Studies on Polynucleotides Containing Hybrid Sequences. Synthesis of Oligonucleotides Possessing a Single Ribonucleotidyl-(3′–5′)-deoxyribonucleotide Linkage†

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ABSTRACT: In the presence of the appropriate deoxyribonucleoside 5′-diphosphate and Mn^{2+} ions *Micrococcus luteus* polynucleotide phosphorylase catalyzes the addition to adenosine trinucleotide of up to two residues of the 5′-phosphates of deoxycytidine, deoxyguanosine, and thymidine, and up to three residues of deoxyadenosine 5′-phosphate. The rates of the first and second addition in each case are sufficiently different to allow the isolation of the mono- and diaddition products with yields of 70–100%. The hybrid tetranucleotides containing a single terminal deoxyribonucleotide residue are also capable of undergoing a second addition reaction in the presence of the diphosphate of another deoxyribonucleoside to form hybrid pentanucleotides containing two different deoxyribonucleotide residues and, in one case, the reaction of pA-A_2-dG with deoxyadenosine 5′-diphosphate, it was possible to add up to two residues of deoxyadenosine phosphate. The limited addition reaction displayed by the deoxyribonucleoside diphosphates contrasts with the extended polymerization that has been reported for the diphosphates of ribonucleosides and a number of deoxyribonucleoside derivatives. Each of the nucleoside diphosphates that are known to undergo polymerization in the presence of the phosphorylase possesses an electronegative atom attached to its 2′ carbon, and the absence of such atoms near the terminals of oligoribonucleotides containing two terminal deoxyribonucleotide residues may be responsible for the observed difficulty experienced by these molecules in further extending their chains. A suggested function of the 2′-substituent groups in an oligonucleotide undergoing chain extension involves the formation of hydrogen bonds between the electronegative atoms and the enzyme in order that the terminus of the oligonucleotide may assume the specific orientation required for further internucleotide bond formation.

The current interest in hybrid polynucleotides containing covalently linked ribo- and deoxyribonucleotide chains has resulted from the discovery that such polynucleotide structures can occur during *in vivo* DNA synthesis and, in order to study some of the properties of the hybrid internucleotide linkage, it has become necessary to develop new synthetic techniques for the preparation of oligonucleotides containing specific ribo- and deoxyribonucleotide sequences. Recent studies in this laboratory have been concerned with the synthesis of oligonucleotides that contain the deoxyribonucleotidyl-(3′–5′)-ribonucleotide linkage and this work arose from the observation that an oligodeoxyribonucleotide containing a single 3′-terminal ribonucleotide moiety could serve, together with ribonucleoside 5′-diphosphates, as substrates for polynucleotide phosphorylase in the addition of further ribonucleotides to its 3′ terminus (McCutchan and Gilham, 1973). In this study these hybrid oligonucleotides were also shown to act as acceptor molecules for "single addition" reactions catalyzed by polynucleotide phosphorylase in the presence of 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates (Mackey and Gilham, 1971; Bennett *et al.*, 1973), thus providing a route to the synthesis of hybrid oligonucleotides of specified sequence. The present work concerns the study of the use of polynucleotide phosphorylase in the synthesis of specific oligonucleotides with the alternative hybrid structure, that is, hybrid oligonucleotides containing a single ribonucleotidyl-(3′–5′)-deoxyribonucleotide linkage. In 1969, Kaufmann and Littauer reported that, in the presence of deoxyadenosine 5′-diphosphate, *Escherichia coli* polynucleotide phosphorylase could catalyze the addition of up to two deoxyadenosine phosphate residues to the dinucleoside phosphates, A-A and A-U, and Bon *et al.* (1970) have studied the kinetics of the addition of one and two residues of deoxyadenosine phosphate to various adenosine oligonucleotides using the same enzyme. Similarly, the enzyme from *Micrococcus luteus* has been reported to readily add a single deoxyadenosine phosphate residue to adenosine oligonucleotides (Chou and Singer, 1971) and, in this study, the addition reaction was shown to proceed at a faster rate in the presence of Mn^{2+} than in the presence of Mg^{2+}. More recently, the enzyme from *M. luteus* has been used to add up to two thymidine phosphate residues to adenosine hexanucleotide (Feix, 1972).

