PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Individual Macromolecules: Preparation and Recent Results with DNA

D. Lang

Phil. Trans. R. Soc. Lond. B 1971 **261**, 151-158
doi: 10.1098/rstb.1971.0046

Phil. Trans. Roy. Soc. Lond. B. **261**, 151–158 (1971) [151]
Printed in Great Britain

Individual macromolecules: preparation and recent results with DNA

By D. Lang
Division of Biology, The University of Texas at Dallas, Dallas, Texas 75230, *U.S.A.*

[Plates 30 and 31]

Protein–monolayer techniques are described which permit visualization of individual nucleic acid molecules by electron microscopy. The range of application is demonstrated by examples of quantitative observations concerning intrinsic and artificially introduced properties of DNA molecules, namely size and shape; binding of the drug ethidium bromide; diffusion coefficient; physical mapping of genetic deletions, adenine–thymine rich regions, single-strand breaks, and sites of messenger RNA synthesis.

Protein film methods

The central role of deoxyribonucleic acids (DNA) in biology has stimulated the application of a wide variety of methods for studying these macromolecules. Electron microscopy was not at first very useful for the simple fact that the drying of droplets containing DNA on specimen grids caused artefacts, lateral aggregation and preferential orientation as shown in figure 1, plate 30. It is often not realized that it was precisely these drying artefacts that were eliminated by Kleinschmidt & Zahn (1959) by using the idea of spreading a monomolecular cytochrome *c* surface film to which the DNA molecules would become attached. Subsequent transfer of portions of the film to grids by simply touching the surface did not cause any obvious major distortions of the macromolecules.

Figure 3 summarizes the appropriate procedures. They have in common the formation of a cytochrome *c* surface-film to which DNA adsorbs. Cytochrome *c* satisfies the following necessary conditions: (i) solubility in aqueous solutions, (ii) surface denaturation at the solution–air interface, (iii) insolubility of the denatured molecules in the film, and (iv) net positive electric charge at neutral pH (for adsorption of negatively charged nucleic acids). Ammonium acetate solutions are used because the crystals of this salt do not remain on grids after drying. Finally, excess solution adhering to grids after film transfer is removed by ethanol, and the DNA is usually contrasted by shadowing or staining.

In the well-known spreading technique of Kleinschmidt & Zahn (1959) (figure 3*a*) a DNA–cytochrome *c* mixture is slowly delivered down a wet glass slide. The protein film starts forming on the slide, thereby adsorbing DNA, and spreads out further over the solvent in the trough. Proper film formation usually requires that the salt concentration in the spreading solution is higher than that in the hypophase. This fact has been used to spread, instead of DNA, T2 bacteriophages which are sensitive to osmotic shock. They release DNA on contact with the low-salt hypophase. The released DNA is then adsorbed in the immediate vicinity of the empty bacteriophage (Kleinschmidt, Lang, Jacherts & Zahn 1962). This one-step release is the main advantage of the spreading technique.

Somewhat simpler is the diffusion method (figure 3*b*; Lang, Bujard, Wolff & Russell 1967), in which the DNA is dissolved in the hypophase. After temperature equilibration, dry cytochrome *c* is spread from a needle. DNA is transported by diffusion to the protein film where it adsorbs irreversibly (Lang & Coates 1968). The advantages are that the salt concentration during DNA adsorption is defined and that the system is free of convection.

A new, very simple and sensitive method is shown in figure 3*c* (Lang & Mitani 1970). Droplets (40 μl) of a solution as specified in figure 3*c* are deposited on a Teflon-coated, flat surface. A cytochrome *c* film develops spontaneously at the interface with air. After about 2 min, DNA adsorbs to this film and accumulates proportional to $(\text{time})^{0.75}$. The cytochrome *c* concentration is such that not more than about one monomolecular layer of protein can be formed at the droplet surface. Hence, times of 4 min to 5 h between droplet deposit and film transfer to grids can be chosen without loss of DNA contrast in micrographs. Delaying transfer permits higher accumulation of DNA. This is important when only very small amounts of DNA are available. The sensitivity limit for an average viral DNA is 2×10^{-5} μg or about one million molecules per droplet. Virus particles can also be prepared in the same way. Bacteria, however,

