Role of Molecular Conformation in Determining the Electrophoretic Properties of Polynucleotides in Agarose–Acrylamide Gels. II†

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ABSTRACT: Polynucleotides of different size and conformation may be separated one from another in many instances by electrophoresis in Agarose–acrylamide gels. In this report we show that linear double-stranded polynucleotides of different molecular weights are resolvable when their weights range from about 0.3×10^6 (or possibly lower) to 2×10^6. In the range of 1 to 3×10^6, circular single-stranded polynucleotides may be resolved from linear single-stranded molecules having the same molecular weight, and circular double-stranded polynucleotides may be resolved from linear double-stranded molecules having the same molecular weight. In this same weight range, "nicked" open circular double-stranded DNA may be separated from intact supercoiled circular double-stranded DNA. Furthermore, linear double-stranded polynucleotides and linear and circular single-stranded polydeoxyribonucleotides, unlike linear single-stranded polyribonucleotides, show an increase in mobility as the voltage gradient is increased. These attributes of polynucleotides, together with their different sensitivities to particular endonucleases and their different affinities for certain dyes, could enable one to determine in many cases the nature of a previously uncharacterized polynucleotide (*i.e.*, whether it is RNA or DNA, whether it is single or double stranded, and whether it is circular or linear).

Electrophoresis of nucleic acids in polyacrylamide gels or composite Agarose–acrylamide gels has been found a useful analytical technique capable of high resolution (*e.g.*, Loening and Ingle, 1967; Dingman and Peacock, 1968). For ribonucleic acids, evidence has accumulated that this technique may be useful for defining some of the physical properties of the molecules under investigation (*e.g.*, Bishop *et al.*, 1967; Peacock and Dingman, 1968; Loening, 1969). We have recently presented evidence that this technique was also capable of distinguishing between single- and double-stranded polynucleotides, provided their molecular weight exceeded 3×10^5 to 4×10^5 (Fisher and Dingman, 1971). That is, unlike single-stranded molecules, double-stranded molecules exhibited an increase in mobility with an increase in temperature and an increase in mobility with an increase in voltage gradient. We also suggested that these results might reflect "end-on" migration of linear double-stranded polynucleotides (Fisher and Dingman, 1971). We felt that, under some conditions at least, Agarose–acrylamide gel electrophoresis might prove a convenient method for determining whether a newly isolated polynucleotide was RNA or DNA, whether it was single or double stranded, and possibly, whether it was linear or circular. In this report we present evidence that, within certain limitations, this technique is capable of providing such information.

Experimental Section

Materials

DEAE-Dextran with an approximate molecular weight of 2×10^6 and an intrinsic viscosity of 0.70 was purchased from Pharmacia (solutions of this material were filtered through an HA Millipore filter prior to use). Pancreatic deoxyribonuclease I, DNase I (electrophoretically purified, RNase free), was the product of Worthington Biochemical Corp. Ethidium bromide was a gift from the Boots Pure Drug Co., Ltd., and actinomycin D was a gift of Merck and Co., Inc. *Escherichia coli* rRNAs were a gift from Dr. A. E. Dahlberg, and rat liver cytoplasmic RNAs were isolated as described previously (Dingman *et al.*, 1970). Rat liver DNA was isolated from rat liver nuclei using preparative isopycnic centrifugation in CsCl (Dingman and Sporn, 1967). MS2 and ϕX174 bacteriophages were purchased from Miles Laboratories, Inc. λ (CI−857), λ (b2b5c), and T7 (wild type) bacteriophages were a gift from Dr. Martin Gellert. T4 (wild type) bacteriophage was a gift from Dr. Walter Keller. SV40 virus and Adenovirus, type 2, were gifts from Dr. James Rose, and Reovirus was a gift from Dr. Aaron Shatkin.

Methods

Isolation of Nucleic Acids. Reovirus RNA was isolated as described previously (Fisher and Dingman, 1971); all bacteriophage nucleic acids were isolated by treating the intact virus (5×10^{10} to 5×10^{11} particles per ml, usually in 0.05 M NaCl–2 mM Na_3EDTA, pH 7.0) with 0.2% sodium dodecyl sulfate for 5 min at room temperature (with gentle mixing) followed by a 15- to 20-min treatment at room temperature with 1 volume of water-saturated phenol (with gentle mixing). Following centrifugation at 4°, the aqueous phase was either diluted 4:5 with sucrose and Bromophenol Blue (Peacock and Dingman, 1967) and then used directly for gel electrophoresis (Dingman *et al.*, 1970) or dialyzed for 24 hr *vs.* 0.05 M NaCl–2 mM Na_3EDTA (pH 7.0) and treated as described above. The mobilities of the various polynucleotides were not affected by the presence of phenol in the aqueous phase. Adenovirus and SV40 virus DNAs were isolated by incubating the virus for 30 min at 50° in 1% sodium dodecyl sulfate (Trilling and Axelrod, 1970), following which the solutions were treated with phenol as described above for the bacteriophages. Another sample of SV40 virus was incubated for 2 hr at 37° in the presence of 0.3% sodium dodecyl sulfate and 1 mg/ml of Pronase (Calbiochem, B grade) followed by treatment with

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phenol as described above for the bacteriophages (J. Rose, personal communication). This latter preparation of SV40 DNA was more degraded than that isolated without the use of Pronase (see Plate I and Results).

The T4 DNA used in these studies had a sedimentation coefficient ($s_{20,w}$) of 59.6 S at a concentration of less than 4 μg/ml. The DNAs designated SV40 I, SV40 II, and SV40 III had sedimentation coefficients ($s_{20,w}$) of 22.2, 15.9, and 14.5 S, respectively. This last value corresponds to a molecular weight of about 2.8×10^6 (Eigner and Doty, 1965).

Table I lists the polynucleotides used in this study; their abbreviations and their assumed molecular weights are also given in this table.

Gel Electrophoresis and the Measurement of Mobilities. Composite Agarose–acrylamide gels of different monomer concentrations were prepared and used as previously described (Peacock and Dingman, 1968; Fisher and Dingman, 1971). All electrophoretic runs were performed at 21° in Tris–EDTA–borate buffer (pH 8.3). Following electrophoresis the gels were stained with Stains-all as described by Dahlberg *et al.* (1969) and scanned at 570 nm using a Gilford recording spectrophotometer, Model 240, with a Gelscan attachment. Electrophoretic mobility (M) defined as $M = (d)\cdot(k)/(i)\cdot(t)$, where d is the distance migrated in centimeters, k is the specific conductance of the buffer at the temperature of the gel, i is the current density in A/cm^2, and t is the time in seconds, was measured as previously described (Fisher and Dingman 1971).

Zone Sedimentation in Sucrose Gradients. Zone sedimentation in linear 4–20% (w/v) sucrose gradients containing 0.03 M NaCl and 5 mM Na_3EDTA (pH 7.0) was performed in an SW 25.1 Spinco rotor in the Spinco Model L2-65B ultracentrifuge. Sedimentation coefficients ($s_{20,w}$) were determined as described by Sporn and Dingman (1963).