Addition of Deoxyribonucleotide Residues to Adenosine Trinucleotide. *M. luteus* polynucleotide phosphorylase is capable of catalyzing the addition of each of the four common deoxyribonucleotides to adenosine trinucleotide. In the presence of the diphosphate of thymidine, deoxycytidine, or deoxyguanosine the enzyme can add up to two residues of the nucleotide to the trinucleotide whereas, with deoxyadenosine 5′-diphosphate, up to three residues of the nucleotide can be added. The products may be readily separated and isolated by chromatography on a polystyrene ion-exchange resin using ammonium chloride elution solvents containing 40% ethanol, and the yields of the products obtained for various reaction times are listed in Table I. In the case of the reaction with deoxyadenosine diphosphate the elution patterns obtained from the chromatographic analyses of the products formed at various incubation times are shown in

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TABLE I: Reaction of Deoxyribonucleoside Diphosphates with Adenosine Trinucleotide.

Deoxyribonucleoside Diphosphate	Products	$10^{-3}\epsilon_{260}$	% Yield of Products after Reaction Time of 1 hr	3 hr	5 hr	7 hr	24 hr
dADP	pA-A_2-dA	45.0	72	33		9	
	pA-A_2-dA-dA	54.6	28	67		84	75
	pA-A_2-dA_2-dA	65.6				7	25
dCDP	pA-A_2-dC	41.9	90	24	5		
	pA-A_2-dC-dC	47.6	10	76	95		100
dGDP	pA-A_2-dG	45.9	95	84		26	18
	pA-A_2-dG-dG	54.2	4	15		74	82
dTDP	pA-A_2-dT	43.8	23[a]		84		8
	pA-A_2-dT-dT	50.8			16		92

[a] Remainder of product was unchanged adenosine trinucleotide (77%).

Figure 1. It will be noted that the rate of addition of a particular deoxyribonucleotide is dependent on both the nucleotide base present in the diphosphate substrate and the number of deoxyribonucleotide residues that are already located at the terminus of the acceptor oligonucleotide. For each diphosphate the rates for single and double addition (as well as triple addition in the case of deoxyadenosine diphosphate) are sufficiently different to allow isolation of the intermediate products in reasonably high yields. Under the conditions specified, the optimum reaction times for the preparation pA-A_2-N, where N = dA, dC, dG, and dT, are 0.75, 1.0, 1.0, and 4.0 hr, respectively. In each reaction the addition of the second deoxyribonucleotide residue is considerably slower than the addition of the first, although there is a significant difference between the order of the reaction rates of the various nucleoside diphosphates for the second addition and that of the rates for the first. Of the four deoxyribonucleotides the second pdC residue displays the most rapid rate of addition, with the second pdA being added somewhat more slowly, while the additions of the second pdG and pdT residues proceed at a much slower rate. The slowest addition is that of the third pdA residue in that it requires some 20 hr to convert 25% of the pentanucleotide, pA-A_2-dA-dA, to pA-A_2-dA_2-dA.

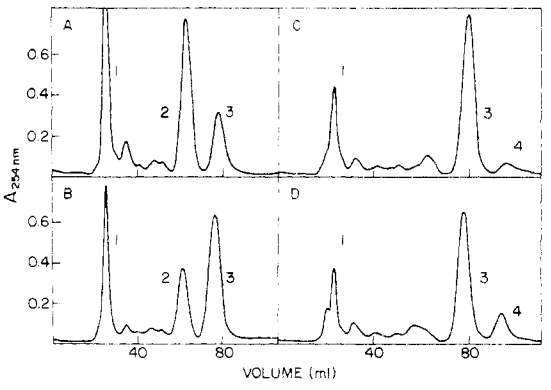

FIGURE 1: Elution patterns from the chromatographic analyses of the reaction of dADP with pA-A-A. Each reaction was carried out as described in the Experimental Section and the incubation times were: (A) 1 hr; (B) 3 hr; (C) 7 hr; (D) 24 hr. Peaks were identified as: (1) dADP; (2) pA-A_2-dA; (3) pA-A_2-dA-dA; (4) pA-A_2-dA_2-dA. Separation of the products was effected on a column (100 × 0.4 cm) of Dowex 1-X2 resin by elution at 12 ml/hr, under pressure, with 200 ml of a 40% ethanol solution containing a linear gradient of 0.3–0.5 M ammonium chloride which had been brought to pH 8 with ammonia.