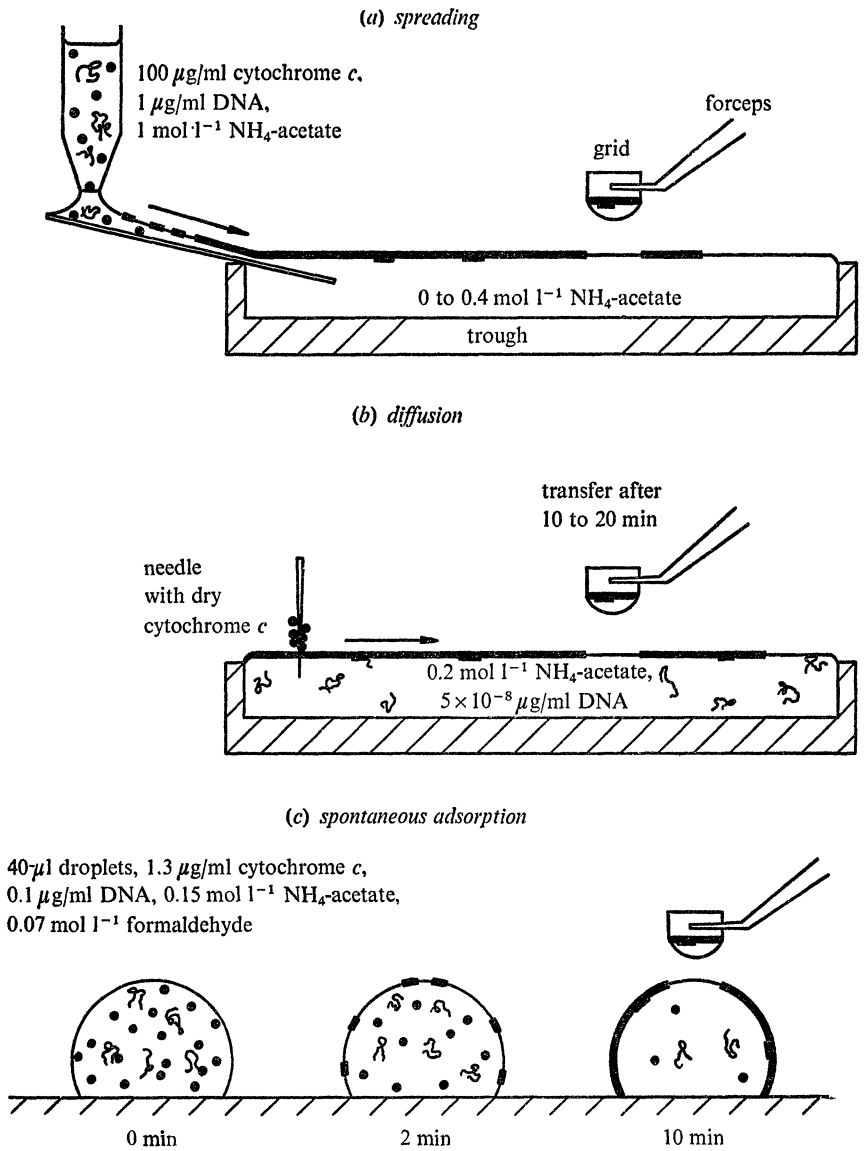


FIGURE 3. Proved and new protein monolayer techniques for transfer of nucleic acids to specimen grids without drying artefacts. The worm-like symbols represent DNA molecules, cytochrome *c* is indicated by dots (in solution) or by thick lines (surface-denatured). Arrows show the direction of spreading. The specimen grids (Siemens-type) are briefly touched face down to the surface film, then to ethanol, and then dried on filter-paper with subsequent shadowing or staining.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

sediment away from the upper droplet surface too rapidly; but the Teflon support may be turned upside down for 10 min as soon as the droplets are deposited and then turned back for about 2 h before transfer of the surface film to grids.