Treatment of Molecules with DNase, Dyes, and Heat. Treatment with DNase I, sufficient to nick a significant proportion of intact SV40 DNA molecules (see Plate I) was carried out in the presence of 0.0025 μg/ml of DNase I and 5 mM Tris, 2.5 mM $MgCl_2$ (pH8.0), and 20 μg/ml of bovine serum albumin (Calbiochem, A grade) (Vinograd *et al.*, 1965). Incubation was for 5 min at 22° and the reaction was stopped by the addition of 0.25 volume of 1% sodium dodecyl sulfate followed 1 min later by 0.1 volume of water-saturated phenol.

Polynucleotides were treated with either 0.1 volume of actinomycin D or 0.1 volume of ethidium bromide. Stock solutions of these dyes contained 100 μg of dye/ml of H_2O. If polynucleotides were to be treated with ethidium bromide, then the 4:5 dilution with sucrose and Bromophenol Blue was omitted; instead, solid sucrose was added to raise the density of the sample. These dyes were not included in either the gels or the buffer.

Heat denaturation of Reovirus RNA was performed as described previously (Fisher and Dingman, 1971). Otherwise, heat denaturation involved heating to 100° for 5 min in 0.05 M NaCl–2 mM Na_3EDTA (pH 7.0) followed by quenching in ice.

Electron Microscopy. Solutions containing DNA were diluted with 2 × SSC (0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.4) to give 2–10 μg/ml of final concentration. An aliquot of this solution was added to a tenth of a volume of DEAE-Dextran solution (0.5% DEAE-Dextran–0.5 M Tris-HCl, pH 7.4) (T. Kakefuda, M. Hatanaka, and E. A. O. Callan, in preparation). An amount, 25 μl, of one of these solutions containing the DNA sample to be examined was dropped onto a water surface in a Teflon-coated aluminum tray. In some cases the spreading area was restricted to an area as small as 5 × 5 cm to concentrate the molecules. The film was picked up on a grid covered by a carbon-coated collodion membrane. The specimens were washed first with 50% ethanol, then 90% ethanol, and air-dried. DEAE-Dextran, which is of low molecular weight and free from nuclease contamination, provided a fine-grained background and maintained the integrity of the nucleic acid molecules. The specimens were rotary

TABLE I: Abbreviations and Molecular Weights for the Polynucleotides Referred to in This Report.

Polynucleotide	Abbreviations[m]	Assumed Mol Wt ($\times 10^{-6}$)	Ref
E. coli 5S RNA	Ec5S	0.036	*a*
E. coli 16S RNA	Ec16S	0.56	*b*
E. coli 23S RNA	Ec23S	1.1	*b*
MS2 phage RNA	MS2	1.05	*c*
Rat liver 18S RNA	RL18S	0.69	*d*
Rat liver 28S RNA	RL28S	1.5	*d*
ϕX174 phage DNA	ϕX174	1.7	*e*
Rat liver DNA	RLDNA		
λ (CI-857) DNA	λ	33	*f*
λ (b2b5c) DNA	λ^m	25	*f*
Adenovirus Type 2 DNA	Ad2	25	*g*
T4 phage DNA	T4	125	*h*
T7 phage DNA	T7	25	*h*
SV40 DNA, intact double-stranded circles	SV40 I	2.8	*i*
SV40 DNA, nicked double-stranded circles	SV40 II	2.8	*i*
SV40 DNA, double-stranded linear	SV40 III	2.8	*i*
SV40 DNA, single-stranded linear	SV40 IV	1.4	*j*
SV40 DNA, single-stranded circles	SV40 V	1.4	*j*
Reovirus RNA, double-stranded light	RVDSLb	0.8	*k*
Reovirus RNA, double-stranded medium	RVDSMa	1.3	*k*
Reovirus RNA, double-stranded heavy	RVDSHa	2.3	*k*
Reovirus RNA, single-stranded light	RVSSL	0.4	*l*
Reovirus RNA, single-stranded medium	RVSSM	0.65	*l*
Reovirus RNA, single-stranded heavy	RVSSH	1.15	*l*

[a] Rosset *et al.* (1964). [b] Kurland (1960). [c] Strauss and Sinsheimer (1963). [d] Fisher and Dingman (1971). [e] Sinsheimer (1959). [f] MacHattie and Thomas (1964). [g] Green *et al.* (1967). [h] Eigner and Doty (1965). [i] Determined from the $s_{20,w}$ of SV40 III using the relationship $s_{20,w} = 0.116M^{0.325}$ (Eigner and Doty, 1965). [j] These values were assumed to be one-half that of the double-stranded SV40 DNA. [k] Bellamy *et al.* (1967). [l] These values were assumed to be one-half those of their corresponding double-stranded form of Reovirus RNA. [m] Small letters at the end of an abbreviation are used to designate subspecies in the order of increasing mobility.

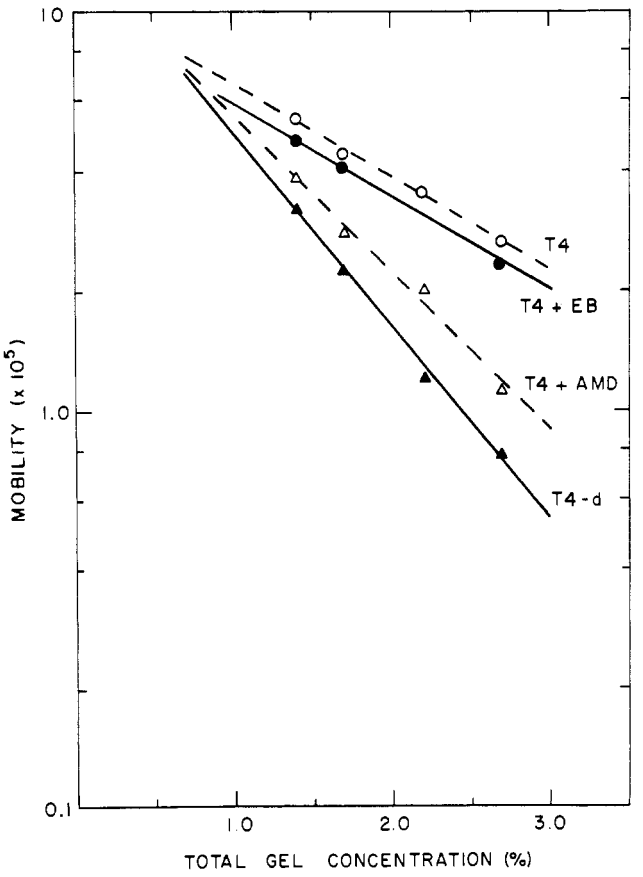

FIGURE 1: Effect of gel concentration on the electrophoretic mobility of T4 DNA (○), after treatment with ethidium bromide (EB) (●), actinomycin D (AMD) (Δ), and heat (d) (▲). These gels were run at 21° and 8.8 V/cm. Mobility = $cm^2 \cdot V^{-1} \cdot sec^{-1}$; the lines were calculated to be the best linear fit by least-squares regression analysis.

