INTERACTION BETWEEN DENATURED DNA, POLYRIBONUCLEOTIDES, AND RIBOSOMAL RNA: ATTEMPTS AT PREPARATIVE SEPARATION OF THE COMPLEMENTARY DNA STRANDS*

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The buoyant density of DNA in the CsCl equilibrium density gradient depends on its composition and secondary structure. Denatured DNA's usually form a single band (Fig. 1*B* and *F*) at a density 0.013 (high % G + C) to 0.017 (low % G + C) gm/cm^3 higher than that of the same DNA in its native helical form (Fig. 1*A* and *E*). Experience with *Escherichia coli* (Fig. 1*F* and *H*) and several other organisms had indicated that the band profile and density of denatured and rapidly cooled DNA are only slightly altered, if at all, by the presence of RNA during the denaturation and centrifugation procedures. It was somewhat surprising, therefore, to observe, in addition to the denaturedlike DNA band (dN′), a DNA- and RNA-containing heavy band (DR) during CsCl gradient centrifugation of a thermally denatured and rapidly cooled DNA + RNA mixture extracted from *Bacillus subtilis* (Fig. 1*D*).[1] Earlier[2] and current studies on the origin, properties, and composition of the materials contained in the DR and dN′ bands are the subject of this communication.

Materials and Methods.—Nucleic acids extracted from the wild type and several mutant strains of *B. subtilis*,[3] *Clostridium perfringens*,[4] and *E. coli* strain B were chiefly employed. The *DNA + RNA extract* was prepared by lysis of the cells with lysozyme (100 μg/ml) and sodium lauryl sulfate (1%) followed by exhaustive deproteinization with a chloroform + butanol mixture (4:1), precipitation with 1.5 volumes of ethanol, and prompt solution of the precipitate in SSC. *Pure DNA* was obtained from this mixture by treatment with RNase (100 μg/ml, 30 min, 37°C), deproteinization, and selective isopropanol precipitation.[5] The molecular weight of the DNA thus prepared was usually above 30×10^6 daltons. *RNA* was extracted from a centrifuged pellet of exponentially growing cells by treatment with lysozyme and sodium lauryl sulfate, and grinding in Tris buffer (0.01 *M* Tris, 0.1 *M* NaCl, 0.01 mg $MgCl_2$, pH = 7.0) with bentonite (1%), sand, DNase (50 μg/ml) and liquefied phenol (Mallinckrodt, A. R.); all these operations, including subsequent phenol deproteinization, were carried out at 0°C. Ribosomal RNA fractions (16S and 23S) were isolated by chromatography at 35°C on a methyl-esterified albumin-kieselguhr column,[6] or by the sucrose gradient centrifugation procedure.

Poly A, poly C, poly G, poly I, and poly U are commercial products of the Miles Chemical Company, Elkhart, Ind. Poly G was purchased as a copolymer of inosinic and guanylic acids (1:1), but upon analysis was found to contain less than a few per cent inosinic acid residues; thus it will be referred to as poly G.

Analytical or preparative CsCl or Cs_2SO_4 density-gradient centrifugation was employed for the identification and isolation of the DNA, RNA, and DR and dN′ bands.[7] Stepwise chromatography on Dowex-1 resin served to determine the