It should be noted that the above methods have a common feature that the nucleic acid molecules are always bound to cytochrome *c* before transfer to grids. Whether this fact introduces significant changes in the nucleic acid structure is not known. A clearer understanding would be desirable.

Applications

Methods such as those described are powerful tools in quantitative study of nucleic acids. They are unique in permitting the observation of individual molecules. Certain molecular changes existing in only a small part of the population cannot be detected by conventional averaging methods, but can be seen or measured on the single molecule. In general, intrinsic properties or deliberately introduced changes of nucleic acids, if represented by geometric or topologic parameters, can be investigated. Straightforward results can obviously be obtained on size and shape of macromolecules and on physical mapping of biologically important interactions of nucleic acids with other macromolecules. In addition, less obvious types of measurements can be made, for example, of diffusion coefficients of DNA or of binding constants for DNA complexes. In the following some results will be presented which demonstrate the range of application.

(*a*) *Topology*

It is easy to recognize whether nucleic acid molecules are double- or single-stranded (figure 2), branched or unbranched, linear or circular, with or without superstructure. For instance, the effect of DNA solvent is shown in figure 4, plate 30. A single DNA molecule collapses drastically into a compact asymmetric structure when water is replaced by ethanol (Lang 1969).

(*b*) *Molecular mass*

The molecular mass can be obtained from contour-length measurements if the molar linear density is known for nucleic acids as prepared on grids. This quantity is not necessarily equal to the one for the B configuration (1.913×10^{10} daltons/cm for duplex Na–DNA). Preparative steps may distort the DNA. Reducing the ionic strength of the hypophase below 0.1 (duplex DNA; Lang *et al.* 1967; Inman 1967) or 0.3 (simplex DNA; Bujard 1969*b*) increases contour lengths. This may also be observed if the molecules are under stress arising from mechanical instability of the protein film. Therefore, a calibration is desirable and was done using DNA from bacteriophages T4, T5, and T7 of known molecular masses as recently determined by other methods by Dr D. Freifelder of Brandeis University. A molar linear density of 2.07×10^{10} daltons/cm was found when prepared under standard conditions (diffusion method; 0.2 mol l^{-1} ammonium acetate). In addition, the molecular masses of T4 and T5 DNA seem to be smaller than generally assumed; a revision of experimental correlations between molecular mass of DNA and sedimentation coefficient as well as intrinsic viscosity would be indicated.

Molecular masses of intact viral DNA usually show a sample standard deviation between 1 and 4 % which is the range of experimental errors; any biological variation of molecular mass, if present at all, must be within that range.

Distributions of molecular masses, important for example in hydrodynamic or degradation studies, can be obtained with similar precision. This includes small DNA fragments often

escaping detection by methods such as sedimentation analysis which evaluate by weight rather than by number of molecules. Transforming DNA always has a wide size distribution which, if known, helps to interpret transformation experiments (Harm 1970).

(*c*) *Hydrodynamic parameters*

The only attempt to measure directly the distribution of end-to-end distances has been done using the diffusion method and a uniform, viral DNA sample (Lang *et al.* 1967). The two-dimensional distribution obtained, when transformed back into the three-dimensional state in