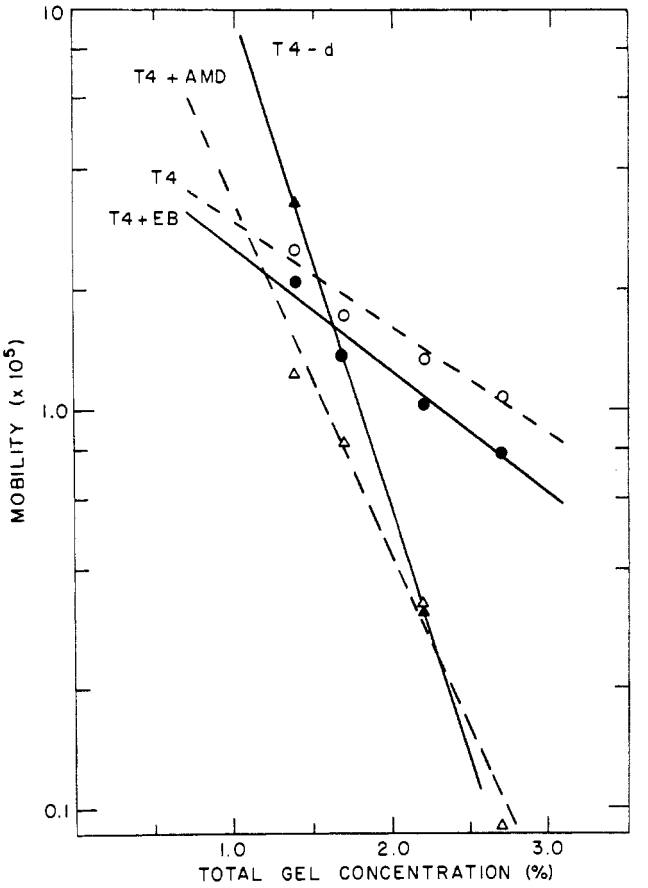

FIGURE 2: Same as Figure 1 except that these gels were run at 2.2 V/cm.

shadowed with platinum/carbon at an angle of 8° and examined with a Phillips EM200 electron microscope at a direct magnification of 9000–15,000. The magnification was calibrated with a grating replica.

Results

Effect of Voltage Gradient and Gel Concentration. In a previous report (Fisher and Dingman, 1971) we presented data showing that high molecular weight, double-stranded polynucleotides exhibited an increase in mobility when the voltage gradient was increased in Agarose–acrylamide gel electrophoresis. Single-stranded polyribonucleotides did not exhibit this effect. In this report we have presented further data relevant to this phenomenon. We first determined the mobility at two different voltage gradients of a number of polynucleotides of different structure in gels of different monomer concentration as illustrated in Figures 1, 2, and 3. From such plots a retardation coefficient, K_R, may be calculated as log M = log $M_0 - K_R T$, where M is mobility at gel concentration T, M_0 is the apparent mobility at zero gel concentration, and T is the concentration of acrylamide plus *N,N'*-methylenebisacrylamide (Ferguson, 1964; Rodbard and Chrambach, 1970; Fisher and Dingman, 1971). (T refers to the total concentration of acrylamide only and does not include the agarose, thus, in these studies, M_0 will not be a measure of the true free mobility.) Table II gives the retardation coefficients calculated for a variety of polynucleotides at two different voltage gradients. The polynucleotides listed in Table II have been subdivided into six groups according to structure, and the data presented there allow one to suggest the following generalizations. (1) Single-stranded RNAs (groups A and A') show little change in K_R with an increase in voltage gradient (and have little change in mobility as well, as shown by Fisher and Dingman, 1971), and have K_R's proportional to molecular weight (see Figure 4 in Fisher and Dingman, 1971). (2) Linear double-stranded RNAs and linear double-stranded DNAs (group B) also show little change in K_R with an increase in voltage gradient (in spite of an increase in mobility, compare T4 DNA in Figures 1 and 2), but their K_R's show little dependence upon molecular weight. (3) Linear single-stranded DNAs (group C), especially those derived by heat denaturation of double-stranded molecules, show a large reduction in K_R with an increase in voltage gradient (they also exhibit an increase in mobility with an increase in voltage gradient, except in very dilute gels, like group B molecules and unlike groups A and A' molecules, compare T4-d in Figures 1 and 2). (4) Circular single-stranded DNAs (group D) have unusually high K_R's (for their molecular weight) and these K_R's show a modest decrease with an increase in voltage gradient. (5) Finally, circular double-stranded DNAs (group E), whether "nicked" or not, show a marked reduction in K_R and an increase in mobility with an increase in voltage gradient. It is obvious that not all possible combinations of molecular weight, composition, and structure have been examined here. We feel, therefore, that the above generalizations should be viewed as tentative. In particular, circular molecules (whether single or double stranded) would be expected to

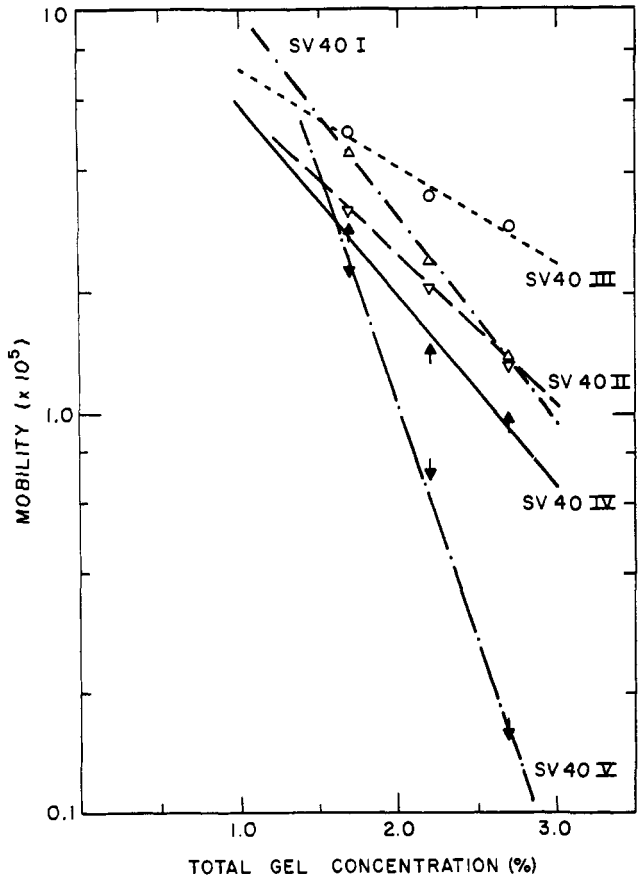

FIGURE 3: Same as Figure 1 except the effect of gel concentration on the electrophoretic mobility of various configurations of SV40 DNA was examined. These gels were run at 21° and 8.8 V/cm. See Table I for a description of the conformation represented by each of the Roman numerals.