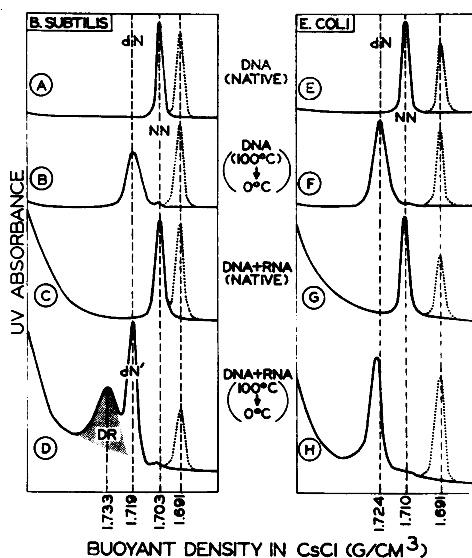

FIG. 1.—Microdensitomer tracings of photographs taken after 22 hr of CsCl density-gradient centrifugation (44,770 rpm, 25°C) of *B. subtilis* (*A–D*) and *E. coli* (*E–H*) DNA (1–3 μg DNA per band per 12 mm 4° centrifuge cell) in pure (RNase-treated) form (*A, B, E, F*) or in the form of a total nucleic acid extract (DNA + RNA), deproteinized but not RNase-treated (*C, D, G, H*). Samples *A, C, E,* and *G* were centrifuged in the native state, while the other samples (*B, D, F, H*) were denatured by heating in SSC for 6 min (approximately 20 μg DNA ± 100 μg RNA per ml SSC) and quenching in ice water before centrifugation. Under condition *D* only, the DNA separates into two bands, denoted henceforth as DR and dN' bands. Under condition *H*, the DNA band seems to be slightly shifted toward the higher density. The dotted lines indicate the position of the native *C. perfringens* DNA employed as the "density marker" (1.691 gm/cm³).

nucleotide composition of P^{32}-labeled RNA, digested in 0.3 *M* KOH (18 hr, 37°C).[8] A low-background (less than 2 cpm) counter was used for P^{32} assays.

Results.—Formation of the DR band: Two alternative procedures, as adopted for *B. subtilis* nucleic acids, lead to the formation of a DR band: (*a*) thermal denaturation (6 min, 96–100°C) followed by rapid cooling (0°C) of the DNA + RNA mixture (Fig. 1*D*); or (*b*) mixing of RNA (10–100 μg/ml) with the denatured, rapidly cooled DNA (10–20 μg/ml) at 0–25°C (Fig. 2*E*), both procedures carried out in SSC and followed by CsCl density-gradient centrifugation at 0° or 25°C. The denatured state of the DNA is obligatory, since slow cooling of the DNA + RNA mixture or its exposure to renaturation conditions (3–6 hr at 65°C) results in partial or complete disappearance of the DR and dN' bands and appearance of a progressively lighter band formed by renatured DNA. The conditions of the DR complex formation are rather critical, since the DR band is not formed when SSC is diluted more than tenfold or when formaldehyde is added (1–2%). The effect of nucleases and the identity of the RNA component are discussed in the following section.

Composition and properties of the DR and dN' materials: The DR and dN' components (Figs. 1*D* and 2*E*) were isolated by preparative CsCl gradient centrifugation and analyzed by various methods. Band dN' contains only denatured DNA, most probably only one of the two complementary strands since it cannot be renatured under conditions which result in renaturation of the nonfractionated

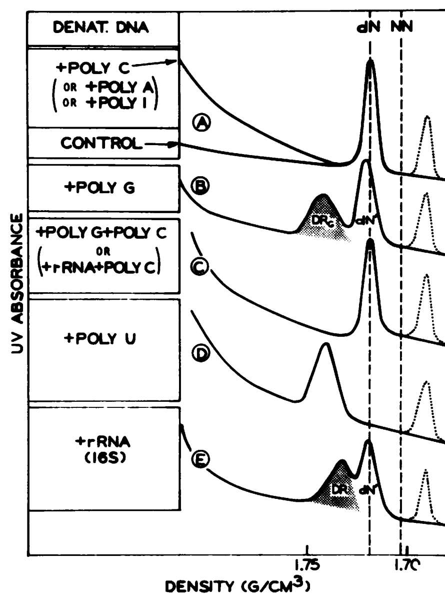

FIG. 2.—Microdensitometer tracings of photographs taken after 20 hr of CsCl density-gradient centrifugation (44,770 rpm, 25°C) of the denatured *B. subtilis* DNA, alone (*A: Control*) or mixed with various polyribonucleotides and/or 16S ribosomal RNA (20 μg DNA $\times$ 10–50 μg polyribonucleotides and/or rRNA per ml SSC), as indicated on the left margin of the diagram (*A–E*). The dotted lines indicate the position of the native *C. perfringens* DNA (cf. Fig. 1).