Addition of Deoxyribonucleotides to Hybrid Oligonucleotides. In order to test the generality of the restricted addition of deoxyribonucleotides to oligoribonucleotides a study of the synthesis of hybrid oligonucleotides containing two different deoxyribonucleotide residues was carried out. These were prepared by treating the appropriate hybrid tetranucleotide containing a single terminal deoxyribonucleotide residue with the enzyme and the diphosphate of a different deoxyribonucleoside. Each of the combinations of oligonucleotide and deoxyribonucleoside diphosphate that were tested resulted in the formation of the desired pentanucleotide and the yields obtained for specific reaction times are listed in Table II. Only single addition was effected in each case except for the reaction of pA-A_2-dG with dADP where a small yield of the diaddition product, pA-A_2-dG-dA-dA, was apparent after 24 hr. The rates of synthesis appear to be dependent on both the type of terminal deoxyribonucleoside residue in the acceptor molecule and the type of deoxyribonucleoside diphosphate used in the addition reaction. Further additions to the tetranucleotide, pA-A_2-dT, as well as additions involving the pdT residue, tend to proceed rather slowly, while all other combinations of substrates result in good yields of product within reasonable raction times. A direct comparison of the rates of synthesis in these reactions is somewhat complicated in that, in some cases, products other than the expected pentanucleotides are formed. These side products apparently arise from the concomitant phosphorolysis of the acceptor oligonucleotide during synthesis, resulting in the release of a small amount of a second deoxyribonucleoside diphosphate into the reaction mixture. The degraded acceptor molecule (pA-A-A) can then undergo addition with the diphoshate that was originally added to the reaction mixture. Thus, in the reaction of pA-A_2-dA with dGDP, small amounts of the oligonucleotides, pA-A_2-dG and pA-A_2-dG-dG, are formed in addition to the desired product, pA-A_2-dA-dG (Table II). The problem of the rearrangement of the acceptor molecule is somewhat more serious in the case of the reaction of pA-A_2-dC with dTDP where larger amounts of the side products, pA-A-A, pA-A_2-dT, and pA-A_2-dT-dT, are formed. Side reactions resulting from phosphorolysis do not appear to be a problem with syntheses involving acceptor molecules containing a dG or dT terminus.

Characterization of Hybrid Oligonucleotides. Each of the oligonucleotides listed in Tables I and II has been isolated and characterized. The chromatographic system

TABLE II: Reaction of Deoxyribonucleoside Diphosphates with Hybrid Oligonucleotides.

Hybrid Oligonucleotide	Deoxyribonucleoside Diphosphate	Product	$10^{-3}\ \epsilon_{260}$	% Yield (Reaction Time, hr)[a]
pA-A_2-dA	dCDP	pA-A_2-dA-dC	50.3	77 (5), 84 (24)
pA-A_2-dA	dGDP	pA-A_2-dA-dG	53.4	52 (5), 78 (24)[b]
pA-A_2-dC	dADP	pA-A_2-dC-dA	50.3	100 (5)
pA-A_2-dC	dTDP	pA-A_2-dC-dT	49.6	17 (5), 28 (24)[c]
pA-A_2-dG	dADP	pA-A_2-dG-dA	53.4	52 (7), 64 (24)
pA-A_2-dG	dADP	pA-A_2-dG-dA-dA	59.7	13 (24)
pA-A_2-dG	dCDP	pA-A_2-dG-dC	50.9	47 (10)
pA-A_2-dT	dADP	pA-A_2-dT-dA	52.2	32 (7), 43 (24)
pA-A_2-dT	dCDP	pA-A_2-dT-dC	49.6	22 (5), 36 (24)

[a] Remainder of product in each case consisted solely of the starting material except as noted. [b] Other products were pA-A_2-dG (4%) and pA-A_2-dG-dG (7%). [c] Other products were pA-A-A (2%), pA-A_2-dT (7%), and pA-A_2-dT-dT (10%).

TABLE III: Characterization of Hybrid Oligonucleotides.