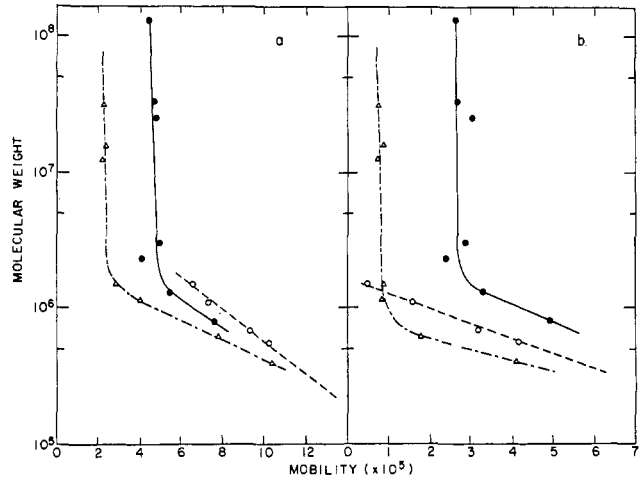

FIGURE 4: The relationship between the molecular weight (Table I) and mobility in Agarose–acrylamide gels for three groups (see Table II) of polynucleotides. Normally linear single-stranded RNAs, group A (----), normally linear double-stranded polynucleotides, either RNA or DNA, group B (——), and linear single-stranded polynucleotides (either RNA or DNA) produced by heat denaturation of normally double-stranded molecules, groups A′ and C (-·-·-). Circular molecules have not been included. (a) Data from 1.7% gels run at 21° and 8.8 V/cm; (b) data from 2.7% gels run at 21° and 8.8 V/cm. Mobility = $cm^2 \cdot V^{-1} \cdot sec^{-1}$.

become less distinguishable from their corresponding linear forms as their molecular weight increased. For example, the circular and linear forms of λ DNA, when electrophoresed as a mixture, could be separated; however, the resolution of these two forms was insufficient to use this technique as a method for determining whether the structure of a molecule of this size (33×10^6) was linear or circular.

Heat treatment of double-stranded RNAs caused a marked change in their mobilities and an increase in their K_R's at both 2.2 and 8.8 V per cm, similar to the effect of heat treatment on double-stranded DNAs. However, heat treatment of these double-stranded RNAs did not result in a molecule which exhibited a marked decrease in K_R with an increased voltage gradient (Table II, group A′) unlike the effect of heat treatment on double-stranded DNAs (Table II group C). Heat treatment of single-stranded RNAs (rRNAs from *E. coli*) did not affect their mobilities significantly although it did cause some band broadening.

The behavior of three of these groups of polynucleotides (A, B, and A′ + C) with respect to the molecular weight dependence of their electrophoretic mobilities in 1.7 and 2.7% gels are illustrated in Figures 4a,b. The data presented in Figure 4 indicate that the molecular size above which Agarose–acrylamide gel electrophoresis fails to achieve any significant separation of polynucleotides of different molecular weight is a function of both gel concentration and molecular conformation (see also Figure 2 in Peacock and Dingman, 1968). These data also explain why a DNA preparation of very heterogeneous size (*e.g.*, RL DNA) migrates as a single, fairly discrete band during electrophoresis in Agarose–acrylamide gels (Dingman *et al.*, 1970). Such bands, however, are not as sharp as those obtained with DNA preparations of homogeneous size (*e.g.*, T4 DNA).

Electrophoretic Properties of SV40 DNA. The resolving power of Agarose–acrylamide gel electrophoresis was strikingly illustrated in the case of SV40 DNA. Log mobility versus gel concentration plots for the various forms of SV40 DNA are shown in Figure 3, and the computed retardation coefficients at both 2.2 and 8.8 V per cm are given in Table II. The mobility of form III, the linear duplex form, in gels of different monomer concentration is very similar to that of other linear double-stranded polynucleotides. In contrast, the circular double-stranded forms of SV40 DNA (I and II) have slower mobilities than form III, particularly at the higher gel concentrations. Furthermore, as the gel concentration is decreased, the mobility of the intact twisted circular form I increases relative to that of the nicked, open-circular form II (Figure 3). The best resolution of the different configurations of SV40 DNA was achieved in a 1.7% gel. Spectrophotometric scans of electrophoregrams from a 1.7% gel are shown in Figure 5. Figure 5e illustrates the degree of separation obtained between the linear and circular single-stranded halves of SV40 DNA II (herein called components IV and V, respectively).

A feature peculiar to intact circular double-stranded polynucleotides is their ability to reanneal spontaneously at low temperatures in suitable solvents following disruption of the double-stranded helix by heat or alkali (Josse and Eigner, 1966). This phenomenon was readily observed in the case of component I of SV40 DNA. When these molecules were heat denatured (100° for 5 min) then "quenched" in ice, they, unlike other double-stranded polynucleotides, returned to their native configuration (compare Figure 5b,f). The retardation coefficients at 2.2 and 8.8 V per cm remained essentially unchanged (Table II), and they continued to stain blue

TABLE II: Effect of Voltage Gradient on the Retardation Coefficient (K_R) of Some Polynucleotides.

Group	Polynucleotide		Conformation[a]	No. of Strands	K_R[b] 2.2 V/cm	K_R[b] 8.8 V/cm
A	Ec5S	RNA	L	1	0.06 ± 0.02	0.07 ± 0.02
	Ec16S	RNA	L	1	0.38 ± 0.02	0.41 ± 0.02
	Ec23S	RNA	L	1	0.51 ± 0.07	0.63 ± 0.03
	MS2	RNA	L	1	0.71 ± 0.11	0.77 ± 0.08
	RL18S	RNA	L	1	0.46 ± 0.02	0.51 ± 0.04
	RL28S	RNA	L	1	0.99 ± 0.07	1.01 ± 0.13
A′	RVSSL	RNA	L	1	0.38 ± 0.04	0.35 ± 0.09
	RVSSM	RNA	L	1	0.63 ± 0.08	0.56 ± 0.12
	RVSSH	RNA	L	1	0.89 ± 0.07	0.79 ± 0.03
B	RVDSLb	RNA	L	2	0.24 ± 0.03	0.22 ± 0.03
	RVDSMa	RNA	L	2	0.25 ± 0.03	0.22 ± 0.03
	RVDSHa	RNA	L	2	0.24 ± 0.03	0.21 ± 0.05
	RL	DNA	L	2	0.33 ± 0.05	0.24 ± 0.03
	SV40 III	DNA	L	2	0.24	0.24 ± 0.08
	λ	DNA	L	2	0.20 ± 0.03	0.26 ± 0.03
	T7	DNA	L	2	0.37	0.22 ± 0.05
	T4	DNA	L	2	0.26 ± 0.09	0.23 ± 0.02
	Ad2	DNA	L	2	0.43	0.40 ± 0.08
C	λ^m-d[c]	DNA	L	1	1.56	0.67 ± 0.11
	T7-d[c]	DNA	L	1	0.94 ± 0.39	0.56 ± 0.08
	T4-d[c]	DNA	L	1	1.18 ± 0.42	0.49 ± 0.04
	SV40 IV	DNA	L	1	0.80 ± 0.24	0.48 ± 0.15
	φX174	DNA	L	1	1.06 ± 0.14	0.89 ± 0.15
D	SV40 V	DNA	C	1	1.62 ± 0.36	1.16 ± 0.17
	φX174	DNA	C	1	1.86 ± 0.25	1.39 ± 0.19
E	SV40 I	DNA	C	2	1.13 ± 0.11	0.51 ± 0.05
	SV40 I-d[c]	DNA	C	2	1.17 ± 0.12	0.53 ± 0.09
	SV40 II	DNA	C	2	1.21 ± 0.09	0.37 ± 0.01