denatured DNA (10–20 μg DNA/ml of 0.3 *M* NaCl + 0.015 *M* Na_3 citrate, pH = 7.6, 6 hr, 65°C). Combining of the dN′ material with ribosomal RNA (procedure *b*) does not result in formation of a DR-like band upon another round of analytical centrifugation. Band DR contains two components: denatured DNA and approximately 5 per cent RNA,[9] designated earlier as cRNA.[2] The denatured DNA component of the DR band most probably contains a population of strands complementary to the dN′ material, since it does not renature alone but re-forms nativelike material when renatured in mixture with dN′ DNA. The RNA component of the DR band consists of ribosomal RNA, or of its fragments, as indicated by its base composition (Table 1; compare second and third line of numerals) and by the formation of a DR band by a mixture of denatured DNA and rRNA, the latter extracted from purified ribosomes or from actinomycin-treated (1 hr, 37°C) *B. subtilis* cells. Both the 16S and the 23S rRNA, fractionated on a methyl-

TABLE 1

BASE COMPOSITION OF *B. subtilis* DNA, rRNA, AND RNA COMPONENT IN THE RNASE-TREATED AND UNTREATED DR MATERIAL

	Per Cent			
	C	A	U(T)	G
DNA*	21.5	28.5	28.5	21.5
rRNA†	21	24	25	30
RNA† in DR	22	25	25	28
RNA† in DR + RNase‡	9	25	24	42

* Cf. Schildkraut *et al.*[7]
† Nucleotide composition of alkaline digest (cf. *Materials and Methods*) of the P^{32}-labeled purified rRNA or of the DR material formed from "cold" denatured DNA and P^{32}-labeled rRNA, isolated by preparative CsCl gradient centrifugation. P^{32}-labeled rRNA was extracted from bacteria grown for 3–4 cell generations in the presence of P^{32}-orthophosphate (50 μc/m/P).
‡ 5 μg RNase per ml SSC, 15 min, 37° C.

esterified albumin-kieselguhr column, are active in formation of the DR band, with the former somewhat more effective than the latter; purified transfer RNA seems to be inactive under the same conditions. The foregoing results, and the observation that this RNA labels late in cells exposed to the P^{32} pulse, suggest that so-called messenger RNA is not a component of the DR material.

Both the DR and the dN′ band completely disappear upon DNase treatment. On the other hand, the ribosomal RNA contained in the DR band is only partially sensitive to RNase (1–100 μg/ml): digestion for periods of 1–15 min results in a progressive decrease in the buoyant density of the DR band toward a value only slightly higher (*ca.* 0.003 gm/cm^3) than that for the dN′ band. The percentage of RNA in the DR material decreases from about 5 to 0.5–1 per cent after extensive RNase digestion. This RNase-resistant residue exhibits an exceptionally low cytosine and high guanine content (Table 1; fourth line of numerals), the latter reaching in some experiments a value above 50 per cent. The intact DNA component of the DR material can be freed of the complexing RNA by heating to 100°C in 10^{-3} *M* sodium citrate and repeated preparative fractionation in the CsCl gradient. The DR material can also be separated from the bulk of the RNA (but not from the denatured DNA) by adsorption on cellulose nitrate filters.[10]

Interaction between denatured DNA and polyribonucleotides: The high guanine content of the RNase-resistant RNA residue in the DR material prompted us to study the interaction between denatured DNA and poly G. As suspected, poly G binds half of the denatured high molecular weight DNA, forming a DR_G band at a density up to 0.03 gm/cm^3 (CsCl) (Fig. 2*B*) or 0.02 gm/cm^3 (Cs_2SO_4) higher than the dN′ band. The material contained in the dN′ band does not contain any components that form complexes with either poly G or ribosomal RNA and cannot be renatured unless combined with the DR or DR_G component. The DR_G material obtained with poly G is completely resistant to ribonuclease, in agreement with the known RNase resistance of purine polynucleotides.