Oligonucleotide	$\bar{V}$[a] (ml)	Degradation Products
pA-A_2-dA	64	pAp:Ap:dA, 1.00:1.95:0.98[b]
pA-A_2-dA-dA	79	pAp:Ap:dA-dA, 1.00:2.05:1;[b] A:dA, 3.15:2.00[c]
pA-A_2-dA_2-dA	96	pAp:Ap, 1.00:2.01;[b] A:dA, 1.00:1.07[c]
pA-A_2-dC	61	pAp:Ap:dC, 1.00:2.07:1.04[b]
pA-A_2-dC-dC	74	pAp:Ap:dC-dC, 1.00:1.98:1;[b] A:pA:pdC, 1.00:2.01:0.94[d]
pA-A_2-dG	75	pAp:Ap:dG, 1.00:2.02:0.94[b]
pA-A_2-dG-dG	96	A:pA:pdG, 1.00:1.97:1.76;[d] A:dG, 3.00:2.01[c]
pA-A_2-dT	59	pAp:Ap:dT, 1.00:2.04:0.93[b]
pA-A_2-dT-dT	77	pAp:Ap:dT-dT, 1.00:2.05:1;[b] A:pA:pdT, 1.00:2.14:2.04[d]
pA-A_2-dC-dA	82	Ap:dCp:dA, 2.96:1.00:1.07;[e] A:dA:dC, 3.00:0.96:0.92[c]
pA-A_2-dC-dT	76	A:pA:pdC:pdT, 1.00:2.07:1.02:0.96[d]
pA-A_2-dG-dA	92	Ap:dGp:dA, 3.08:0.91:1.00;[e] A:dG:dA, 3.06:0.99:1.00[c]
pA-A_2-dG-dA-dA	105	A:dG:dA, 2.79:1.00:1.89[c]
pA-A_2-dG-dC	86	A:pA:pdG:pdC, 1.00:2.02:0.86:1.04;[d] A:dG:dC, 3.04:0.95:1.00[c]
pA-A_2-dT-dA	79	Ap:dTp:dA, 2.91:0.96:1.00;[e] A:dT:dA, 3.02:1.00:1.09[c]
pA-A_2-dT-dC	70	Ap:dTp:dC, 2.89:1.04:1.00[e]
pA-A_2-dA-dC	74	Ap + dAp:dC, 3.76:1.00;[e] A:dA:dC, 2.86:0.94:1.00[c]
pA-A_2-dA-dG	94	Ap + dAp:dG, 3.84:1.00;[e] A:dA:dG, 2.89:1.08:1.00[c]

[a] Chromatographic retention volumes on a column (100 × 0.4 cm) of Dowex 1-X2 (−400 mesh) using 200 ml of 40% ethanol containing a linear gradient of 0.3–0.5 M ammonium chloride (pH 8) at 12 ml/hr. [b] Products and molar ratios of products obtained by alkaline hydrolysis. Dinucleoside phosphate values were calculated to one significant figure. [c] Products and molar ratios of products obtained by digestion with snake venom phosphodiesterase and alkaline phosphatase. [d] Products and molar ratios of products obtained by digestion with snake venom phosphodiesterase after removal of terminal phosphate group with phosphatase. [e] Products and molar ratios of products obtained by digestion with spleen phosphodiesterase after removal of terminal phosphate group with phosphatase.

(McCutchan and Gilham, 1973) used for the fractionation of the products from each reaction consists of a column of polystyrene ion-exchange resin with elution solvents containing 40% ethanol and a linear gradient of chloride ion, and, in this system, the various products have distinctive retention volumes (Table III). The structures of the products were determined by a number of different degradative procedures. Alkaline hydrolysis was used to determine the ribonucleotide content of the oligonucleotide product together with its terminal deoxyribonucleoside or di(deoxyribonucleoside) phosphate. In some cases, the total nucleoside composition of the oligonucleotide was determined by complete degradation with a mixture of snake venom phosphodiesterase and alkaline phosphatase. Further characterization of the products consisted of the determination of their 5′- and 3′-terminal nucleotides together with their chain lengths and base compositions. These analyses were carried out by the method of Ho and Gilham (1973) in which an oligodeoxyribonucleotide is treated with phosphatase to remove its terminal phosphate groups and then with alkali to inactivate the enzyme *in situ*. The mixture is then treated with either snake venom phosphodiesterase or spleen phosphodiesterase and the products are separated by ion-exchange chromatography. In the application of this technique to the analysis of hybrid oligonucleotides it was necessary to show that the short alkaline treatment required to

dispose of the phosphatase is not sufficient to cause measurable hydrolysis of any of the ribointernucleotide linkages. The results of the degradative analyses are listed in Table III.