[a] Conformation: linear (L), circular (C). [b] K_R values ± two standard deviations were determined by least-squares regression analysis. [c] These molecules have been heat treated (see Methods).

with Stains-all like other double-stranded polynucleotides. A small percentage of these molecules apparently sustain a single nick during the heat treatment and these then separate into two bands with mobilities characteristic of components IV and V of SV40 DNA (Figure 5f) which now stain a purple-blue similar to other single-stranded polynucleotides. The analogous components of Polyoma DNA have been electrophoretically separated in agar gels by Thorne (1967), although the order of migration differed and the resolution achieved was not as good as that found here with Agarose–acrylamide gels.

Effect of Actinomycin D and Ethidium Bromide. Because actinomycin D and ethidium bromide have been shown to exhibit a high degree of specificity in their binding to polynucleotides (Haselkorn, 1964; Waring, 1966), we were interested to examine the effects of these dyes on the mobilities of various polynucleotides. Our experimental results are given in Table III; Figures 1 and 2 also illustrate the effect of these dyes on T4 DNA.

It is important to note that it is possible for some treatment to affect markedly the mobility of a molecule without significantly affecting the computed retardation coefficient, K_R, but it is not possible for the K_R to be altered without a corresponding change in mobility. For example, the mobilities of linear double-stranded polynucleotides are markedly affected by changing the voltage gradient (Table III and Fisher and Dingman, 1971), whereas the K_R's are not affected (compare plots of T4 DNA in Figures 1 and 2). It was found that actinomycin D had a greater effect on the mobilities of linear double-stranded DNAs at 2.2 V/cm than at 8.8 V/cm (Table III), whereas the opposite appeared to be true for the circular double-stranded DNAs (SV40 I and II). Treatment of DNAs with either actinomycin D or ethidium bromide at the concentrations used here resulted not only in changes in mobility but also in broadening of the migrating zone. This was particularly true for DNAs treated with ethidium bromide and for SV40, components I and II, treated with either ethidium bromide or actinomycin D.

Ethidium bromide affected the mobility (at 8.8 V/cm) of SV40 I more than any of the other polynucleotides examined (Table III). This might have been expected considering the ability of this dye to bring about unwinding of the superhelical form of Polyoma virus DNA (Crawford and Waring, 1967). We obtained electron microscopic evidence that this is what happened in the case of SV40 I (Plate ID). Aside from this effect, ethidium bromide did not affect the mobilities of other

TABLE III: Effect of Actinomycin D and Ethidium Bromide on the Mobilities of Some Polynucleotides.

Polynucleotide	Gel Concn	Mobility[a] at 2.2 V/cm			Mobility[a] at 8.8 V/cm		
		No Treatment	+ Actinomycin D	+ Ethidium Bromide	No Treatment	+ Actinomycin D	+ Ethidium Bromide
λ	2.7	1.2		1.0	2.7	0.7	2.8
T4	1.4	2.5	1.3	2.1	5.4	3.9	4.8
T4	1.7	1.8	0.8	1.4	4.4	2.8	4.1
T4	2.2	1.4	0.3	1.1	3.5	2.1	
T4	2.7	1.1	0.1	0.8	2.7	1.1	2.4
SV40 I	1.7	3.3			4.5	2.1	2.6
SV40 I	2.2	0.9	0.5	0.9	2.4	0.9	1.9
SV40 I	2.7		0.1	0.3	1.4	0.2	0.8
SV40 II	1.7	2.0	1.4	1.6	3.2	2.7	
SV40 II	2.2	0.6	0.5	0.6	2.3	1.1	2.1
SV40 II	2.7		0.1	0.3	1.4	0.3	1.2

[a] These values have been multiplied by 10^5. Replicate measurements of mobilities (done in different gels of the same composition and run at the same voltage and temperature) gave values that differed usually by less than 5%.

DNAs to any large extent. It had even less effect on *E. coli* RNAs.

Actinomycin D proved to be very selective in its effect: it did not alter the mobility of heat-denatured DNAs, single-stranded RNAs, or double-stranded RNAs (from Reovirus); only double-stranded DNAs were affected in its presence. Thus, only those polynucleotides known to bind actinomycin D (Haselkorn, 1964) had their electrophoretic mobilities affected by actinomycin D. We are not certain why actinomycin D caused this effect. However, electron microscopic examination of T4 DNA in the presence of actinomycin D (Plate II) showed evidence of side-to-side aggregation of these molecules. This phenomenon might well have accounted for the observed effect. Aflatoxin B_1, which also binds to DNA (Sporn *et al.*, 1966), did not induce any change in the mobility of T4 DNA or human DNA (C. W. Dingman and M. P. Fisher, unpublished observations).