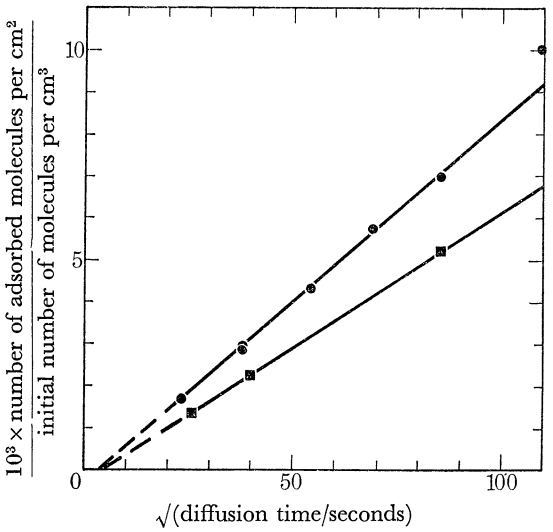
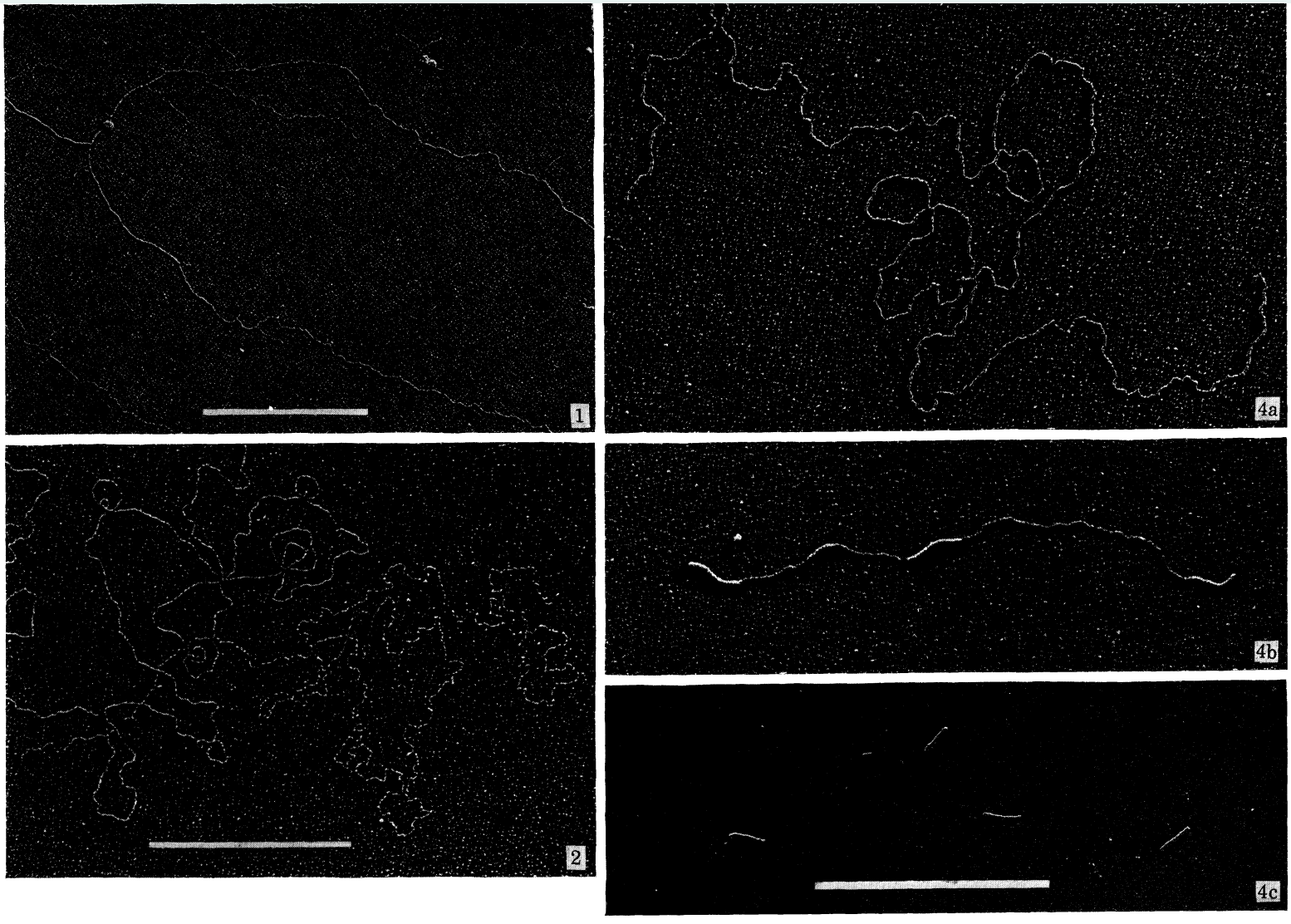


FIGURE 5. Kinetics of diffusion-controlled adsorption of T3 DNA (●) and of χ DNA (■). Diffusion coefficients are measured by the slopes. T3 and χ DNA are 12 and 20 μm long, respectively.

solution, is identical with a statistical chain configuration indicating that DNA is a random coil in solution. The Kuhn statistical element was found to be considerably longer than obtained by other methods (Hearst, Schmid & Rinehart 1968); the difference may be explained either by an effect of the cytochrome *c* film, or by the extremely low DNA concentration used (5×10^{-2} μg/ml) which permits a DNA coil to extend freely without significant restriction by other molecules.