Conclusion

This study has shown that polynucleotide phosphorylase can be used to prepare hybrid oligonucleotides containing any of the four common deoxyribonucleotide residues at the 3′ terminus of the molecule. The reaction conditions permit the synthesis of either the mono- or diaddition product in each case and it is possible to isolate the products in yields of 70–95 and 80–100%, respectively. Oligonucleotides containing a single terminal deoxyribonucleotide have also been shown to be efficient acceptors for the addition of a second residue containing a different deoxyribonucleotide base. These methods thus provide a route to the synthesis of model oligonucleotides of defined sequence for the study of the properties of the hybrid internucleotide linkage. In addition, the synthetic system can be employed for the preparation of primer molecules for use in studies on nucleic acid sequence by the complementary copying technique. The present methods have been used to prepare longer oligoribonucleotides containing one or two terminal deoxyribonucleotide residues, and these have been shown to serve as primers for the repair synthesis of DNA using *E. coli* DNA polymerase I (H. L. Weith, personal communication).

The problem of the formation of side products in some of these reactions is thought to arise from the phosphorolysis of the acceptor oligonucleotides, which results from the small amount of inorganic phosphate that is released during the synthetic step. This problem has also been recognized recently in this laboratory in other studies that are concerned with stepwise synthesis of certain oligoribonucleotides using the single addition reaction catalyzed by polynucleotide phosphorylase in the presence of 2′-*O*-(α-methoxyethyl)nucleoside 5′-diphosphates (Mackey and Gilham, 1971; Bennett *et al.*, 1973). In these cases the formation of side products arising from the concomitant phosphorolysis of the acceptor molecule can be prevented by enzymatically removing the inorganic phosphate as it is released in the reaction mixtures (J. J. Sninsky and P. T. Gilham, unpublished results). It is expected that the application of this technique to the synthesis of hybrid oligonucleotides will also eliminate the production of undesired sequences.

