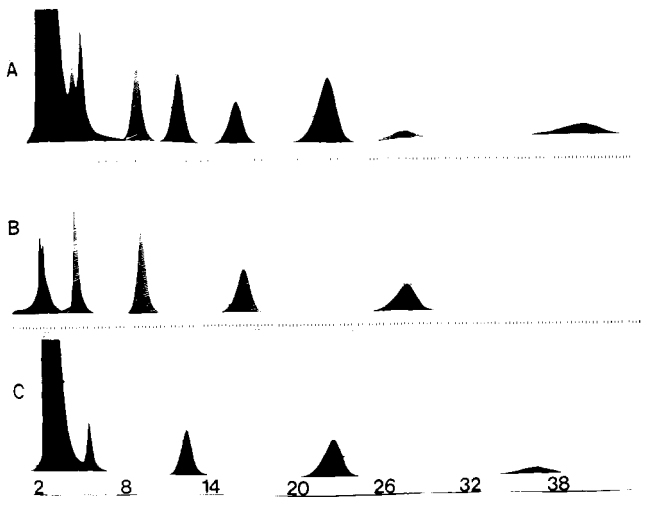

FIGURE 4: Separation of a mixture of ribonucleosides (hatched) and deoxyribonucleosides (black). Abscissa in minutes, chart movement from right to left. (A) An aliquot of the isostich of heptanucleotides was digested to nucleosides according to Duch and Laskowski (1971). The digest was chromatographed on a cation exchange column of Varian LCS-1000. (B) Another aliquot was digested with ammonia and separated into mono- and heptanucleotides as described in the legend to Figure 5. The first double-headed peak (mononucleotides) was treated with prostatic phosphatase to form nucleosides and chromatographed as before. Only ribonucleosides were found. (C) The second peak of Figure 5 was treated as under Figure 4A. This time, the remaining heptanucleotide contained only deoxyribose nucleosides.

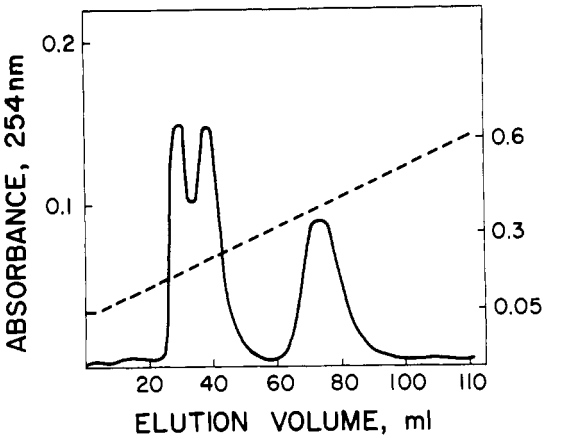

FIGURE 5: Elution profile of a hydrolysate of an aliquot of heptanucleotides (3.5 A_{254} units). The hydrolysis was performed in 1 M NH_4OH according to Richards and Laskowski (1969a). The hydrolysate was diluted 1:10 with water and applied to a DEAE-cellulose column (0.8 × 24 cm). The material was eluted with a linear gradient from 0.05 to 1 M $(NH_4)_2CO_3$. Total volume of the gradient was 200 ml; flow rate, 50 ml/hr. (—) Absorbance at 254 nm;(---) gradient.

Since in the experiment shown in Table III all available mono- and dinucleotides were used for dephosphorylation studies, another experiment was performed. The results (Table V) are essentially identical with those of Table III, but different from the experiments performed with preparation A. This time mono- and dinucleotides were analyzed, providing the opportunity to speculate on the origin of mononucleotides. Their composition resembled the ω terminus of penta- or hexanucleotides and suggested that they could have originated from a cleavage of the ω terminus at that level. An experiment was then performed using an aliquot of the trinucleotide isostich. It was dephosphorylated with alkaline phosphatase and redigested with a tenfold amount of salmon testes DNase. The results were inconclusive since the appearance of nucleosides and mononucleotides was almost simultaneous.