High molecular mass DNA diffuses very slowly as compared to more compact macromolecules. Moreover, dependence upon concentration is high at those concentrations required for conventional methods. The diffusion method, however, is sufficiently sensitive to enable the diffusion coefficient of DNA to be measured at 'zero' concentration. The number of adsorbed DNA molecules as counted on micrographs per unit area is linear with respect to the square root of the diffusion time, that is between film formation and film transfer to grids (figure 5). Theoretical analysis shows that diffusion coefficients are given by the slope, and the average diameter of the randomly coiled DNA by the extrapolated intercept with the ordinate (Lang & Coates 1968). The diffusion coefficient was found to be independent of concentration between 3×10^{-3} and 2×10^{-1} μg/ml.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



(*Facing p.* 154)

FIGURE 1. DNA dried onto a specimen grid and shadowed with platinum. Lateral aggregation and preferential orientation in one direction are typical drying artefacts. The bar represents 1 μm. (Magn. $\times$ 26 000.)

FIGURE 2. Double- and single-stranded DNA prepared on the same grid by the diffusion method. Single-strand DNA has less contrast and is more kinked. (Magn. $\times$ 32 000.)

FIGURE 4. DNA appears as a loose coil when prepared from aqueous solution (*a*, diffusion method). In ethanol, a DNA molecule collapses upon itself into a compact, asymmetric rod structure (*c*) if the DNA concentration is sufficiently low to prevent formation of a macroscopic precipitate. The middle illustration (*b*) shows an intermediate state. The average volume of a rod is equal to the calculated volume of a T3 DNA molecule (3.8×10^{-17} cm^3). The DNA in the top and middle figures appear much thicker as they really are because they were prepared with cytochrome *c* and shadowed under 7°, whereas the rods were obtained on the grid without cytochrome *c* and shadowed under 20°. (Magn. $\times$ 37 000.)