Discussion

Relationship of Retardation Coefficient to Molecular Radius and Molecular Size. Rodbard and Chrambach (1970, 1971) have discussed the possible relationship between the retardation coefficient (K_R), a measure of molecular surface area, and the geometric mean radius ($\bar{R}$) of spherical molecules migrating in polyacrylamide gels under the influence of an electric field (where $\bar{R} = [(0.396)(\bar{v})(MW)]^{1/3}$. We have briefly explored the applicability of this relationship to polynucleotides by first assuming that this relationship had the form: $(K_R)^{1/2} = C_1(\bar{R} + r)$ (Rodbard and Chrambach, 1970). In this relationship C_1 is a constant related to the specific electrophoretic system used and r is related to the effective radius of the composite gel fiber. If we next assume that the *E. coli* RNA species designated 23, 16, and 5 S had partial specific volumes ($\bar{v}$) of 0.5 and the molecular weights given in Table I, then the geometric mean radii ($\bar{R}$) of the molecules (assuming sphericity) are computed to be 60.2, 48.0, and 19.2 Å, respectively. A plot of the square root of the measured K_R at 8.8 V/cm *vs.* $\bar{R}$ for these molecular species is shown in Figure 6. Linear double-stranded polynucleotides under these conditions have retardation coefficients (K_R's) in the neighborhood of 0.24 (Table II); thus they are behaving in these gels as if they had molecular radii of about 35 Å. This is consistent with the hypothesis that they migrate end-on, a suggestion we made in a previous report (Fisher and Dingman, 1971). This value of 35 Å is also surprisingly close to the 30 Å cross-sectional radius of DNA in solution determined from low-angle X-ray scattering measurements by Bram and Beeman (1971). Circular double-stranded molecules have retardation coefficients indicating a

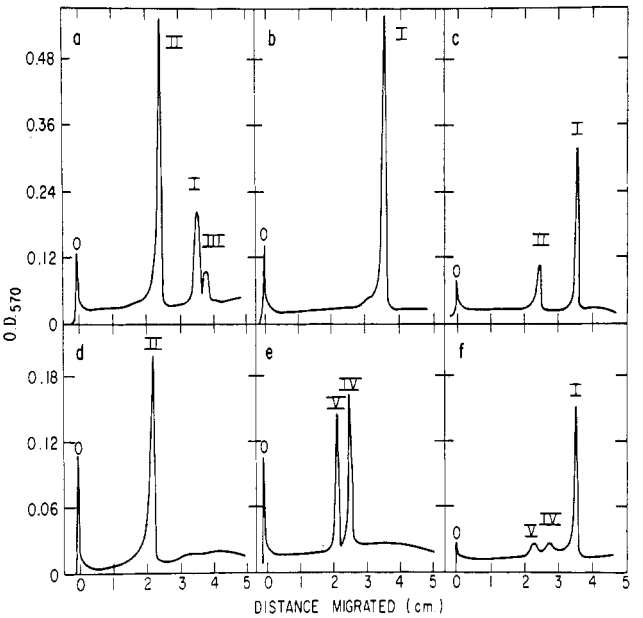

FIGURE 5: Scans (at 570 nm) of Agarose–acrylamide gel electrophoregrams of SV40 DNA run in 1.7% acrylamide–0.5% Agarose gels at 21° and 8.8 V/cm. (a) SV40 DNA isolated in the presence of Pronase (see Methods); (b) SV40 DNA after fractionation by zone sedimentation in a sucrose gradient (this material had a sedimentation coefficient of 22.2); (c) SV40 DNA isolated without pronase and treated briefly with DNase I (see Methods); (d) same as (b) except this material had a sedimentation coefficient of 15.9; (e) same as (d) following heat denaturation; and (f) same as (b) following heat denaturation. See Table I for a description of the conformation represented by each of the Roman numerals.

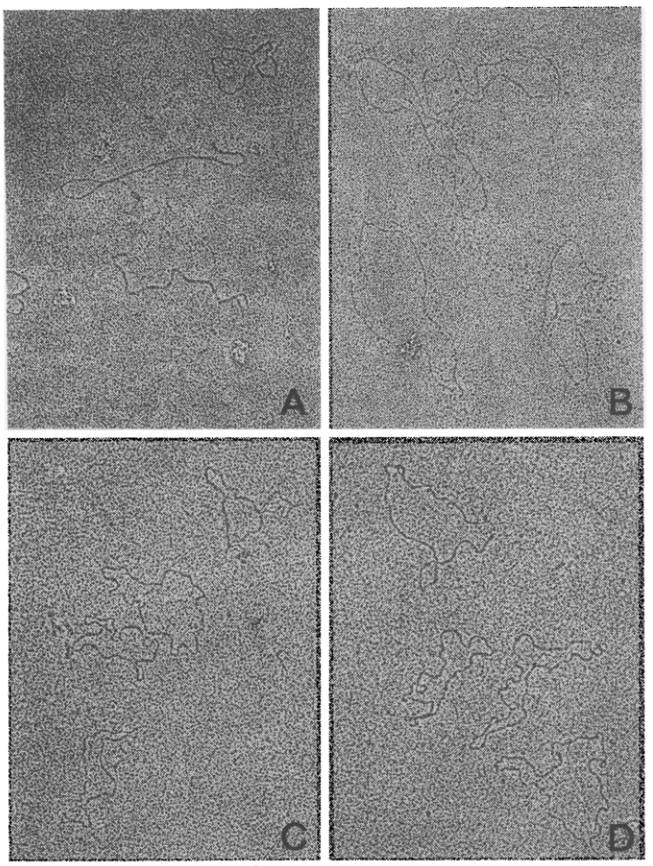

PLATE I: Electron micrographs of SV40 DNA. ×56,300. (A) SV40 DNA isolated without the use of Pronase, most of these molecules were very tightly supercoiled; (B) SV40 DNA isolated in the presence of Pronase, most of these molecules were open, relaxed circles; (C) SV40 DNA isolated without Pronase, as in (A), and treated briefly with DNase I (see Methods); and (D) SV40 DNA isolated without Pronase, as in (A) and treated with ethidium bromide. In (C) and (D) many of the molecules are open, relaxed circles.

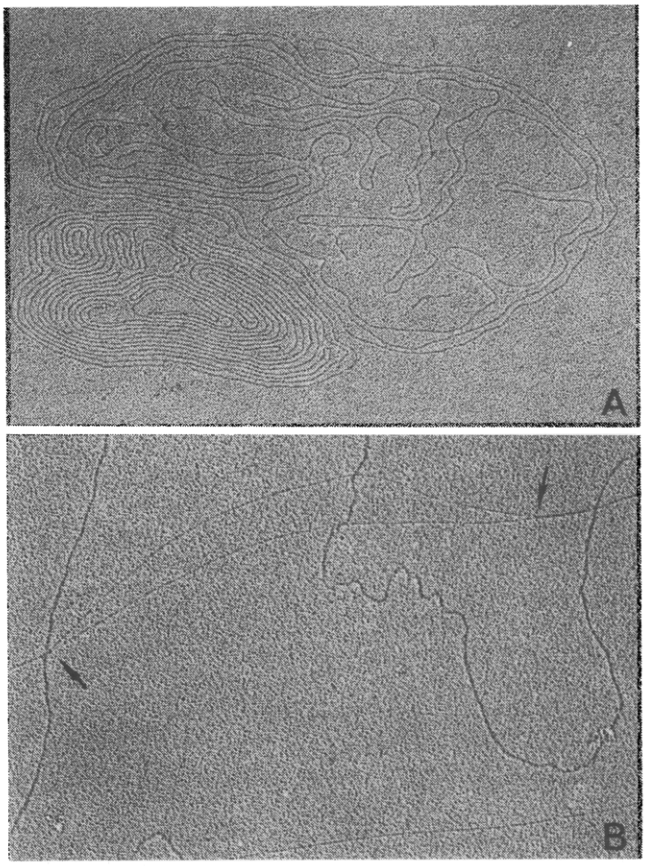

PLATE II: Electron micrographs of T4 DNA. ×56,300. (A) Untreated T4 DNA; (B) T4 DNA treated with actinomycin D, many of these molecules show partial or full side-to-side aggregation (see arrows) with another double-stranded molecule.

significantly larger apparent molecular radius (about 50 Å at 8.8 V/cm and about 80 Å at 2.2 V/cm). Much more thorough discussions of the relationships between molecular size, molecular radius, and retardation coefficient (particularly for globular proteins) have been presented elsewhere (Richards and Lecanidou, 1971; Rodbard and Chrambach, 1971; Chrambach and Rodbard, 1971).