One of the differences between preparation A and preparation B was the appearance of ribonucleosides. With preparation A, ribonucleosides were observed with short fragments (di- to tetranucleotides). In no case did they constitute a significant portion of the total isostich and were, therefore, neglected. With preparation B, ribo derivatives were either absent or barely detectable except in the case of heptanucleotides. To ascertain that the isostich of heptanucleotides was contaminated with ribose containing derivatives, an aliquot was digested to nucleosides (Figure 4). After the digestion, ribonucleotides accounted for approximately 50% of the isostich. One of the possible explanations for this observation was that the contaminating RNA, which was still present in the preparation of DNA, was originally composed of heptanucleotides and higher oligomers. Neither preparation A nor preparation B contained easily detectable RNase activity (Yamamoto and Bicknell, 1972); however, traces of this activity might have been present. Preparation B was presumably the less contaminated of the two, and allowed heptaribonucleotides to remain unhydrolyzed.

To ascertain that heptanucleotides were indeed a mixture of heptaribo- and heptadeoxyribonucleotides, another aliquot was digested with 1 M ammonia at 100° according to Richards and Laskowski (1969a). This procedure was cho-

sen because it opened essentially all cyclic phosphates. After hydrolysis, the mixture was neutralized, diluted 1:10, passed through Tomlinson and Tener's column (1963), and eluted as shown in Figure 5. The first double-headed peak was a mixture of pyrimidine and purine mononucleotides as verified by chromatography of an aliquot on a Varian anion exchange column. Another aliquot was digested with prostatic phosphatase (Ostrowski, 1968) and chromatographed for nucleosides (Figure 4B). No deoxyribonucleoside was observed. The second peak (Figure 5), assumed to be deoxyriboheptanucleotide, was treated with crude venom according to Duch and Laskowski (1971), and an aliquot was chromatographed on a cation exchange column (Figure 4C). Only deoxyribose-containing nucleosides were detected.

Discussion

As mentioned in the Introduction, some properties are common to both salmon testes DNase and spleen DNase II. Because of this, salmon testes DNase was originally classified as a DNase II like nuclease (Yamamoto and Bicknell, 1972). We are now avoiding this term for two reasons. First the term is losing its sharp delineation (see Laskowski, 1967); second, the detailed analysis of products disclosed significant differences between the two enzymes.

Salmon testes DNase produces oligonucleotides terminating with each of the four possible nucleotides. However, quantitative differences are pronounced. At the ω terminus, dG is favored. It is therefore concluded that the preferential cleavage is Gp-N, particularly when short oligonucleotides are being formed.

Another characteristic of digests of thymus DNA with salmon testes DNase is the reluctant formation of mononucleotides (Figure 2). Only with high doses of enzyme are small amounts of mononucleotides formed (Figure 3). In this respect, DNase from salmon testes resembles pancreatic DNase I (Vanecko and Laskowski, 1961) and venom endonuclease (Georgatsos and Laskowski, 1962) and differs from nucleases like DNase II of thymus (Privat de Garilhe and Laskowski, 1955), micrococcal nuclease (Sulkowski and Laskowski, 1962), and mung bean nuclease (Sung and Laskowski, 1962; Wechter, *et al.*, 1968). With the previously studied enzymes the change to exonucleolytic attack was reflected in a decrease in specificity, and the composition of termini of shorter fragments becomes more random. No indication of such a change was detected for salmon testes DNase. A systematic variation was observed at the ω terminus after prolonged digestion, and this sharpened rather than lowered specificity (Tables II, IV, and V). As the length of the fragment decreased, the frequency of dpGp at the ω terminus increased.

Finally, methodological issues should be mentioned. As already found by Duch *et al.*, 1973 (addendum) the removal of uv absorbing contaminant from acid potassium phosphate allowed the development of a system which clearly separated each of the four monophosphates and each of the four 3′, 5′-diphosphates (Figure 1). The purity of the venom exonuclease was also satisfactory as attested by the results shown in Table IV. The length of an isostich could be determined regardless of whether the α or ω terminus was used for calculation. Precautions must be taken not to store phosphorylated derivatives for an extended period of time even at sub-zero temperatures (Table I).

