Biopolymers 6, 793.
Sussman, M., and Sussman, R. (1969), *Symp. Soc. Gen. Microbiol. 19,* 403–435.
Verma, I. M., and Baltimore, D. (1973), *Methods Enzymol. 29,* 125–130.
Verma, I. M., Temple, G. F., Fan, H., and Baltimore, D. (1972), *Nature (London), New Biol. 235,* 163–167.
Verma, I. M., Temple, G. F., Fan, H., and Baltimore, D. (1973), *in* Control of Transcription, Vol. 3, Biswas, B., Mandal, R., Stevens, A., and Cohn, W., Ed., London, Plenum Publishing Co., pp 355–372.
Vogt, V. (1973), *Eur. J. Biochem. 33,* 192–200.
Zassenhaus, P., and Kates, J. (1972), *Nature (London), New Biol. 238,* 139–141.

Characterization of a Pair of Isopycnic Twin Crustacean Satellite Deoxyribonucleic Acids, One of Which Lacks One Base in Each Strand†

Dorothy M. Skinner* and Wanda G. Beattie

ABSTRACT: Two double-stranded DNAs (satellites I and II) of identical density, both containing unusually large porportions of deoxyguanylate and deoxycytidylate, have been isolated from four tissues of the hermit crab, *Pagurus pollicaris.* They have been separated from each other by the use of Ag^+ + Hg^{2+} – Cs_2SO_4 gradients and characterized as to t_m; the densities of the native double stranded and denatured single stranded DNAs have been determined. Base compositional analyses indicate that satellite DNA I has one strand containing 50% deoxyguanylate residues but is essentially free of deoxycytidylate (<3%); compositional analyses of the other strand give 47% deoxycytidylate and less than 2% deoxyguanylate. The distribution of the radioiodinated deoxyguanylate-rich strand of satellite I in alkaline CsCl indicates that deoxycytidylate is present in only trace amounts, if at all, in that strand. Assuming complementarity, the opposite strand should therefore be lacking in deoxyguanylate residues. In the other satellite (II), the divergence of the composition of the two strands is less severe (dG:dC = 36%:26% and 28%:32%, respectively).

We have previously described crustacean "satellite" DNAs rich in either dA + dT or dG + dC residues, the single strands of which are either nonbiased (Skinner, 1967; Beattie and Skinner, 1972) or biased (Beattie and Skinner, 1972) in base composition. We report here the physical and chemical characteristics of two (dG + dC)-rich satellites that make up less than 1% of the total DNA of various tissues (gonads, epidermis, hemocytes, and nerve) of the hermit crab, *Pagurus pollicaris.* Although the two satellite DNAs have identical densities in neutral CsCl gradients (such that we refer to them as "isopycnic twins"), they can be completely separated from each other in Cs_2SO_4 gradients containing both mercury and silver salts (Skinner and Beattie, 1973). The complementary strands of satellite I have very different base compositions, one being essentially free of guanine residues, the other of cytosine residues; the base-compositional bias of the individual strands of satellite II is less extreme.

Included here are data on the characterization of the iospycnic twins, including thermal dissociation and reassociation characteristics and the densities of the separated strands and their base compositions. In another paper (Skinner *et al.,* 1974), we present the nucleotide sequence of satellite I which is a repeating tetramer (5′-T-A-G-G-3′), and its complement, identical with part of the hexameric sequence that accounts for 50% of the sequences of the α satellite of the guinea pig, *Cavia porcellus* (Southern, 1970), and that may also be present in the HS-α satellite of the kangaroo rat, *Dipodomys ordii* (W. Salser and K. Fry, in press).

Materials and Methods

Animals. Hermit crabs were obtained from the staff of the Marine Biological Laboratory, Woods Hole, Mass. Guinea pig tissues were the kind gift of Dr. M. G. Hanna.

Isolation of DNAs. DNA was isolated from nervous tissue (eyestalk ganglia), testes, and hemocytes of hermit crabs according to methods previously described (Skinner, 1967; Skinner *et al.,* 1970) and from ovaries and epidermis by a modification of the Marmur (1961) method. Ovaries and epidermis were homogenized in 0.1 M NaCl–0.05 M EDTA (pH 8) and centrifuged at 9750*g* for 15 min to collect nuclei, which were resuspended in 0.1 M NaCl–0.05 M EDTA and made 8% in sodium dodecyl sulfate and 1 M in $NaClO_4$. Repeated deproteinizations with isoamyl alcohol–chloroform (Sevag *et al.,* 1938) followed. Preparations were then treated with RNase and Pronase as described previously (Skinner, 1967). Guinea pig DNA was isolated according to the procedure of Britten *et al.* (1970). Liver and kidney (20 g) from an adult male were mixed, as were testes and vas deferens (5 g). Tissues were finely minced and rinsed three times in 0.1 M NaCl–0.05 M EDTA to remove blood. They were then homogenized in an Omnimixer (Sorvall) in 5 volumes of a solution containing 8 M urea, 0.2 M sodium phosphate buffer (pH 6.8), 0.25% sodium dodecyl sulfate, and 3 mM EDTA. To the homogenate was added 5 volumes of hydroxylapatite (Bio-Rad, Bio-Gel HT). The mixture was shaken for 5 min to permit the DNA to bind to the hydrox-

†From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830. *Received April 8, 1974.* This research was sponsored by the U. S. Atomic Energy Commission under contract with the Union Carbide Corporation.

ylapatite, distributed into 40-ml conical centrifuge tubes, and centrifuged for 15–30 sec at 1000 rpm. The hydroxylapatite was washed repeatedly with 0.14 M sodium phosphate buffer (pH 6.8) and 8 M urea (Britten *et al.*, 1974) until the supernatants were free of absorbance at 260 nm. DNA was eluted from the hydroxylapatite by six washings in 0.4 M sodium phosphate buffer (pH 6.8) and four low-speed, short-term centrifugations as above; 50 mg of DNA was recovered from the liver–kidney mix and 7 mg from testes. The DNA from the liver and kidney was shaken with isoamyl alcohol–chloroform (1:24) and centrifuged.