Poly A, poly C, and poly I do not exhibit any pronounced interaction with denatured DNA (Fig. 2*A*). However, poly C interferes with the DNA:poly G and DNA:rRNA interaction (Fig. 2*C*). In separate experiments it was found that an excess of cytosine mono- or diphosphates also inhibits the formation of the DR_G and DR complex between denatured DNA and poly G or ribosomal RNA. Poly U forms complexes with both strands of denatured *B. subtilis* DNA (Fig. 2*D*), but this reaction can be inhibited by simultaneous addition of poly A.

Formation of DR_G and DR bands among nucleic acids derived from different sources: Denatured DNA from all the Bacillaceae tested—*C. perfringens* (31% G + C), *B. megaterium* (38% G + C), *B. cereus* (39% G + C), and various mutants of *B. subtilis* (43% G + C),[7] including asporogenic variants—produces the DR band with ribosomal RNA in all the inter- or intraspecific combinations. However, RNA isolated from *B. subtilis* or *B. cereus* spores does not have this property. Low-density DR bands, poorly separated from the dN′ material, were formed with RNA isolated from "old" bacteria, held overnight in the postexponential, stationary growth phase. Although no separate dN′ and DR bands were observed with nucleic acids extracted from *E. coli* (50% G + C), DNA denatured in the presence of RNA bands at a somewhat higher density (Fig. 1*H*) than RNA-free denatured DNA (Fig. 1*F*). It is likely that a similar phenomenon was operative

in earlier studies on renaturation of *E. coli* DNA in the presence of ribosomal RNA.[11] As in the case of *B. subtilis* (Fig. 2*B*), poly G effects separation of the DNA strands for all the species mentioned above, with very wide separation between the dN′ and DR_G bands for *C. perfringens* DNA (up to 0.05 gm/cm³), lesser separation for *B. megaterium*, *B. cereus*, or *B. subtilis* DNA (up to 0.03 gm/cm³), and a moderate separation (*ca.* 0.01 gm/cm³) for *E. coli* DNA. The ribosomal RNA of *E. coli* forms a DR band with denatured *B. subtilis* DNA.

The DNA of several other bacterial species, including *Cytophaga johnsonii* (34% G + C), *Sarcina lutea* (71% G + C), and *Micrococcus lysodeikticus* (72% G + C),[7] did not exhibit any perceptible change in CsCl banding profile when heated and rapidly cooled in the presence (versus absence) of ribosomal RNA; in the presence of poly G some density increase (0.003, 0.020, and 0.011 gm/cm³, respectively) but no strand separation into separate bands was observed. In preliminary studies with bacteriophage DNA, poly G appeared to interact with only one strand of coliphage T7 DNA, essentially in the same manner as with *E. coli* or *B. subtilis* DNA. Under the same conditions, however, all the denatured coliphage λ DNA formed complexes with poly G. Poly U interacted with the total denatured DNA of most of the species, but not with T7, *S. lutea*, or *M. lysodeikticus* DNA. Detailed studies on the interaction between poly G (and poly U) and the denatured DNA of various viral, bacterial, and mammalian species will be reported elsewhere.