Although two different molecules with closely similar mobilities in gels of different monomer concentration will have nearly the same retardation coefficient, molecules with similar retardation coefficients (and thus similar apparent molecular radii) are not necessarily unresolvable. For example, all the species of Reovirus RNA have similar K_R's, but they can be easily resolved be gel electrophoresis (Bellamy *et al.*, 1967; Fisher and Dingman, 1971), and this is reflected in their different apparent M_0 values. We would expect that asymmetric molecules such as these would exhibit a change in K_R in very dilute gels, in which case their computed M_0 values then would more nearly reflect their mobilities in free solution (a change in K_R at lower gel concentrations has been observed for high molecular weight proteins treated with sodium dodecyl sulfate, see Figure 2 in Neville, 1971).

The Relationship between the Molecular Size of Polynucleotides and the Resolution Obtained by Gel Electrophoresis. In our experience, the following generalizations (with respect to composite Agaroseacrylamide gel electrophoresis) seem to hold true. (1) Linear single-stranded polyribonucleotides of different molecular weight are resolvable when their molecular weights range from a few hundred to a few million (above this upper limit we have insufficient data to make predictions) (see also Bishop *et al.*, 1967, Peacock and Dingman, 1968, and Loening, 1969). (2) The linear single-stranded polynucleotides produced by denaturing double-stranded DNAs or RNAs or those linear DNAs which are single stranded in their native configuration (*e.g.*, nicked ϕX174 DNA) behave in a more complex manner. They appear to have mobilities inversely proportional to their molecular weights up to about 2×10^6. Molecules with molecular weights greater than this are not well resolved (see Figure 4). (3) Linear double-stranded polynucleotides of different molecular weight are resolvable when their molecular weights range from about 3×10^5 to 2×10^6, although they will all have nearly the same K_R. Below a molecular weight of 3×10^5 resolution of double-stranded molecules with different molecular weights would be expected; however, we have not yet examined any such molecules. Moreover, they might not be distinguishable from single-stranded species of the same molecular weight by their electrophoretic behavior (Fisher and Dingman, 1971). Above about 2×10^6, linear double-stranded polynucleotides of different molecular weight are not resolvable by this technique (Figure 4). Takahashi *et al.* (1969) have presented evidence that high molecular weight double-stranded DNAs of different size can be resolved by electrophoresis on simple Agarose gels. It would be interesting to explore this latter technique further to determine its advantages and limitations. (4) Finally, in the molecular

TABLE IV: Some Characteristics of Polynucleotides of Different Composition, Strandedness, and Configuration, as Analyzed by Electrophoresis in Agarose–Acrylamide Gels.

Polynucleotide	No. of Strands	Configuration	K_R[a]	Effect on Mobility[b] of Treatment with: Heat	Actinomycin D	RNase	DNase I	Color after Staining
RNA	1	Linear	Variable	−	−	+	−	Purple
RNA	2	Linear	0.2–0.4	+	−	+[c]	−	Blue
DNA	1	Linear	Near 1	−	−	−	+	Purple
DNA	1	Circular	1–2	−	−	−	+	Purple
DNA	2	Linear	0.2–0.4	+	+	−	+[c]	Blue
DNA	2	Circular (nicked)	Near 1	+	+	−	+[c]	Blue
DNA	2	Circular (intact)	Near 1	−[d]	+	−	+[c,e]	Blue

[a] At 21° and 2.2 V/cm; these values are those found for the molecules studied in these investigations and should not be taken at this time to be all inclusive. [b] No effect, −; significant effect, +. [c] These molecules would be expected to be relatively resistant (with respect to their electrophoretic properties) to treatment with very low concentrations of the appropriate endonuclease. [d] This lack of effect is meant to imply rapid renaturation at neutral pH at low temperatures. [e] Treatment of this molecular configuration with very low concentrations of DNase I would result in a molecule with the behavior exhibited by nicked circular DNA.

weight range of 1 to 3 × 10^6 at least, circular single-stranded polynucleotides may be resolved from linear single-stranded molecules of the same molecular weight, and circular double-stranded polynucleotides may be resolved from linear double-stranded molecules of the same molecular weight. Furthermore, in the same molecular weight range, nicked, open circular double-stranded molecules may be resolved from intact supercoiled circular double-stranded molecules.

Determination of Structure and Conformation of Polynucleotides by Gel Electrophoresis. Within the limitations described above, we are now in a position to set up "diagnostic" tests in dealing with newly isolated polynucleotides of unknown structure. These tests should be applicable not only to purified material (such as that from isolated viruses) but also to species found or isolated in the presence of a variety of cellular nucleic acids.

It has been a consistent finding, using Stains-all (Dahlberg *et al.*, 1969), that double-stranded polynucleotides, whether RNA or DNA, stain blue, while single-stranded polynucleotides, whether RNA or DNA (and including denatured DNA) stain purplish to pink-blue. This is a useful test, however, only when one is dealing with sufficient material in the gel to give a reasonably dense band (above 0.1–0.2 μg). As little as 0.01 μg of a single species of polynucleotide may be visually observed after staining with Stains-all, but at that level the color is too faint to be useful in a diagnostic sense.

Our earlier studies (Fisher and Dingman, 1971) indicated that, provided their molecular weight exceeded 0.3 × 10^6 to 0.4 × 10^6, double-stranded polynucleotides were distinguishable from single-stranded polyribonucleotides, particularly by the fact that their electrophoretic mobilities in Agarose–acrylamide gels increased when the voltage gradient was increased. These results, plus the ones reported here taken together with the different susceptibility of polyribo- and polydeoxyribonucleotides to endonucleases, should enable one to determine, in many cases, the structural characteristics of a newly isolated polynucleotide by means of Agarose–acrylamide gel electrophoresis. Some of these distinguishing features of polynucleotides of different structure are listed in Table IV. Gel electrophoresis offers some distinct advantages for such determinations in that it requires relatively inexpensive equipment, very little material, and the necessary data may be obtained quite rapidly.