Purification of Satellite DNAs. The (dG + dC)-rich isopycnic twin satellite DNAs of the crab, *P. pollicaris*, were purified by repeated centrifugations in two-step ("relaxed") preparative CsCl isopycnic gradients (Brunk and Leick, 1969), the lower phases (3.3 ml) of which were adjusted to ρ = 1.684 g/cm^3 and the upper phases (2.6 ml), in which the DNA was dissolved, to ρ = 1.511 g/cm^3. Centrifugations were for 40 hr at 40,000 rpm in a Spinco (L265B) Ti-50 rotor at 25°. The (dG + dC)-rich satellite of the guinea pig was isolated by centrifugation in Ag^+ – Cs_2SO_4 gradients with a ratio of added metal ion to total DNA phosphorus (Davidson *et al.*, 1965) of 0.27 (Corneo *et al.*, 1968). The two native (dG + dC)-rich satellites of *P. pollicarus* were separated from each other by centrifugation in Hg^{2+} + Ag^+ – Cs_2SO_4 gradients as described previously (Skinner and Beattie, 1973). DNA for Cs_2SO_4 gradients was dialyzed exhaustively against 5 mM $Na_2B_4O_7$. Preparative Cs_2SO_4 gradient solutions were made by adding 3 g of Cs_2SO_4 to DNA (100–150 μg in borate buffer per gradient). After the Cs_2SO_4 was in solution, $HgCl_2$ and $AgNO_3$ were added to produce ratios of 0.1 and 0.64; the final volume of each gradient was 4.5 ml. The samples were stirred at room temperature for 1–2 hr, after which the final density was adjusted to 1.549 g/cm^3 (Szybalski, 1968). The gradients were centrifuged 40 hr at 40,000 rpm at 25° in a Spinco Ti-50 rotor. After the centrifugation on preparative Cs_2SO_4 gradients, heavy metals were removed from the samples by exhaustive dialysis against 10 μM EDTA. The four individual components of the two denatured satellites of *P. pollicarus* were collected by centrifugations of purified satellites through CsCl buffered to pH 12.4 with 0.05 M phosphate (Vinograd *et al.*, 1963). The purity of each component was monitored by centrifugation in a Model E analytical ultracentrifuge. Neutral and alkaline analytical gradients were performed as described previously (Skinner, 1967; Beattie and Skinner, 1972). Neutral gradients were buffered to pH 8 and alkaline gradients were made 0.04 M in phosphate (pH 12.45). Samples were centrifuged in a Model E analytical ultracentrifuge at 44,770 rpm for 20 hr at 25°. *Micrococcus luteus* DNA (ρ = 1.727 g/cm^3 at pH 8 or 1.788 g/cm^3 at pH 12.45) or purified *Gecarcinus lateralis* poly(dA-dT)[1] (ρ = 1.677 g/cm^3 at pH 8 or 1.734 g/cm^3 at pH 12.45) was used as a marker. Densities were calculated from microdensitometer (Joyce-Loebl) tracings of photographs taken at 265 nm. Optical-grade cesium chloride was obtained from Harshaw Chemicals or Henley Company; optical grade cesium sulfate was obtained from Gallard-Schlesinger or EM Laboratories (Elmsford, N. Y.).

Thermal Dissociations. DNAs in 15 mM NaCl–1.5 mM sodium citrate (pH 7) or in 120 mM sodium phosphate (pH 6.8) were heated in stoppered 1-ml cuvets in a Gilford spectrophotometer (Model 2400) programmed for a 0.5% temperature increase per min.

In Vitro Synthesis of DNA Complementary to P. pollicaris Isopycnic Twin Satellite I. DNA polymerase isolated from *M. luteus*, an organism with (dG + dC)-rich DNA, was the kind gift of Dr. R. D. Wells; that polymerase is routinely used in the synthesis of the synthetic polymer poly[(dT-dG)·(dA-dC)] (R. D. Wells, personal communication). The reaction mixture contained, in 0.2 ml final volume, 10 nmol of each of the three nonradioactive deoxynucleotides, 0.1 μCi of [^{32}P]dATP, 0.5 μCi of [^{3}H]dTTP, 10 μmol of Tris buffer (pH 7.8), 400 nmol of mercaptoethanol, 1 μmol of Mg^{2+}, and 3.7 μg (12.3 nmol) of satellite I DNA. After incubation at 37° for 1 hr, the reaction was terminated by the addition of 5 M NaCl to 400 mM and 100 mM EDTA (pH 8) to 20 mM. The product was freed of unreacted precursors by passage through a G-75 Sephadex column (2 × 18 cm). The DNA was concentrated by evaporation under vacuum and centrifuged to equilibrium in "two-step" alkaline CsCl gradients. The density of the lower phase was 1.859 g/cm^3, and that of the upper phase was 1.570 g/cm^3. Fractions containing 0.07 ml were collected directly on strips (1 cm × 57 cm) of Whatman No. 17 filter paper, washed twice (10 min each) in 5% trichloroacetic acid, twice in ethanol, and once in acetone, and dried on a slide warmer at 45°. The strips were cut into pieces (~1 cm^2) and placed in shell vials for counting in toluene–2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene (Carrier and Setlow, 1971) in a Nuclear Chicago scintillation counter.

Determination of the Base Composition of the Satellites. DNAs (10–20 μg) were dried at 37° under a stream of nitrogen, dissolved in a small volume (~10 μl) of 200 mM NH_4HCO_3–1 mM MgOAc (pH 8.7), and degraded to nucleosides by the action of a mixture of three enzymes: DNase I (12.5 μg), snake venom phosphodiesterase (12.5 μg), and alkaline phosphatase (7 μg), all purchased from Worthington. Incubations were for 4–7 hr at 37°. Nucleosides (in a volume of 15 μl) were separated by ion exclusion (Singhal and Cohn, 1972) on a column of the cation exchanger Aminex A-6. The column (0.6 cm × 50 cm) was heated to 50°; flow rate was regulated between 0.20 and 0.28 ml/min by a peristaltic pump; the eluent was 0.02 M NH_4HCO_3 (pH 9.5). Under such conditions, the major deoxynucleosides elute in the order dT, dC, dG, and dA; hydroxymethyldeoxycytidineis clearly separated immediately following dC (see Figure 6).

Iodination of DNAs. Preparation of Materials. Hydroxylapatite was prepared according to Tiselius *et al.* (1956) as modified by Miyazawa and Thomas (1965). The separated strands of *Pagurus* satellite I were iodinated according to the method of M. Cohen for single-stranded RNA [M. Cohen, personal communication; modified from Commerford (1971), including an incubation at 90° and pH 8.9 to remove weakly bound ^{125}I (Getz *et al.*, 1972)]. The iodinated DNA was dialyzed against 1 mM sodium phosphate (pH 6.8) overnight, then applied to hydroxylapatite at room temperature and washed with 10 mM sodium phosphate until no further counts were eluted. The DNA was eluted with 0.4 M sodium phosphate.