Discussion.—The experimental results suggest that the DNA of *B. subtilis* and of several other microorganisms contains dG:dC-rich sequences which repeat themselves many times along the whole genome at intervals of between a few and ten million daltons, with dG on one strand and dC on the other. Thus, ribosomal RNA which happens to contain G-rich sequences, or synthetic poly G, interacts only with the DNA strand which contains the dC-rich regions, forming the DR complex. Most probably, the dC-rich regions are always associated with the same DNA strand all along the whole bacterial "chromosome," or at least through its very long sections, since *B. subtilis* DNA of molecular weight approximating 100×10^6 ($^1/_{10}$ of the genome) gives very clear separation into the DR and dN′ bands (peaks), containing roughly the same amounts of DNA (compare Figs. 1*D* and 2*E*). However, one "switch" (inversion) per genome (10^9 daltons) in the continuity of the dC-rich sequences would be difficult to exclude, since the resulting renaturability of 5–10 per cent of the material in the separated dN′ or DR bands (half of the one or two "switch" regions in the linear or circular chromosome) is difficult to detect in analytical centrifugation experiments. Several experiments with rRNA indicate that the distances between the dG:dC-rich regions must be close to 10 million daltons, since progressive shearing of the DNA results in an increased ratio of the dN′ to DR material, indicating that the smaller fragments are deficient in the dC-rich regions. Also, the 5 per cent increase in the buoyant density of the DR band, as compared with the dN material,[9] indicates that one rRNA molecule (16S, 600,000 daltons) should be attached per each 12×10^6 daltons of the DNA strand. A higher frequency of dC-rich regions could be derived by a similar rough calculation based on a density shift of the DNA:poly G complex (DR_G band).

A schematic presentation of the distribution of the dC-rich sequences in portions of the bacterial genome, and of the interaction between these sequences in denatured

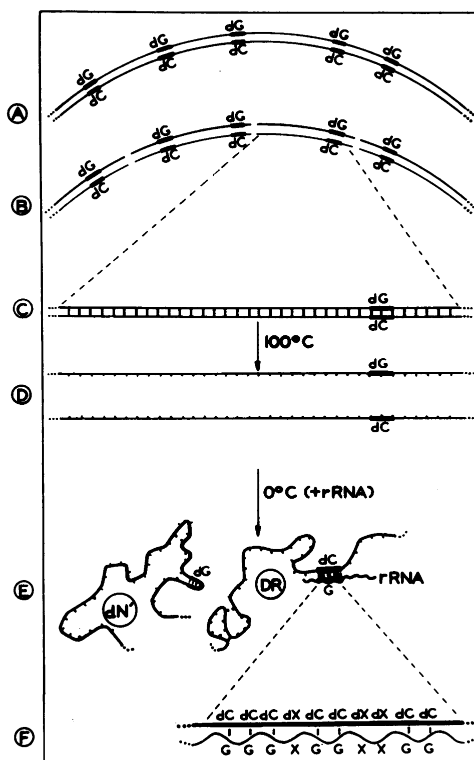

FIG. 3.—Diagrammatic representation of the complex formation between one of the complementary DNA strands (containing dC-rich sequences) and ribosomal RNA (rRNA). (*A*) A section of the *B. subtilis* chromosome corresponding to approximately 120 × 10^6 daltons. (*B*) Fragmentation of this section into 30 × 10^6 dalton fragments during the extraction and deproteinization procedure. (*C*) Enlarged representation of one of the fragments containing one dG:dC-rich sequence. (*D*) Strand separation on heating to 100°C. (*E*) Upon cooling to 0–20°C in the presence of rRNA, the G-rich sequence of rRNA interacts with the dC-rich sequence of one strand, forming the DR complex. The other strand, in which the dG-rich sequence forms intrastrand dG-dG bonds, corresponds to the dN' material (Fig. 1*D*). The region of rRNA not bound to DNA (thin wavy line) is susceptible to RNase digestion. (*F*) Hypothetical sequence within the complex-forming dC:G-rich region. Symbols dX and X indicate any deoxyribonucleotides or ribonucleotides, respectively. The base composition of the G-rich rRNA region (wavy line) corresponds to the RNase-resistant fraction in Table 1 (bottom line of numerals).

DNA with G-rich sequences in ribosomal RNA, is outlined in Figure 3. Admittedly, not all features of the observed phenomena can be easily explained by this simplified model.