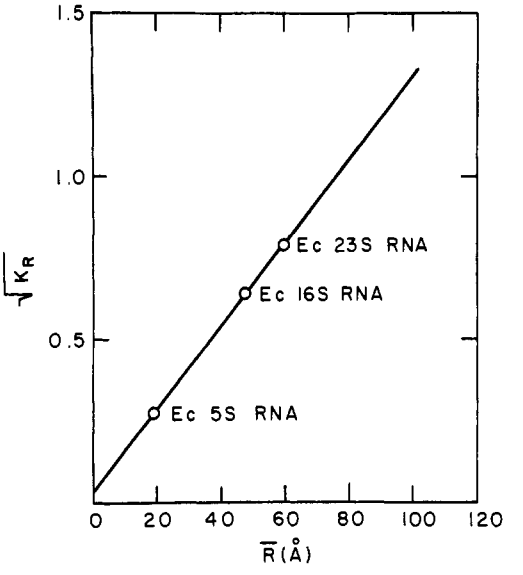

FIGURE 6: The relationship between $(K_R)^{1/2}$, the square root of the measured retardation coefficients at 8.8 V/cm, and $\bar{R}$, the computed geometric mean molecular radii for *E. coli* rRNAs.

Acknowledgments

We thank Dr. A. C. Peacock for his helpful discussions of this work and Mrs. Emily Callan for her fine technical assistance with the electron microscopy.

References

Bellamy, A. R., Shapiro, L., August, J. T., and Joklik, W. K. (1967), *J. Mol. Biol. 29*, 1.
Bishop, D. H. L., Claybrook, J. R., and Spiegelman, S. (1967), *J. Mol. Biol. 26*, 373.
Bram, S., and Beeman, W. W. (1971), *J. Mol. Biol. 55*, 311.
Chrambach, A., and Rodbard, D. (1971), *Science 172*, 440.
Crawford, L. V., and Waring, M. J. (1967), *J. Mol. Biol. 25*, 23.
Dahlberg, A. E., Dingman, C. W., and Peacock, A. C. (1969), *J. Mol. Biol. 41*, 139.
Dingman, C. W., Kakefuda, T., and Aronow, A. (1970), *Biochim. Biophys. Acta 224*, 114.
Dingman, C. W., and Peacock, A. C. (1968), *Biochemistry 7*, 659.
Dingman, C. W., and Sporn, M. B. (1967), *Cancer Res. 27*, 938.
Eigner, J., and Doty, P. (1965), *J. Mol. Biol. 12*, 549.
Ferguson, K. A. (1964), *Metabolism 13*, 985.
Fisher, M. P., and Dingman, C. W. (1971), *Biochemistry 10*, 1895.
Green, M., Piña, M., Kimes, R., Wensink, P. C., MacHattie, L. A., and Thomas, C. A., Jr. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1302.
Haselkorn, R. (1964), *Science 143*, 682.
Josse, J., and Eigner, J. (1966), *Annu. Rev. Biochem. 35*, 789.
Kurland, C. G. (1960), *J. Mol. Biol. 2*, 83.
Loening, U. E. (1969), *Biochem. J. 113*, 131.
Loening, U. E., and Ingle, J. (1967), *Nature (London) 215*, 363.
MacHattie, L. A., and Thomas, C. A., Jr. (1964), *Science 144*, 1142.
Neville, D. M., Jr. (1971), *J. Biol. Chem. 246*, 6328.
Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6*, 1818.
Peacock, A. C., and Dingman, C. W. (1968), *Biochemistry 7*, 668.
Richards, E. G., and Lecanidou, R. (1971), *Anal. Biochem. 40*, 43.
Rodbard, D., and Chrambach, A. (1970), *Proc. Nat. Acad. Sci. U. S. 65*, 970.
Rodbard, D., and Chrambach, A. (1971), *Anal. Biochem. 40*, 95.
Rosset, R., Monier, R., and Julien, J. (1964), *Bull. Soc. Chim. Biol. 46*, 87.
Sinsheimer, R. L. (1959), *J. Mol. Biol. 1*, 43.
Sporn, M. B., and Dingman, C. W. (1963), *Biochim. Biophys. Acta 68*, 389.
Sporn, M. B., Dingman, C. W., Phelps, H. L., and Wogan, G. N. (1966), *Science 151*, 1539.
Strauss, J. H., Jr., and Sinsheimer, R. L. (1963), *J. Mol. Biol. 7*, 43.
Takahashi, M., Ogino, T., and Baba, K. (1969), *Biochim. Biophys. Acta 174*, 183.
Thorne, H. V. (1967), *J. Mol. Biol. 24*, 203.
Trilling, D. M., and Axelrod, D. (1970), *Science 168*, 268.
Vinograd, J., Lebowitz, J., Radloff, R., Watson, R., and Laipis, P. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 1104.
Waring, M. J. (1966), *Biochim. Biophys. Acta 114*, 234.

Subunits of Ribonucleic Acid Polymerase in Function and Structure. I. Reversible Dissociations of *Escherichia coli* Ribonucleic Acid Polymerase†

Akira Ishihama

ABSTRACT: Two systems for reversibly dissociating DNA-dependent RNA polymerase from *Escherichia coli* have been developed by use of *p*-chloromercuribenzoate and urea. A subunit complex which retains the binding activities of ribonucleoside triphosphates and an antibiotic rifampicin was isolated by treatment of the enzyme with *p*-chloromercuribenzoate, and found to be composed of β and β' subunits. Thus, the site on the enzyme participating in the substrate-binding reaction appeared to reside in β and/or β' subunit. On the other hand, low concentrations of urea dissociated the enzyme into α–β and α–β' complexes. The observation that both fragments were capable of binding template DNA suggest that not only β' but also β subunit may possess the DNA-binding site. Although these are all reversible reactions, the retention of the activity to carry out part of the polymerase reactions was essential for reassociations to form active enzyme.

The complex structure of DNA-dependent RNA polymerase of *Escherichia coli* has received considerable attention. The studies of Burgess and coworkers (Burgess *et al.*, 1969; Burgess, 1969) have demonstrated that the enzyme is composed of at least four different polypeptide chains, α, β, β', and σ. There is also the possibility that another component, called ω, is a part of the holoenzyme structure. Based on the known molecular weight and the relative content of these components, it appears that the holoenzyme has the structure $\alpha_2\beta\beta'\sigma(\omega)$. The subunit σ can be dissociated from the enzyme and the resulting core enzyme with the structure $\alpha_2\beta\beta'(\omega)$ has been shown to possess all of the enzymatic activities associated with the polymerase. However, it has been shown that the core enzyme can hardly initiate RNA synthesis from intact

† From the Institute for Virus Research, Kyoto University, Kyoto, Japan. *Received November 5, 1971.* This work was initiated while the author was a postdoctoral research associate at the Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, N. Y., and was supported in part by grants from the National Institutes of Health, the National Science Foundation, the American Cancer Society to Dr. Jerard Hurwitz and the Ministry of Education of Japan.