Results

Densities in Neutral and Alkaline CsCl Gradients. Initially, we isolated the four single strands obtained from preparative alkaline CsCl gradients (Figure 1a) of the twin satellites. Figure 1b–d shows the reband of each of the single-strand components in alkaline CsCl gradients. A 20–50% contamination

[1] Abbreviations used are: poly(dA-dT), naturally occurring satellite DNA composed of more than 90% alternating deoxyadenylate and thymidylate residues and some deoxyguanylate and deoxycytidylate residues (some thymidylate and deoxyadenylate residues are also present in irregular sequences (Skinner, 1967; data given here).); dsDNA, double-strand DNA; ssDNA, single-stranded DNA.

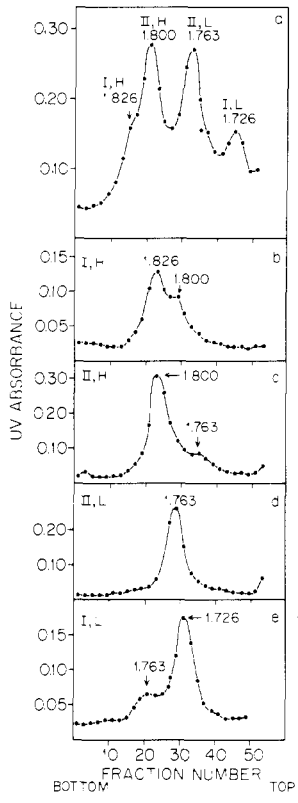

FIGURE 1: Preparative alkaline CsCl gradient of isopycnic twins unseparated by $Ag^+ + Hg^{2+}$-Cs_2SO_4 centrifugation. (a) Satellite DNA (211 μg, purified to a single band in neutral CsCl by two sequential centrifugations in neutral CsCl gradients) was centrifuged in a "relaxed" alkaline CsCl gradient as described. Fractions were collected through a hole in the bottom of the tube, diluted to 0.5 ml, and read at 260 nm. Fractions 5-18, 19-29, 30-37, and 38-49 were pooled, neutralized, dialyzed to remove CsCl, concentrated *in vacuo* at 37°, and rebanded in alkaline CsCl; the densities of each gradient were adjusted to cause the major component of the DNA to band close to the center of the gradient. (b) 5-18; satellite I H, contaminated with satellite II H. (c) 19-29; satellite II H contaminated with satellite II L. (d) 30-37; satellite II L, very slightly contaminated with I L and II H. (e) 38-49; satellite I L, contaminated with II L. Appropriate fractions of gradients (b), (c), (d), and (e) were pooled, neutralized, dialyzed, concentrated, and checked for purity by centrifugation in a Model E analytical ultracentrifuge.

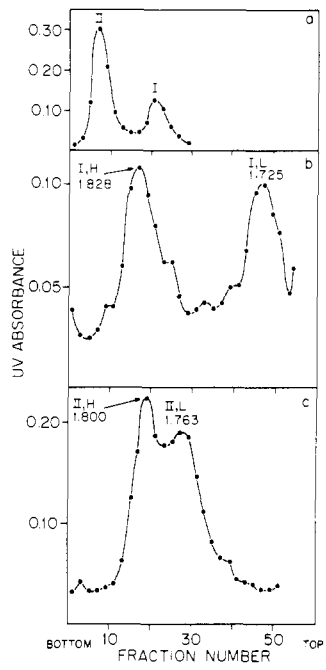

FIGURE 2: Preparative neutral $Ag^+ + Hg^{2+}$-Cs_2SO_4 density gradient of isopycnic twins. Satellite DNA (100 μg), purified by two sequential centrifugations in neutral CsCl gradients, was centrifuged in a Cs_2SO_4 gradient in the presence of mercury at a ratio of 0.1 and silver at a ratio of 0.64. Eight-drop fractions were collected and read as described (Figure 1a). Fractions 2-13 and 18-27 were collected, dialyzed to remove the metals, concentrated, and centrifuged in relaxed alkaline preparative CsCl gradients to separate the single strands. (b) satellite I; (c) satellite II. Fractions from gradients (b) H strand (fractions 6-23); L strand (fractions 44-51) and (c) H strand (fractions 11-19); L strand (fractions 28-35) were pooled, dialyzed and monitored for purity in a Model E ultracentrifuge.

with strands of similar densities is seen for both strands of satellite I and for the dense (H) strand of satellite II. After the method for separating the two satellites by centrifugation in Cs_2SO_4 density gradients in the presence of both Ag^+ and Hg^{2+} had been established (Skinner and Beattie, 1973; Figure 2a), we used it routinely. The purity of the single strands recovered from subsequent alkaline CsCl gradients was much greater, especially for satellite I (Figure 2b). Although the strands of satellite II overlap each other markedly (Figure 2c), they appear to be virtually free of satellite I. After rebanding of selected fractions from the preparative gradients, the ssDNAs were monitored for purity and their buoyant densities determined by centrifugation in a Model E analytical centrifuge.

The results are summarized in Table I. Also included for comparison are the published densities of several synthetic polymers. Note that in both alkaline and neutral CsCl gradients the densities of satellite I H are similar to those of the synthetic single-strand polymer $(dG\text{-}dT)_n$. However, the densities of the partner light strand (satellite I L) are greater than those of the synthetic single-stranded $(dC\text{-}dA)_n$ (Doerfler and Hogness, 1965; Wells and Blair, 1967; Wells and Larson, 1972). Furthermore, the density of I L in alkaline CsCl is only very slightly higher than its density in neutral CsCl. Since increased density in alkaline CsCl is caused by the replacement by Cs^+ of the titratable protons of thymine or guanosine residues (Vinograd *et al.*, 1963), we took this as further evidence that satellite I L contained few dT or dG residues, although these (or other unidentified) bases must be present to some extent to account for the neutral buoyant density greater than that of $(dC\text{-}dA)_n$. The data of Wells and Larson (1972) show that samples of $(dG)_n$·$(dC)_n$ have a large density increase in alkaline CsCl whereas $(dT\text{-}dC)_n$ shows only a 7 mg/ml increase, the same as we found with satellite I L. Our results are therefore consistent with a polymer composed of dA and dC, with dT adjacent to dC (see Skinner *et al.*, 1974).

The densities of the single strands of satellite II H and L are also listed in Table I.

Dissociation and Reassociation. The biphasic thermal dissociation of the unseparated isopycnic twin satellites is shown in Figure 3 as is a remelt of the unfractionated satellites following a period of quick cooling (inset Figure 3a), together with the individual thermal dissociations of satellites I and II. The magnitude of the increase in uv absorbance of the first phase (t_m = 72°) of the unfractionated satellite (Figure 3a) was such that we concluded that it was due to the component made up of the strands of least and greatest density (Figure 1a), while the considerably greater hyperchromicity of the component that dissociated at 83° was attributed to the strands of middle densities (Figure 1a). This was borne out when the t_m's of the separated satellites were determined.