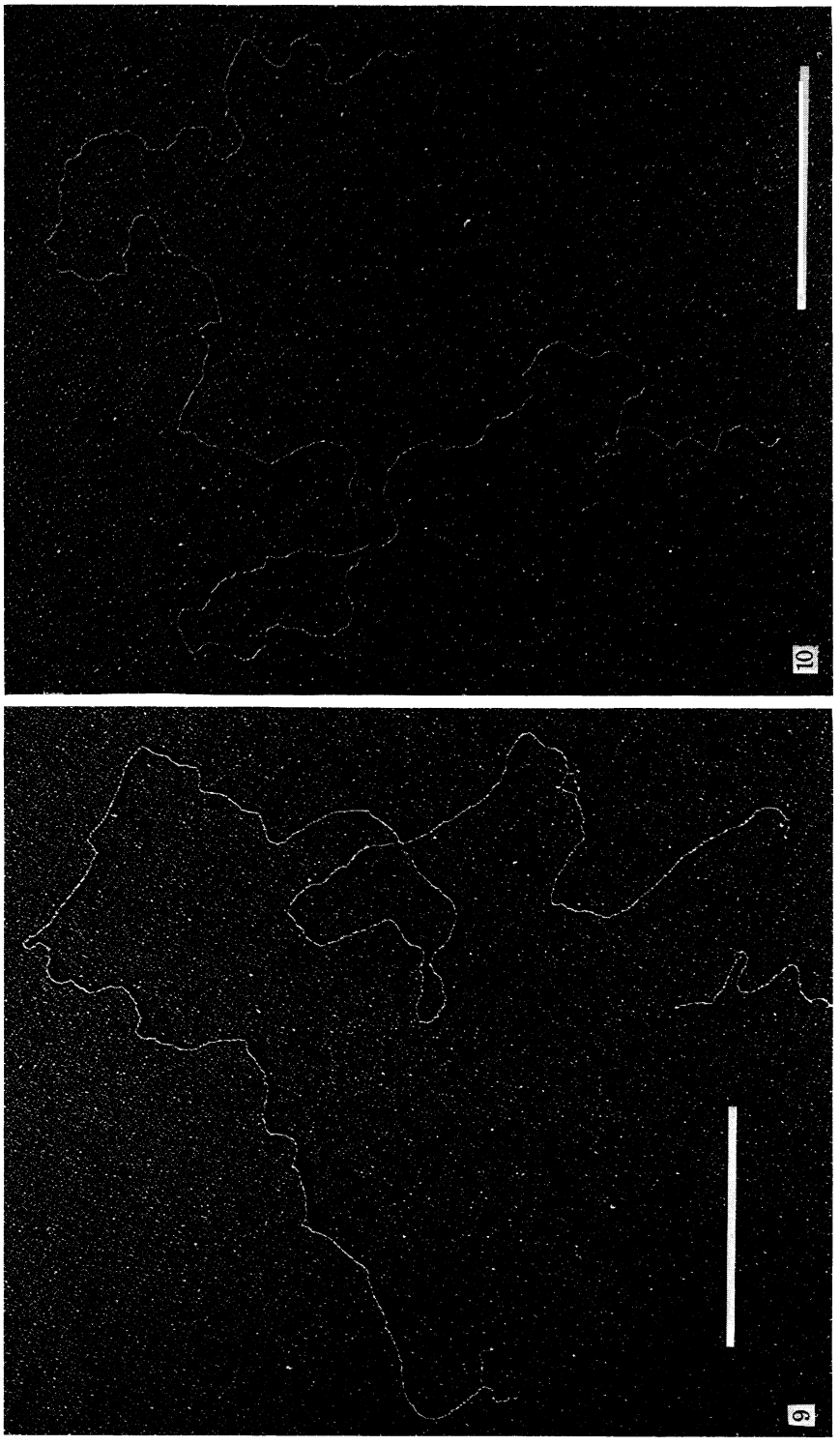


FIGURE 9. The T7 DNA molecule shown has been partially denatured by heating in presence of dimethylsulphoxide and formaldehyde. A collection of such molecules reveals a pattern of adenine-thymine rich regions which melt first. Prepared with the spontaneous-adsorption method. (Magn. × 33 000).

FIGURE 10. *In vitro* synthesis of messenger RNA along the T7 DNA template. The RNA appears as a bush attached to DNA. The DNA was incubated for 5 min with *E. coli* RNA polymerase in buffer containing nucleotides and 0.2 mol l^{-1} KCl at pH 7.5. (Magn. × 33 000.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(*d*) *Association constants*

DNA is believed to bind the drug ethidium bromide by intercalation between base pairs (Fuller & Waring 1964). Accordingly, an increase in contour length of duplex DNA with increasing drug concentration was expected and found (figure 6*a*). Under conditions as