Finally, it is of some interest to compare the behavior of deoxyribonucleoside diphosphates in the presence of the enzyme and an oligonucleotide acceptor molecule with that of ribonucleoside diphosphates. Bon *et al.* (1970) and Chou and Singer (1971) have both considered the possibility that conformation differences at the terminals of acceptor oligonucleotide molecules could be a factor in determining their ability to accept further nucleotide residues. For example, Chou and Singer (1971) have pointed out two specific differences between deoxyadenosine and adenosine residues that they suggest might account for their observation that molecules of the type A-A_n-dA have great difficulty in serving as acceptors for adenosine phosphate residues. One of their suggestions concerns the difference in the conformations of the two furanose rings which could place the 3′-hydroxyl group of the terminal deoxyribose ring in an unfavorable position for further internucleotide bond formation, while the other suggested difference invokes the formation, in the ribose case, of a hydrogen bond between the 2′-hydroxyl group and the N_3 position in the adenine moiety. However, in view of the fact that limited addition is exhibited by all four deoxyribonucleoside 5′-diphosphates, it may be of some advantage to consider other factors that might account for the phenomenon. In recent years there have been a number of reports describing the action of polynucleotide phosphorylase on deoxyribonucleoside 5′-diphosphates containing various substituents at the $C_{2'}$ position. For example, the 5′-diphosphates of 2′-*O*-methyladenosine (Rottman and Heinlein, 1968), 2′-*O*-methylcytidine (Zmudzka *et al.*, 1969), 2′-*O*-methyluridine (Zmudzka and Shugar, 1970), 2′-azido-2′-deoxyuridine (Torrence *et al.*, 1972, 1973), 2′-fluoro-2′-deoxyuridine (Janik *et al.*, 1972), 2′-chloro-2′-deoxyuridine and 2′-chloro-2′-deoxycytidine (Hobbs *et al.*, 1972b), 2′-*O*-ethyladenosine (Khurshid *et al.*, 1972; Tazawa *et al.*, 1972), and 2′-amino-2′-deoxyuridine (Hobbs *et al.*, 1972a) are all capable of polymerizing to high molecular weight molecules in the presence of the enzyme. All of these substrates as well as the common ribonucleoside diphosphates possess electronegative atoms attached to their $C_{2'}$ positions and it is possible that this may be one of the structural requirements for enzymatic polymerization. For example, polymerization may require the presence of such atoms to serve as proton acceptors in the formation of one or more hydrogen bonds between the enzyme and the terminal nucleotide residues of the polynucleotide that is undergoing chain extension. The restricted addition of deoxyribonucleoside diphosphates could then be understood in terms of the absence of such hydrogen bonds preventing an oligoribonucleotide that contains two terminal deoxyribonucleotide residues from assuming the correct conformation on the enzyme surface for the addition of further residues. There is some evidence that the structural factors which might be responsible for the restricted addition of deoxyribonucleotides are operative near the terminals of the oligonucleotide acceptor molecules. It has recently been shown that the hybrid oligonucleotides, pdT-dT_n-A, are able to serve as acceptors for the addition of further ribonucleotides whereas the corresponding deoxyribo species, pdT-dT_n-dT, are not (McCutchan and Gilham, 1973). Finally, it should be pointed out that there are 2′-substituted nucleoside diphosphates (2′-*O*-(α-methoxyethyl)nucleoside diphosphates) which also exhibit restricted addition to oligoribonucleotides. However, in each of these cases, the reaction is limited to one addition only, and it has already been suggested that this activity could be due to a steric hindrance effect involving the 3′-hydroxyl group and the neighboring substituent group at the 2′ position of the oligonucleotide after the first addition has been made (Mackey and Gilham, 1971; Bennett *et al.*, 1973).

Experimental Section

Materials. Deoxyribonucleoside 5′-diphosphates and tris(hydroxymethyl)methylaminopropanesulfonic acid were purchased from Calbiochem, Los Angeles, Calif. Poly(A) and *M. luteus* polynucleotide phosphorylase (type 15) were obtained from P-L Biochemicals, Milwaukee, Wis., and a unit of activity of the enzyme is defined as the amount that catalyzes the incorporation of 1 μmol of ^{32}P into adenosine diphosphate in the presence of poly(A) per 15 min at 37° (Singer, 1966). *E. coli* alkaline phosphatase (Grade BAPF), snake venom phosphodiesterase (Grade VPH), and spleen phosphodiesterase (Grade SPH) were purchased from Worthington Biochemical Corporation, Freehold, N.J. The phosphatase was dialyzed against 0.005 M Tris-Cl (pH 8) prior to use, and the unit of activity is defined as the

quantity required to liberate 1 μmol of *p*-nitrophenol from *p*-nitrophenyl phosphate per minute at 25° (Garen and Levinthal, 1960). The snake venom phosphodiesterase was pretreated according to the procedure of Sulkowski and Laskowski (1971) to remove any 5′-nucleotidase activity. Adenosine trinucleotide was prepared by the method of Bennett *et al.* (1973) from the degradation of poly(A) with pig liver nuclei ribonuclease that had been purified by the procedure of Heppel (1966). The chromatographic materials, Dowex 1-X2, Dowex 1-X4, and beaded polyacrylamide were purchased as AG 1-X2 (−400 mesh), AG 1-X4 (−400 mesh), and Bio-Gel P-2 (200–400 mesh), respectively, from Bio-Rad Laboratories, Richmond, Calif.