A mixture of the unfractionated satellites (*i.e.*, I and II) was

TABLE I: Densities of Synthetic DNA Polymers and Naturally Occurring Satellite DNAs.[a]

Polymer	Density (g/cm³) in CsCl		Crab Satellites	Density (g/cm³) in CsCl	
	Neutral	Alkaline		Neutral	Alkaline
$(dA)_n$	1.622[b]	1.622[b]			
$(dT)_n$	1.739[b,c]	1.771[b]			
$(dC)_n$	1.685[c]	1.722[c]			
$(dG)_n$	1.754[c]	1.780[c]			
$(dA\text{-}dT)_n$	1.672[b,c] 1.679[e,f]	1.722[b,c]	Crabs[d]	1.677[d]	1.734[d]
$(dG\text{-}dT)_n \cdot (dC\text{-}dA)_n$	1.690[b]		I (ds)[g]	1.725	
$(dC\text{-}dA)_n$	1.687[b,c]	1.687[b,c]	I L (ss)	1.718	1.725
$(dG\text{-}dT)_n$	1.771[b]	1.825[b]	I H (ss)	1.771	1.828
	1.794[c]				
			II (ds)[h]	1.725	
			II L (ss)	1.718	1.763
			II H (ss)	1.751	1.800

[a] Single strands of satellites were recovered from alkaline CsCl gradients of the paired isopycnic twins centrifuged together, or from a single "twin" centrifuged alone in alkaline CsCl. Each purified single strand was subsequently centrifuged in the presence of a marker DNA in neutral or alkaline CsCl. Neutral CsCl gradients were at pH 8[b,e,f] or 7.3.[c] Alkaline CsCl gradients were at pH 12.45 or 12.5. Densities were also calculated from alkaline CsCl gradients or mixtures of satellites I and II (see Figure 1a). The error in the densities listed is ±0.003 g/cm³. [b] Wells and Blair (1967). [c] Wells and Larson (1972). [d] Beattie and Skinner (1972). The poly(dA-dT) satellites of various crabs contain 3–8% dG + dC residues (W. G. Beattie and D. M. Skinner, unpublished observations). [e] Schildkraut *et al.* (1962). [f] Erikson and Szybalski (1964). [g] *P. pollicaris* satellite I. [h] *P. pollicaris* satellite II.

melted rapidly, cooled for 30 min, then reheated; the temperature was increased 2.5 times as rapidly as usual. The hyperchromicity of the second melt was equal to that of the first phase of the first melt, and the transition only slightly less sharp (inset Figure 3a). The slightly higher t_m (75°) appears to be due to the rapidity of the temperature increase in the second dissociation. We conclude that satellite I had rapidly reassociated on cooling while satellite II had not. This conclusion was verified by further experiments (see Figure 4).

The dissociation of isolated satellite I (Figure 3b) again resembles that of the synthetic poly(dG-dT)$_n$·(dC-dA)$_n$ in three respects: (1) the temperature of half-dissociation of the naturally occurring satellite in 15 mM NaCl–1.5 mM sodium citrate (pH 7) is 72° as compared with 74° for the synthetic polymer (Wells *et al.*, 1965); (2) it dissociates over a very narrow temperature range, the transition being only 1.5°; and (3) it reassociates rapidly after thermal dissociation and dissociates a second time at a second temperature, almost as abruptly as at the first (see inset, Figure 3a). The last two properties imply, at least, a simple and highly repetitious sequence.

The t_m of satellite II (Figure 3b) is that predicted for a DNA of ρ = 1.725 g/cm³ and of ~60% (G + C) content (De Ley, 1970; Mandel *et al.*, 1970). Its failure to reassociate as readily as satellite I, even though present in higher concentration, implies a somewhat more complex sequence.

The reassociation of mixtures of single strands recovered from alkaline CsCl gradients was also monitored. The H and L strands of satellite I mixed in equal amounts at neutral pH reassociated to form a single band at ρ = 1.725 g/cm³ even at 0° (Figure 4a), and the density remained the same after 2 hr at 76° (Figure 4b). The H and L strands of satellite II mixed under the same conditions (Figure 4c) required a period of heating at 76° before reassociation was complete (Figure 4d), again indicating their greater complexity. There was no detectable interaction of satellite I H or L with either component of satellite II (data not shown).

The extent of reassociation of the two individual satellites was determined with a Model E analytical ultracentrifuge. The experiments were done before enough DNA had been collected for an optical determination of the t_m's of the satellites. t_m's were estimated from the data of Gillis *et al.*, (1970) to be 101° in 0.176 M Na^+, calculating approximately 73% dG + dC from the densities in CsCl gradients (De Ley, 1970). Subsequently we found that these reassociations at 76° had been carried out

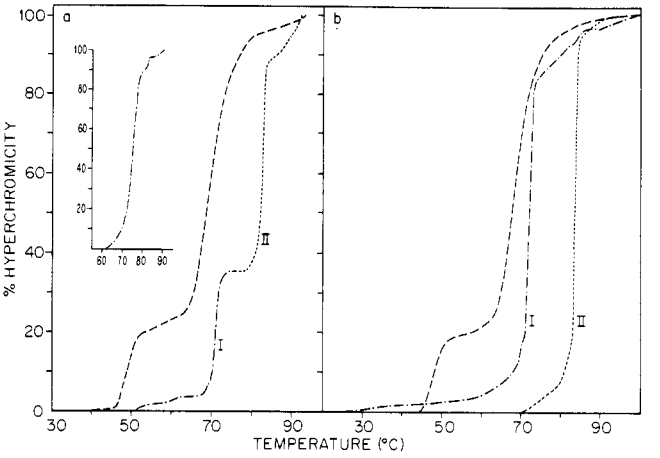

FIGURE 3: Thermal dissociation of DNA of *Pagurus pollicaris* (dG + dC)-rich satellites and Gecarcinus total DNA as a control. Isopycnic twin satellite DNA isolated from the testes of *Pagurus* was purified by 2 rounds of centrifugation in neutral CsCl preparative gradients. A 9-μg sample was dialyzed against 15 mM NaCl–1.5 mM sodium citrate (SSC/10), pH 7, and heated in a Gilford Model 2400 spectrophotometer as described (a); then cooled and reheated (inset, Figure 3a). The isopycnic twins were separated from each other by centrifugation in Hg^{2+} + Ag^+-Cs_2SO_4; 29 μg of satellite I (· · · ·) and 35 μg of satellite II (- - - - -) were heated in SSC/10; as a control, 26 μg of total DNA of *Gecarcinus* was also melted (——) (b). The purity of the satellites was monitored by analytical ultracentrifugation of an excessive amount (>5 μg) of each satellite. No main-band DNA was detected.