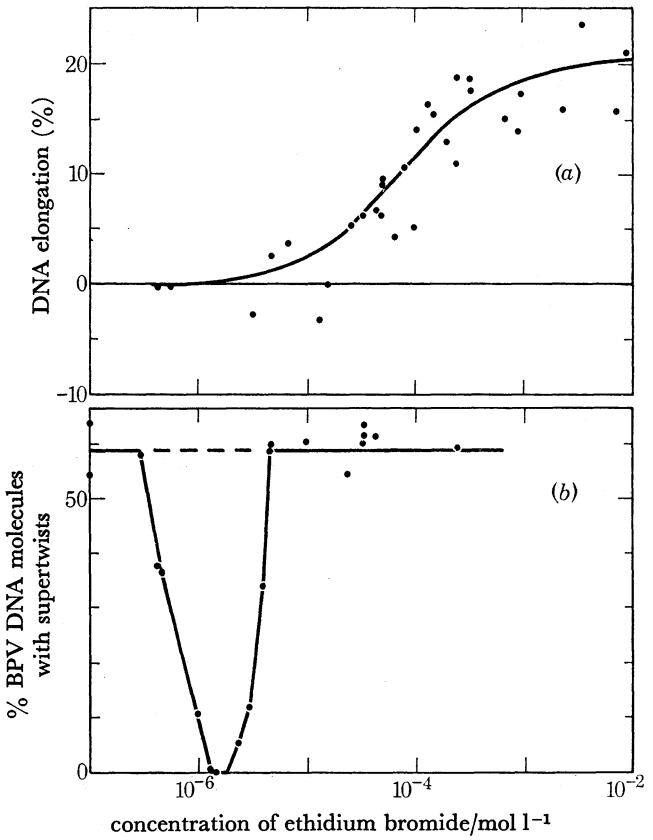


FIGURE 6. (*a*) Effect of the drug ethidium bromide upon contour length of T3 DNA. (*b*) Supertwists of covalently closed circular DNA from bovine papilloma virus (BPV) disappear at about 1.5×10^{-5} mol l^{-1} ethidium bromide and reappear at higher drug concentrations.

applied, the estimated drug concentration at which half of the possible elongation has been reached, is equal to the dissociation constant of the complex with respect to intercalation, about 10^{-4} l/mol, assuming a simple mass-action relationship between complex and constituents. In addition, covalently closed, circular duplex DNA responds by changing its number of superhelical turns (Crawford & Waring 1967). This is confirmed by electron microscopy as shown in figure 6*b*.

(*e*) *Physical mapping along DNA molecules*

(i) Gene mapping by genetic recombination studies can now be correlated with physical mapping on DNA molecules, as proposed by Nomura & Benzer (1961), by electron microscopy of wild-type DNA hybridized with the same DNA containing deletion mutations (figure 7). Davis & Davidson (1968) and Westmoreland, Szybalski & Ris (1969) have pin-pointed deletions in DNA from bacteriophage λ, and Bujard in our laboratory has made similar observations with T4 DNA. The length of a deletion can also be measured if the single-stranded portions are made to unfold.

(ii) After partial heat denaturation of T5 DNA in presence of dimethylsulphoxide and formaldehyde, single-strand portions of reproducible length become detached from specific regions along the DNA molecule as shown by Bujard (1969*a*), thereby identifying intrinsic single-strand interruptions known to be present (Abelson & Thomas 1966). Three interruptions have been mapped. They are on the same strand (figure 8). Region A (7.9 % of total length) is most likely the first-step-transfer portion of T5 DNA which comprises 8.3 % of the genome (Lanni 1968). The model in figure 8 is also consistent with earlier reports on sheared and denatured DNA of T5 (Burgi, Hershey & Ingraham 1966; Rubenstein 1968).

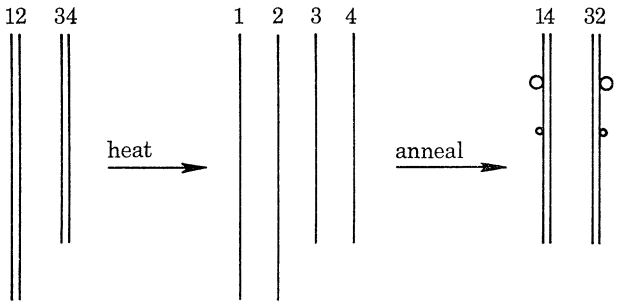


FIGURE 7. Scheme of an experiment to locate DNA deletions and to measure their length. 1, 2: wild-type DNA; 3, 4: double-deletion mutant DNA.

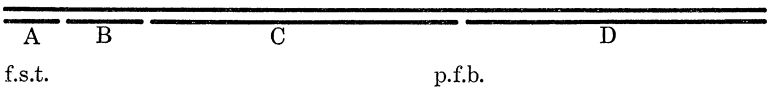


FIGURE 8. Model of T5 DNA proposed by Bujard (1969*a*) showing intrinsic single-strand interruptions, the first-step-transfer section (f.s.t.), an adenine-thymine rich region (B), and the point of first breakage (p.f.b.). The relative lengths of regions A through D are 7.9, 11.1, 41, and 40 %, respectively.