The method of separation of phosphorylated derivatives of deoxyribonucleosides is now as reliable as the separation of nucleosides. However, only at the level of nucleosides can the correction for contaminating ribonucleosides be made. With nucleases nonspecific to sugar, it was shown (Tutas, 1972, Kedzierski *et al.*, 1973) that an error up to 50% can be caused by contaminating ribonucleosides. They appeared predominately in short fragments (mono- to trinucleotides) and were particularly prevalent during the early stages of degradation of DNA. We interpreted these results as an indication that contaminating RNA is present in DNA preparations as short chains which are most likely to be rapidly degraded to the level of mono- to trinucleotides. The finding of riboheptanucleotide among the products of digestion by salmon testes DNase supports this interpretation, except that the contaminating RNA is now believed to be somewhat longer (a minimum of 7, and possibly even 10–20 nucleotides). The fragments of RNA that survive drastic treatment with RNases during the preparation of thymus DNA are presumably perfectly hybridized and thus protected.

A second consequence of this finding is that with the presently available method, each isostich obtained from the Junowicz and Spencer (1971) or Tomlinson and Tener (1963) columns should be analyzed for contaminating ribonucleosides. Even if an overall contamination of DNA with RNA is small, the pattern of distribution of the ribose-containing derivatives among the isostichs may result in a significant error at a specific isostich.

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Further Studies on the Subunit Structure of *Chromatium* Ribulose-1,5-bisphosphate Carboxylase†

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ABSTRACT: Upon alkali exposure *Chromatium* ribulose-1,5-bisphosphate carboxylase dissociates into constituent subunits, a catalytic oligomer of the larger subunit, A_8, and monomeric form of the small subunit B. By sedimentation equilibrium molecular weights of the native enzyme and the catalytic oligomer produced by an alkali treatment were estimated to be 5.11×10^5 and 4.29×10^5, respectively. To provide information on reversibility of the dissociation by determining whether the enzymically inactive small subunit B of the whole enzyme molecule did indeed exchange with exogenously added subunit B a radioisotopic method was used. After initial alkaline dialysis at pH 9.2 of a mixture of a nonlabeled native enzyme preparation and ^{14}C-labeled subunit B, and the subsequent dialysis at pH 7.0, incorporation of ^{14}C into the recovered native enzyme was determined. Without the alkaline treatment there was no detectable exchange, while after alkaline dialysis for 5 and 10 hr the subunit B exchange was 89 and 82%, respectively. Rabbit antiserum prepared against the catalytic oligomer of the spinach ribulose-1,5-bisphosphate carboxylase, anti-[A] (spinach), inhibited the *Chromatium* carboxylase and oxygenase activities. This result together with the identical immunoprecipitation lines on an agar plate formed between the antiserum and the *Chromatium* carboxylase and between the antiserum and the catalytic subunit of the *Chromatium* enzyme strongly indicated structural near identity of the catalytic subunits of the spinach and *Chromatium* carboxylase molecules. Results also show that the catalytic site of the *Chromatium* ribulose-1,5-bisphosphate carboxylase and oxygenase exists in the large polypeptide chain.

Ribulose-1,5-bisphosphate (RuP_2)[1] carboxylase (EC 4.1.1.39) from a photosynthetic purple bacterium, *Chromatium* strain D, dissociated reversibly into constituent subunits upon brief exposure to alkali (eq 1) (Akazawa *et al.*, 1972). The oligomeric form of the larger subunit was shown to retain partial enzyme activities of RuP_2 carboxylase and oxygenase reaction without the aid of the small subunit B (Takabe and Akazawa, 1973a,b). The molecular weights of the catalytic oligomer determined by Sephadex G-200 gel filtration and polyacrylamide gel electrophoresis of different porosities were 4.4×10^5 and 4.2×10^5, respectively. Since a molecular weight value of 5.7×10^4 was obtained for the monomeric large subunit (A) of the enzyme (Akazawa *et al.*, 1972), it has been proposed that the catalytic unit is composed of eight large subunits (A_8) (Takabe and Akazawa, 1974). In spinach leaf RuP_2 carboxylase the octamer of the larger subunit produced by alkaline treatment in the presence of *p*-mercuribenzoate was shown to be the catalytic entity (Nishimura *et al.*, 1973). The absence of cross-contamination between the catalytic oligomer and the small

$$A_{m(=8)}B_n \underset{\text{pH 7.0}}{\overset{\text{pH 9.0}}{\rightleftharpoons}} A_{m(=8)} + nB \qquad (1)$$

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[1] Abbreviations used are: RuP_2, ribulose-1,5-bisphosphate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; ammediol, 2-amino-2-ethyl-1,3-propanediol.