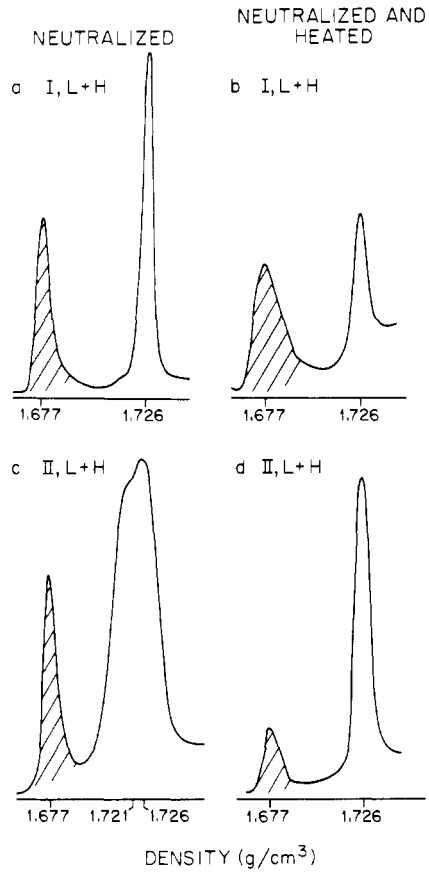

FIGURE 4: Tracings of Model E analytical ultracentrifuge runs of mixtures of components of satellite I and II after reassociation. Satellite I H (3.8 μg/ml) and L (4.0 μg/ml) were (a) mixed at 0°, denatured with alkali, and neutralized or (b) heated in 0.176 M Na^+ at 76° for 2 hr; (c) satellite II H (3.6 μg/ml) and L (4.0 μg/ml) were treated as (a); (d) satellite II H (3.6 μg/ml) and L (4.0 μg/ml) were treated as satellite I, (b). (e) A mixture of satellite I L (4.0 μg/ml) and satellite II H (3.6 μg/ml) was heated at 76° for 2 hr in 0.176 M Na^+.

11 and 20° below the actual t_m's. Since these temperatures are within the broad range in which reassociation may be observed (Bonner *et al.*, 1973) and, more importantly, since reassociation occurred in both cases under these conditions, the experiments were not repeated.

In summary, the density and thermal dissociation data indicate that satellite I is a DNA of simple structure, in some respects similar to $(dG\text{-}dT)_n\cdot(dC\text{-}dA)_n$ (Doerfler and Hogness, 1965; Wells *et al.*, 1965; Wells and Blair, 1967), and that satellite II, although more complex, must also contain many repeated sequences since it reassociates completely within a short time at low DNA concentration.

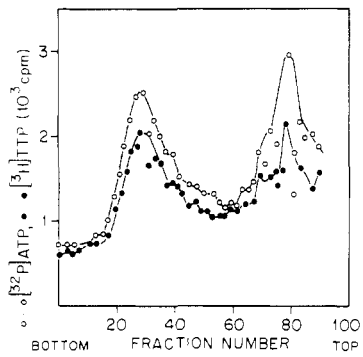

FIGURE 5: Alkaline preparative CsCl gradient of DNA synthesized *in vitro* with satellite I as template. The product of the DNA-synthesizing reaction mixture was desalted, centrifuged in alkaline CsCl gradients, and counted as described in Materials and Methods.

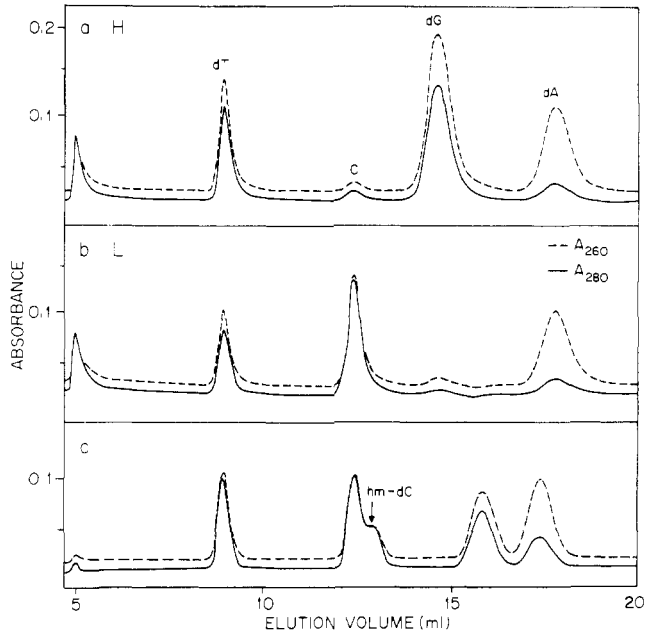

FIGURE 6: Elution pattern of nucleosides from Aminex A-6; 17 μg of satellite I H (a) and 9 μg of satellite I L (b) were degraded enzymatically, applied to an Aminex A-6 column and eluted. Standard nucleosides are shown in (c). hm-dC indicates position of hydroxymethyldeoxycytidine.

Distribution of [^{32}P]Adenosine and [^{3}H]Thymidine Residues in DNA Synthesized with Satellite I as Template. If satellite I H were composed entirely of dG and dT residues and satellite I L of dC and dA residues, then DNA molecules synthesized *in vitro* with satellite I as template in the presence of [^{3}H]dTTP and [α-^{32}P]dATP should distribute at the bottom and top of an alkaline CsCl gradient and contain primarily ^{3}H and ^{32}P label, respectively. Figure 5 shows that, in fact, the two peaks contain approximately equal amounts of ^{32}P and ^{3}H, tentative evidence for the presence of equivalent numbers of dA and dT residues in both strands.

In each of four experiments, less than 1% of the total radioactive precursors was incorporated into the DNA product. To check the fidelity of the *in vitro* incorporating system we used as template the satellite poly(dA-dT) isolated from the land crab, *G. lateralis* (Skinner, 1967); this DNA is known to be efficiently copied in various *in vitro* DNA polymerase systems. In two separate experiments, we found by nearest-neighbor analysis (Josse *et al.*, 1961) of the product that 90 and 92% of the α-^{32}P from labeled dATP was transferred to dTMP. This is in good agreement with evidence that although the majority of dA residues alternate with dT residues, as in the poly(dA-dT) satellite of certain other crustacea (Swartz *et al.*, 1962), 5% of the dT residues of the poly(dA-dT) are adjacent to other dT residues (Skinner, 1967; Allen *et al.*, 1972; Gray and Skinner, 1974) and with our evidence that the satellite contains 4.4% dG and 4.4% dC residues. This control indicated that our DNA polymerase system was yielding faithful copies and strengthened the conclusions given above with respect to the results shown in Figure 5.