Reaction of Deoxyribonucleoside Diphosphates with Adenosine Trinucleotide. Each reaction mixture (total volume, 0.1 ml) contained adenosine trinucleotide (75 nmol), the deoxyribonucleoside 5′-diphosphate (300–400 nmol), sodium tris(hydroxymethyl)methylaminopropanesulfonate, pH 9.0 at 20° (10 μmol), manganese chloride (1 μmol), and polynucleotide phosphorylase (0.1 unit). The mixtures were kept at 37° for the specified times (Table I) and the products were separated by ion-exchange chromatography on a column (100 × 0.4 cm) of Dowex 1-X2 resin using elution at 12 ml/hr, under pressure, with 200 ml of a 40% ethanol solution containing a linear gradient of 0.3–0.5 M ammonium chloride which had been brought to pH 8 with ammonia. The retention volumes and yields of the various products are listed in Tables III and I respectively, and the elution patterns resulting from the treatment of adenosine trinucleotide with the enzyme and deoxyadenosine 5′-diphosphate for various times are shown in Figure 1. For the isolation of the products from each reaction mixture the appropriate fractions from the chromatographic separation were combined and dialyzed against water to remove ammonium chloride or, alternatively, the combined fractions were concentrated to a small volume and desalted by passing them through a column (60 × 1 cm) of Bio-Gel P-2 beaded polyacrylamide using 20% ethanol as the eluting solvent.

The yields of products were calculated by using approximate values for their molar absorptivities. The molar absorptivity of an oligonucleotide at 260 nm and at pH 7 was obtained by dividing the sum of the molar absorptivities of its component nucleotides at 260 nm and pH 7 by its hyperchromicity ratio at that wavelength. The hyperchromicity ratio is defined as the ratio of the optical density of the components of an oligonucleotide to that of the intact oligonucleotide at the same wavelength and pH. These ratios were derived, in part, from the hyperchromicity data reported by Toal *et al.* (1968) for a number of oligoribonucleotides. In the case of a tetranucleotide containing three contiguous adenosine moieties and one other nucleotide it can be shown that the hyperchromicity ratio is near that of adenosine trinucleotide itself and thus, for the present work, the hyperchromicity ratio of a hybrid oligonucleotide containing x residues of adenine was taken as equal to that of an adenosine oligonucleotide of chain length, x. The hyperchromicity ratios used for A-A_n-A, where n = 1, 2, 3, 4, were 1.27, 1.36, 1.40, and 1.40, respectively.

Reaction of Deoxyribonucleoside Diphosphates with Hybrid Oligonucleotides. Enzyme-catalyzed additions to oligonucleotides containing a terminal deoxyribonucleoside residue were carried out as described above for additions to adenosine trinucleotide except that the amount of oligonucleotide used in each case was about 50 nmol. The separation and isolation of the products were also carried out in the same manner, and their retention volumes are listed in Table III. The yields of products were calculated as described above and these are given in Table II together with reaction times.

Characterization of Oligonucleotides. ALKALINE HYDROLYSIS. The oligonucleotide (about 2 OD (optical density) units (260 nm)) was dissolved in 0.25 M sodium hydroxide (0.4 ml) and kept at 37° for 20 hr and the products were separated directly by ion-exchange chromatography.

DEGRADATION WITH SNAKE VENOM PHOSPHODIESTERASE AND PHOSPHATASE. The oligonucleotide (2–4 OD units (260nm)) was dissolved in 0.1 ml of 0.2 M Tris-Cl–0.04 M magnesium acetate (pH 9.0) and added to 0.1 ml of snake venom phosphodiesterase solution (1.0 mg/ml) and 0.1 ml of phosphatase solution (3 units/ml). The mixture was incubated at 37° for 3 hr.

DEGRADATION WITH SNAKE VENOM PHOSPHODIESTERASE AFTER DEPHOSPHORYLATION. The oligonucleotide (2–4 OD units (260 nm)) was dissolved in 0.1 ml of 0.04 M Tris-Cl–0.02 M magnesium chloride (pH 8) and treated with 0.1 ml of phosphatase solution (3 units/ml). After incubation at 37° for 3 hr the phosphatase was inactivated by the method of Ho and Gilham (1973). The solution was treated with 2 M sodium hydroxide (50 μl) and allowed to stand at room temperature for 20 min. In a separate experiment this treatment was shown to cause a negligible amount of hydrolysis of ribointernucleotide linkages. The alkaline solution was adjusted to pH 9 with dilute hydrochloric acid and then evaporated to dryness. The product was dissolved in 0.1 ml of 0.2 M Tris-Cl–0.04 M magnesium acetate (pH 9.0) and incubated at 37° with 0.1 ml of snake venom phosphodiesterase solution (1 mg/ml) for 2 hr.