Failure of the other DNA strand, containing dG-rich sequences, to react with poly C (Fig. 2*A*) could be expected, since dG sequences have a strong tendency to interact within themselves[12] (Fig. 3*E*), and since in at least one instance a complex between complementary polyribopurine and polydeoxyribopyrimidine nucleotides (poly I:poly dC) is considerably more stable than a corresponding complex between polydeoxyribopurine and polyribopyrimidine nucleotides.[13] The guanine-rich sequences in rRNA should be no longer than 200–400 nucleotides, since only 10–20 per cent of the 16S ribosomal RNA (16S RNA ≅ 2000 nucleotides) remains associated with the DNA after RNase treatment of the DR material. All these proper-

tics of the DR aggregates distinguish them clearly from the so-called DNA:messenger-RNA hybrids[10] formed by slow annealing, a process which usually destroys the DR band. Complexes formed with the dC-rich sequences in DNA are not very specific, since both the poly G and rRNA, the latter derived from closely or distantly related species including even mammalian cells (unpublished results), attach to the DNA of any of the Bacillaceae tested. Furthermore, there seems to be no obvious relationship between the base composition of the DNA and the formation of the DR or DR_G band. However, a similarity is observed in the modes of DNA:rRNA, DNA:poly G, and DNA:poly U interactions among several bacterial species within one family, while each family tested is characterized by its own distinct pattern of interactions.

What could be the biological function of these repeating dG:dC-rich sequences in DNA and of the G-rich sequences in rRNA? Although no answer to this question is at present available, one could easily postulate that the G-rich sequences in rRNA (synthesized under the guidance of the dC-rich sequences on one of the DNA strands) provide the ribosomal binding sites for the initial attachment of the C-rich regions of "messenger" RNA (synthesized on the DNA strand containing dG-rich sequences). The interference with this DR complex formation by poly C or even by cytidine mono- or diphosphates might reflect some general regulatory role of cytosine nucleotides in the synthetic processes.

Whatever the origin and function of these dG:dC-rich sequences in DNA, they permit preparative separation of the complementary DNA strands by CsCl gradient centrifugation. This type of fractionation has been reported previously only for a few cases[14] in which a natural bias in the base composition differentially affected the buoyant density or chromatographic behavior of the denatured strands. Attempts at strand fractionation by chromatography of the denatured DNA on columns containing immobilized poly G or rRNA, and studies on hybridization between the separated individual DNA strands and the ribosomal, soluble, and various fractions of pulse-labeled RNA will be described elsewhere.

Summary.—Ribosomal RNA (rRNA) forms complexes with only one of the two complementary DNA strands of *Bacillus subtilis*. Similar complexes are formed when poly G substitutes for rRNA, indicating that dC-rich sequences on one of the DNA strands (most probably on the same strand along the whole genome) and G-rich sequences on rRNA are the bonding sites. Formation of such complexes permits preparative separation of the complementary DNA strands, since the mixture of denatured DNA and poly G or rRNA splits into two well-separated bands when subjected to CsCl equilibrium density-gradient centrifugation. The "heavier" band is composed of DNA strands which contain dC-rich sequences in association with a few per cent poly G or rRNA, whereas the "lighter" fraction (0.01–0.04 gm/cm^3 buoyant density differential) contains the complementary strands characterized by the corresponding dG-rich sequences. Similar patterns of DNA:poly G interaction are observed for all Bacillaceae and for *Escherichia coli* but not necessarily for all the other organisms studied. Poly U forms complexes with both DNA strands, whereas poly I, poly C, and poly A do not interact directly with denatured DNA. The last two, however, inhibit the interaction of denatured DNA with poly G and poly U, respectively.