Since the observed data shown in that figure differed from the prediction, indicating that the satellite differs from synthetic $poly(dG\text{-}dT)_n\cdot(dC\text{-}dA)_n$, we purified sufficient quantities of the H and L strands of satellite I to determine their base com-

TABLE II: Base Compositions of the (dG + dC)-Rich Satellites of *P. pollicaris* and the α Satellite of *C. porcellus*.

Satellite	Amount analyzed (μg)	Base (%) dT	dC	dG	dA	% dG + dC	% dG + dT
P. pollicaris							
I H	17	22.4	1.8	49.8	25.9		
						53	73
I H	7	22.8	3.4	51.7	22.3		
I L	9	23.5	47.0	1.9	27.8	49	25
II H	17	27.5	26.7	36.4	9.4		
						63	65
II H	15	28.1	25.7	36.9	9.2		
II L	9	11.6	32.6	29.0	26.8		
						60	41
II L	11	14.1	31.2	27.7	27.0		
C. porcellus							
α H	20	39.3	3.7	35.5	21.5	40	75
α L	10	20.9	36.4	2.9	39.8	39	24

positions directly. H and L strands of satellite II were purified at the same time.

Base Compositions of Satellites I and II. Figure 6 shows the elution pattern of the nucleosides in DNase digests of the L and H strands of satellite I. The nucleosides are sufficiently separated and the monitoring device is sufficiently sensitive to permit the quantitative determination of less than 1 μg of a single nucleoside. The position of hydroxymethyldeoxycytidineis indicated in the calibration data of Figure 6c. The H strand (ρ = 1.828 g/cm^3) of satellite I is extremely rich in dG residues; the 2–3% dC may well be due to contamination by another species of DNA (see below); its dC-rich partner contains corresponding quantities of the opposite bases (Table II). The base compositions of the L and H strands of satellite II are less extreme. No odd bases were detected in either satellite.

The base composition of the separated strands of the guinea pig α satellite reported here agree quite well with those previously reported (Corneo *et al.*, 1968; Yunis and Yasmineh, 1970). As in crab satellite I, each strand is very low in dC or dG residues.

Source of Small Amounts of dC and dG in Opposite Strands of Satellite I. To determine whether the small amount of dC in the H strand and the equally small amount of dG in the L strand of satellite I were due to contamination by trace amounts of the opposite strands, several types of experiments were performed with either H- or L-strand DNAs labeled by iodination with Na^{125}I. Prefatory to the experiments with the satellite DNAs, *Escherichia coli* DNA was iodinated, degraded to free bases by incubation with 90% formic acid at 175° for 45 min, and chromatographed on Whatman No. 1 paper in two solvent systems, butanol–water (86:14) and water. The ^{125}I, presumably in the form of 5-iodocytosine (Commerford, 1971), migrated as a single peak in both solvent systems (data not shown). There is thus no indication of bases other than cytosine being labeled by this technique.

It has been reported that the presence of a large number of 5-iodocytosine residues causes an increase in density; 21% 5-iodocytosine residues in calf thymus DNA increases the density by 35 mg/cm^3 (Commerford, 1971). As a control on our own technique we centrifuged iodinated *E. coli* DNA (see Figure 8a) and found its density to be identical with that of cosedimented nonradioactive *E. coli* marker DNA. We assume that iodination did not change the density of the H-strand satellite since its cytosine content, if any, is so low.

We then studied the behavior of the H and L strands of iodinated satellite I in alkaline CsCl gradients. Three possibilities were considered. If H strand was contaminated by a small amount of iodinated L strand DNA, then a band should form at ρ = 1.724 g/cm^3 (Figure 7a). Even if the DNA were completely iodinated, its density would not be expected to shift to more than ρ = 1.794 g/cm^3. If the dC residues form an integral part of the H strand, a band should appear at ρ = 1.828 g/cm^3 (Figure 7b). If the dC residues are contained in a species of DNA other than the H and L strands of satellite I, a band should form with a density different from either of the strands of satellite I or II (Figure 7c).

Iodinated H-strand DNA preparations formed two bands, one superimposed on the main band of *P. pollicaris* (ρ = 1.760 g/cm^3; Figure 8b) and the other at ρ = 1.775 g/cm^3. It is possible that the lighter of the two components was contaminating L strand DNA, since the increase in density (35 mg/cm^3) is that expected if 10.5% of the dC residues of a DNA containing

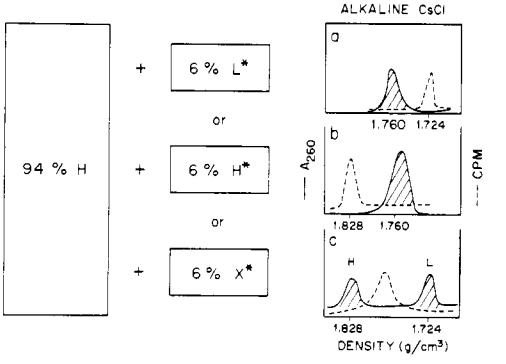

FIGURE 7: Hypothetical sources of cytosine residues in H strand preparations and their predicted positions in alkaline CsCl gradients; 94% of the single-stranded DNA is dC free H strand. The remainder is either (a) contamination with 6% L strand, or (b) dC residues that are an integral part of the H strand, or (c) contamination with another DNA (X*) rich in dC residues. The dC residues are labeled with ^{125}I. Hatched peaks indicate positions of marker DNAs. In (a) and (b) marker DNA is main component DNA of *P. pollicaris* (ρ = 1.760 g/cm^3); in (c) markers are H and L strands of satellite I.