DEGRADATION WITH SPLEEN PHOSPHODIESTERASE AFTER DEPHOSPHORYLATION. The oligonucleotide (2–4 OD units (260 nm)) was treated with phosphatase and the enzyme was inactivated as described above. The alkaline solution was then adjusted to pH 6.5 with dilute hydrochloric acid and evaporated to dryness. The product was dissolved in 0.1 ml of 0.3 M ammonium succinate–0.05 M EDTA (pH 6.5) and incubated at 37° with 0.1 ml of spleen phosphodiesterase solution (1 mg/ml) for 2 hr.

SEPARATION OF DEGRADATION PRODUCTS. The products from the various digestions were separated by the method of Ho and Gilham (1973). The column of Dowex 1-X4 (−400 mesh, chloride form) had dimensions 100 × 0.2 cm and the following additions were made by means of a sample injection loop: 0.4 ml of M sodium hydroxide (0.3 ml in the case of the alkaline digestions), 1 ml of 10% ethanol, the digestion mixture made up to 1 ml with 10% ethanol, and 1 ml of 10% ethanol. Elution was then effected under pressure with the appropriate solvent system at 8–10 ml/hr. In the case of the digestions involving the degradation of the oligonucleotides to nucleosides with snake venom phosphodiesterase and phosphatase the elution solvent was 200 ml of 10% ethanol containing a linear gradient of 0–0.5 M ammonium chloride which had been brought to pH 10 with ammonia. The retention volumes (in milliliters) were: dC, 16.0; dA, 22.9; A, 36.4; dT, 39.9; dG, 62.6. For all other digestions the solvent consisted of 200 ml of 10% ethanol containing a linear gradient of 0–0.5 M ammonium chloride which had been brought to pH 9 with ammonia. With this system retention volumes (in milliliters) were: dC, 16.6; dA, 23.4; dT, 35.7; A, 36.1; dG, 44.7; dC-dC, 48.1; dI,

54.6; dT-dT, 59.6; pdC, 60.9; dCp, 64.5; pdT, 66.5; dTp, 68.3; pA, 76.3; pdA, 77.3; dAp, 79.8; Ap (2′,3′), 86.1, 96.3; pdG, 88.8; dGp, 91.2; pAp (2′,3′), 110, 114.8. The amount of each degradation product was determined spectrophotometrically.

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Ultracentrifugal Characterization of the Mitochondrial Ribosome and Subribosomal Particles of Bovine Liver: Molecular Size and Composition†

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ABSTRACT: We have measured the molecular weights of the bovine liver mitochondrial ribosome and subribosomal particles by high-speed equilibrium centrifugation. The formaldehyde-fixed ribosome, large subunit, and small subunit had sedimentation coefficients ($s_{20,w}$) of 56.3, 44.9, and 30.1 S, and had buoyant densities in CsCl of 1.42, 1.43, and 1.43 g/cm^3, respectively. These buoyant densities correspond to RNA contents of 31 and 33%. Partial specific volumes, $\bar{v}$, of 0.674 and 0.671 ml/g were calculated for these compositions and used to compute molecular weights of 2.83×10^6, 1.65×10^6, and 1.10×10^6 for the three species. Despite its low sedimentation rate, the 55 S ribosome is thus about the size of the bacterial ribosome.

Mitochondrial ribosomes are considered to be of the prokaryotic variety primarily on the bases of antibiotic susceptibility, sedimentation coefficient, and sizes of their RNAs. Generally it has been assumed that these ribosomes are about the size of typical bacterial ribosomes with the notable exception, of course, of the 55S ribosomes that occur in mammalian mitochondria. These ribosomes, thought to be considerably smaller than other prokaryotic ribosomes because of their unusually low sedimentation coefficient and very small RNA molecules, have been called "mini-ribosomes" in recent years (see review of Borst and Grivell, 1971). We were interested, therefore, in measuring the molecular weight (MW) of a representative 55S ribosome from mammalian mitochondria to define better the relation of these ribosomes to others, and also to form the basis for studies of their protein content (O'Brien *et al.*, 1974).

We have measured the molecular weight of the bovine liver mitoribosome and its subribosomal particles by high-speed equilibrium centrifugation, one of the few absolute

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