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The following abbreviations are used in this paper: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; poly A, poly C, poly G, poly I, poly U, homopolymers of adenylic, cytidylic, guanylic, inosinic, and uridylic acids, respectively; DNase, deoxyribonuclease I; RNase, pancreatic ribonuclease (Worthington) heated 10 min to 96°C; NN, a symbol for native, double-stranded DNA; dN, a symbol for denatured DNA; DR (or DR_G), symbol for materials composed of a complex between rRNA (or poly G) and one DNA strand, which contains dC-rich sequences; dN′, a symbol for material containing the other complementary DNA strand with dG-rich sequences; rRNA, ribosomal RNA; G, guanine or guanosine; dG, deoxyguanosine; C, cytosine or cytidine; dC, deoxycytidine; SSC, 0.15 *M* NaCl + 0.015 *M* trisodium citrate, pH = 7.6.

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[1] This formation of two bands in the CsCl gradient upon denaturation and rapid cooling of deproteinized nucleic acid extract from *B. subtilis* was first observed by Dr. V. N. Iyer and one of the present authors (W. S.) in controls for experiments designed for a different purpose [Iyer, V. N., and W. Szybalski, these PROCEEDINGS, **50**, 355 (1963)].

[2] Opara-Kubinska, Z., and W. Szybalski, *Abstracts*, 8th Annual Meeting, Biophysical Society. WA9 (1964).

[3] Szybalski, W., Z. Opara-Kubinska, Z. Lorkiewicz, E. Ephrati-Elizur, and S. Zamenhof, *Nature*, **188**, 743 (1960).

[4] Obtained from the Department of Medical Microbiology, University of Wisconsin.

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

[6] Mandell, J. D., and A. D. Hershey, *Anal. Biochem.*, **1**, 66 (1960); Kubinski, H., G. Koch, and O. Drees, *Biochim. Biophys. Acta*, **61**, 322 (1962).

[7] Meselson, M., F. W. Stahl, and J. Vinograd, these PROCEEDINGS, **43**, 581 (1957); Szybalski, W., *Experientia*, **16**, 164 (1960); Erikson, R. L., and W. Szybalski, *Virology*, **22**, 111 (1964); Opara-Kubinska, Z., Z. Borowska, and W. Szybalski, *Biochim. Biophys. Acta*, **72**, 298 (1963); Schildkraut, C. L., J. Marmur, and P. Doty, *J. Mol. Biol.*, **4**, 430 (1962).

[8] Koch, G., and H. Kubinski, *Z. Naturforsch.*, **19b**, 683 (1964).

[9] The fraction of complexed RNA was calculated on the basis of the buoyant density shift (CsCl) of the DR band (0.010–0.015 gm/cm^3) from the position of the denatured DNA, as compared with the ribosomal RNA (0.2–0.3 gm/cm^3).

[10] Nygaard, A. P., and B. D. Hall, *Biochem. Biophys. Res. Commun.*, **12**, 98 (1963).

[11] Schildkraut, C. L., J. Marmur, J. R. Fresco, and P. Doty, *J. Biol. Chem.*, **236**, PC2 (1961); Brieger, I., M.Sc. thesis, University of Wisconsin, 1962.

[12] Ralph, R. K., W. J. Connors, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 2265 (1962).

[13] Chamberlin, M., personal communication. The "melting" temperatures of 64 and 78° for poly dG:poly C and poly C:poly dG "hybrids," respectively, reported by S. Lee-Huang and L. F. Cavalieri [these PROCEEDINGS, **51**, 1022 (1964)], are also consistent with these results.

[14] Marmur, J., and S. Cordes, in *Informational Molecules*, ed. H. J. Vogel *et al.* (Academic Press, 1963), p. 79; Marmur, J., and C. M. Greenspan, *Science*, **142**, 387 (1963); Aurisicchio, S., E. Dore, C. Frontali, F. Gaeta, and G. Toschi, *Biochim. Biophys. Acta*, **80**, 514 (1964); Tocchini-Valentini, G. P., M. Stodolsky, A. Aurisicchio, M. Sarnat, F. Graziosi, S. B. Weiss, and E. P. Geiduschek, these PROCEEDINGS, **50**, 935 (1963); Guild, W. R., and M. Robison, these PROCEEDINGS, **50**, 106 (1963).