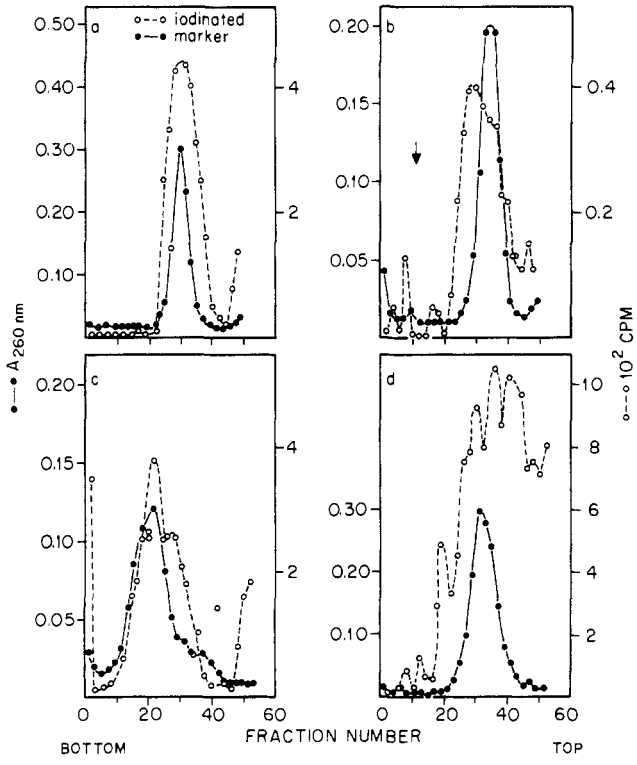

FIGURE 8: Preparative alkaline CsCl density gradient mixtures of ^{125}I-labeled DNAs and marker DNAs. (a) *E. coli* DNA (55 μg) (ρ = 1.764 g/cm^3) was added to trace amounts of ^{125}I-labeled *E. coli* DNA and centrifuged on a "relaxed" gradient. The density of the lower phase was 1.859 g/cm^3 and of the upper phase 1.570 g/cm^3. (b) Main-component DNA (47 μg) (ρ = 1.760 g/cm^3) of *P. pollicaris* was centrifuged with less than 1 μg of ^{125}I-labeled H-strand DNA. The upper phase had an original ρ = 1.570 g/cm^3. The position of H-strand DNA is indicated by an arrow. (c) Main-component DNA (47 μg) of *P. pollicaris* was centrifuged with less than 1 μg of ^{125}I-labeled L-strand DNA. The upper phase had an original ρ = 1.496 g/cm^3. (d) As (c) except 60 μg of main-component DNA and density of upper phase was 1.576 g/cm^3.

50% dC residues were iodinated. The other band may be a DNA contaminant that is neither H nor L strand. More importantly, there is essentially no radioacitivity at the density of H-strand DNA (ρ = 1.828 g/cm^3; arrow, Figure 8b). From these experiments we conclude that the dC residues are not an integral part of the H-strand DNA.

Iodination of the dC-rich L strand yielded more than 25 times as many counts on the DNA, which banded principally in the expected region of the gradient (*i. e.*, it was less dense than the main band marker; Figure 8c). When centrifuged in CsCl of increased density, the iodinated L-strand DNA was distributed as shown in Figure 8d. The broad peak may indicate partial degradation of the iodinated DNA. A small peak (defined by only a single point) was observed on the dense side of the main band. This again may represent a DNA contaminant that is neither H or L strand.

Discussion

It was of interest to determine whether each of the individual strands of satellite I was made up of only three bases. Given complementarity, it was only necessary to show that one base was absent from one strand; it could then be assumed that its complement was absent from the other. It is not possible rigorously to exclude the presence of one base, but the data on the buoyant densities of the radioiodinated H strand suggest that fewer than 3% of the total cytosines in the preparation sediment with the H strand, and fewer than 3% of the bases in the preparation are cytosines; hence, less than one base in 1000 in this strand is dC, and presumably dG is equally rare if not altogether absent from the L strand.

The method used here for nucleoside analysis distinctly separates 5-hydroxymethyldeoxycytidine from deoxycytidine. We may therefore say with some certainty that neither the crab nor guinea pig satellites contain detectable quantities of 5-hydroxymethyldeoxycytidine. This agrees with the conclusion suggested by Corneo *et al.* (1968).

Experiments with radiolabeled H strand of the guinea pig α satellite should indicate whether the small amount of dC residues (and, by complementarity, dG residues in the L strand) are contaminations or are an integral part of the H strand.

On the basis of dissociation and reassociation characteristics, it is now clear that satellite DNAs are highly variable with respect to their complexity. This is true not oly in comparison of one satellite to another, but also within the structure of a given satellite. For example, the simplest eukaryotic DNA yet described is the "crab poly(dA-dT) satellite" constituting 6–30% of various crustacean DNAs; although more than 90% of this molecule is alternating dA and dT residues, the remainder (which may consist of more than 10^8 nucleotides in some *Cancer* genomes) is of unknown structure and genetic significance. Similarly, the repeating hexanucleotide sequence of the guinea pig satellite, as determined by analysis of pyrimidine tracts (Southern, 1970), is again a simple structure but one that comprises only 50% of the satellite. The faithful reassociation and sharp melting transition of the reassociated *P. pollicaris* satellite I described here suggest that a relatively simple repeating sequence makes up a large fraction of the molecule. As such, it seemed reasonable to attempt a sequence analysis. The results, described in a companion paper (Skinner *et al.*, 1974), corroborate this conclusion; the principal repeat, making up virtually all of the total molecule, is the tetramer -T-A-G-G- on one strand, and its complement on the other. Preliminary studies on the reassociation properties of the (dG + dC)-rich satellite of *G. lateralis* and the (dA + dT)-rich satellite of the blue crab, *Callinectes sapidus*, show that they are more complex than the *P. pollicaris* satellite I.

From studies with bacterial DNAs, De Ley (1970) has concluded that there is a linear relationship between base composition, buoyant density, and t_m in the range of 40–70% dG + dC. Conversely, it is well known that the densities of $(dA\text{-}dT)_n$ and $(dG\text{-}dC)_n$ are respectively considerably greater and less than predicted from this relationship (Schildkraut *et al.*, 1962); and other polydeoxynucleotides of extreme base compositions, whether synthetic polymers or naturally occurring DNAs, also deviate from the simple relation. The reason for the deviation lies in the fact that the properties of the individual bases in the polynucleotides are strongly influenced by their neighbors (see Gray and Tinoco, 1970), and in DNAs of simple sequence the nearest neighbor pattern may not approach the nearly random distribution commonly observed in the more complex bacterial DNAs.

A clear example of this behavior is seen in satellite I H. Although poly(dA) is the least dense polymer and shows no density change in alkali (Table I), whereas polymers of dT and dG are the most dense and show the greatest change, the presence of 25% dA in satellite I H does not result in a lower density in neutral CsCl or a smaller increase in alkaline CsCl than those of (dT-dG) (Table I) (Doerfler and Hogness, 1965; Wells and Blair, 1967). It is clear then that estimating the base compositions of simple DNAs from buoyant density and melting temperature alone is hazardous and may be highly misleading even

if the total base composition itself is not very extreme.

Gall and Atherton (1974) have shown that several pairs of *Drosophila virilis* satellites differ from each other by the substitution of a single base; they have then postulated that similar pairs of satellites differing by a single base may be found in other organisms. In the case of *Pagurus pollicaris,* however, the distinctly different base compositions of satellites I and II make it unlikely that they are so related. Given the known sequence of satellite I, no combination of altering one base in the repeating tetramer or one base in every other tetramer (generating a repeating octomer), or deleting one base from each tetramer (generating a trimer) or one base from every other tetramer (generating a heptamer) yields a base composition within the confidence limits of the known base composition of either strand of satellite II.

Previously we studied the degree of relatedness between the (dG + dC)-rich satellite of *G. lateralis* (the strands of which are unbiased with respect to base composition) and DNAs of other crustaceans. In that case, the homology decreased markedly with evolutionary divergence (Graham and Skinner, 1973), the satellite being 5–10% homologous with the DNAs of five true crabs, 3% with the DNA of the lobster, an animal in a different taxonomic Section, and 0.4% with the very primitive crustacean, the brine shrimp, *Artemia salina.* Because of the extreme interstrand bias of the *P. pollicaris* satellite I described here, it is doubtful that these sequences are represented to any very significant extent in the unbiased *G. lateralis* satellite. The less extreme *P. pollicaris* satellite II has a base composition closer to that calculated for the *G. lateralis* satellite and may represent in part yet another homologous case among the Crustacea. This point has not yet been determined.

Acknowledgments

We thank Mr. John Dohrmann for providing some frozen tissues of *Pagurus,* Dr. M. G. Hanna for guinea pig tissues, Mr. W. L. Carrier for *E. coli* DNA, Dr. R. P. Singhal for running several of the nucleoside analyses, Drs. Donald Kelmers and Bimal Pal for the use of the Aminex A-6 column setup, and Drs. W. E. Cohn and R. Litman for helpful discussions.

References

Allen, F. S., Gray, D. M., Roberts, G. P., and Tinoco, I. (1972), *Biopolymers 11,* 853.

Beattie, W. G., and Skinner, D. M. (1972), *Biochim. Biophys. Acta 281,* 169.

Bonner, T. I., Brenner, D. J., Neufeld, B. R., and Britten, R. J. (1973), *J. Mol. Biol. 81,* 123.

Britten, R. J., Graham, D. E., and Neufield, B. (1974), *Methods Enzymol. 29E,* 363.

Britten, R. J., Pavich, M., and Smith, J. (1970), *Carnegie Inst. Washington, Yearb. 68,* 400.

Brunk, C. F., and Leick, V. (1969), *Biochim. Biophys. Acta 179,* 136.

Carrier, W. L., and Setlow, R. B. (1971), *Anal. Biochem. 43,* 427.

Commerford, S. L. (1971), *Biochemistry 10,* 1993.

Corneo, G., Ginelli, E., Soave, C., and Bernardi, G. (1968), *Biochemistry 7,* 4373.

Davidson, N., Widholm, J., Nandi, U. S., Jensen, R., Olivera, B. M., and Wang, J. C. (1965), *Proc. Nat. Acad. Sci. U. S. 53,* 111.

De Ley, J. (1970), *J. Bacteriol. 101.* 738

Doerfler, W., and Hogness, D. S. (1965), *J. Mol. Biol. 14,* 237.

Erikson, R. L., and Szybalski, W. (1964), *Virology 22,* 111.

Gall, J. G., and Atherton, D. C. (1974), *J. Mol. Biol.* (in press).

Getz, M. J., Altenburg, L. C., and Saunders, G. F. (1972), *Biochim. Biophys. Acta 287,* 485.

Gillis, M., De Ley, J., and DeCleene, M. (1970), *Eur. J. Biochem. 12,* 143.

Graham, D. E., and Skinner, D. M. (1973), *Chromosoma 40,* 135.

Gray, D., and Skinner, D. M. (1974), *Biopolymers 13,* 843.

Gray, D. M., and Tinoco, I. (1970), *Biopolymers 9,* 223.

Josse, A., Kaiser, A. D., and Kornberg, A. (1961), *J. Biol. Chem. 236,* 864.

Mandel, M., Igambi, L., Bergendahl, J., Dodson, M. L., Jr., and Scheltgen, E. (1970), *J. Bacteriol. 101,* 333.

Marmur, J. (1961), *J. Mol. Biol. 3,* 208.

Miyazawa, Y., and Thomas, C. A., Jr. (1965), *J. Mol. Biol. 11,* 223.

Schildkraut, C. L., Marmur, J., and Doty, P. (1962), *J. Mol. Biol. 4,* 430.

Sevag, M. G., Lackman, D. B., and Smolens, J. (1938), *J. Biol. Chem. 124,* 425.

Singhal, R. P., and Cohn, W. E. (1972), *Biochim. Biophys. Acta 262,* 565.

Skinner, D. M. (1967), *Proc. Nat. Acad. Sci. U. S. 58,* 103.

Skinner, D. M., and Beattie, W. G. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 3108.

Skinner, D. M., Beattie, W. G., Blattner, F. R., Stark, B. P., and Dahlberg, J. E. (1974), *Biochemistry 13,* 3930.

Skinner, D. M., Beattie, W. G., Kerr, M. S., and Graham, D. E. (1970), *Nature (London) 227,* 837.

Southern, E. M. (1970), *Nature (London) 227,* 794.

Sueoka, N., and Cheng, T. Y. (1962), *Proc. Nat. Acad. Sci. U. S. 48,* 1851.

Swartz, M. N., Trautner, T. A., and Kornberg, A. (1962), *J. Biol. Chem. 237,* 1961.

Szybalski, W. (1968), *Methods Enzymol. 12B,* 330.

Tiselius, A., Hjerten, S., and Levin, O. (1956), *Arch. Biochem. Biophys. 65,* 132.

Vinograd, J., Morris, J., Davidson, N., and Dove, W. F., Jr. (1963), *Proc. Nat. Acad. Sci. U. S. 49,* 12.

Wells, R. D., and Blair, J. E. (1967), *J. Mol. Biol. 27,* 273.

Wells, R. D., and Larson, J. E. (1972), *J. Biol. Chem. 247,* 3405.

Wells, R. D., Ohtsuka, E., and Khorana, H. G. (1965), *J. Mol. Biol. 14,* 231.

Yunis, J. J., and Yasmineh, W. G. (1970), *Science 163,* 263.