(iii) Patterns of nucleotide sequences rich in adenine and thymine emerge when a sample of duplex DNA is partially denatured by controlled heating as demonstrated for λ DNA by Inman (1966). Figure 9, plate 31, shows T7 DNA with three denatured regions where the two strands are separated. Such patterns may be used to identify the ends of the molecule, particularly if the nucleotide sequence of the collection of molecules is not circularly permuted.

(iv) A fundamental activity of DNA in living cells is the synthesis of other macromolecules (RNA) along segments of the DNA structure, thereby transcribing information into RNA. The micrographs of Miller & Beatty (1969) obtained by dispersing nucleoli of amphibian oocytes, beautifully illustrate this part of molecular biology. The enzyme DNA-dependent RNA polymerase is involved in this process. Dr Beatriz Gomez in our laboratory, in collaboration with Dr P. Witonsky, is studying *in vitro* transcription of T7 DNA by *E. coli* RNA polymerase using the diffusion method and the spontaneous-adsorption method (figures 2*b* and *c*). The aim is to locate sites and patterns of RNA synthesis along a clearly visible DNA template. Figure 10, plate 31, shows a T7 DNA molecule, 12 μm long, which has synthesized RNA for 5 min in presence of enzyme and nucleotides. Under the conditions applied, the RNA is collapsed and appears as a 'bush'.

The question whether or not these bushes are attached to random sites along the DNA was answered by measuring the distribution of all fractional DNA lengths as they were defined by bushes (figure 11). By comparison with the calculated distribution (smooth line in figure 11)

of distances defined by random events along a linear structure of finite length, it follows that the measured distribution is non-random. This type of test, not requiring the identification of one end of the DNA molecule, is objective. A relative distance of about 0.12 is clearly prominent (or 0.88 with respect to the other end). Apparently, the peaks in figure 11 are not sharp. The

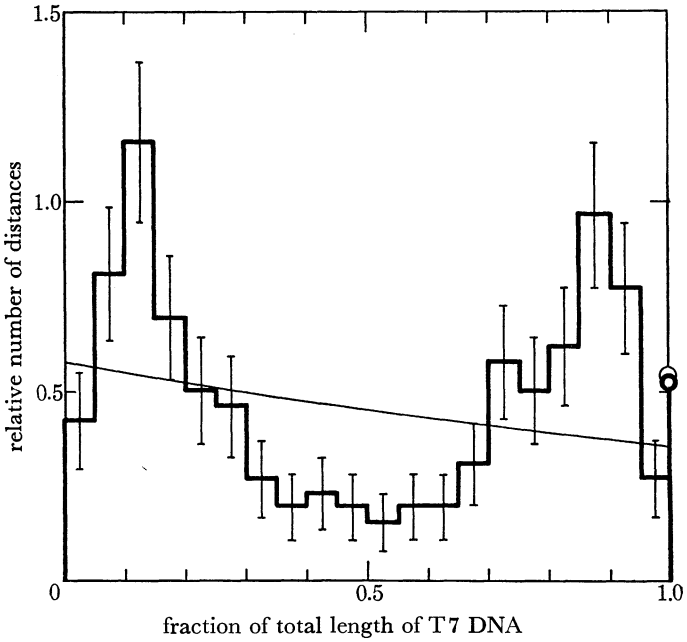


FIGURE 11. Distribution of all fractional DNA distances as defined by RNA bushes. 70% of the molecules had no bush, 28% one bush, and 2% two or three bushes. On the average, 0.33 bushes were found per DNA molecule; 518 distances were measured.

growth rate of RNA chains is probably not uniform, and there may be superposition of specific and non-specific (single-strand breaks in DNA?) polymerase sites on the DNA. Further studies are in progress including the attempt to unfold the RNA and to correlate specific sites with regions rich in adenine–thymine content.

CONCLUSION

It is likely that more results will be obtained within the general framework outlined by the above examples and that polymer chemists will apply these methods to synthetic polymers.

Prepared with assistance of Mrs B. Flores da Cunha, Mr L. Lewis, Jr, and Miss B. Bruton and supported by the U.S. Public Health Service (GM-34,964; GM-13,234) and by the National Science Foundation (GB-4388; GB-6837).

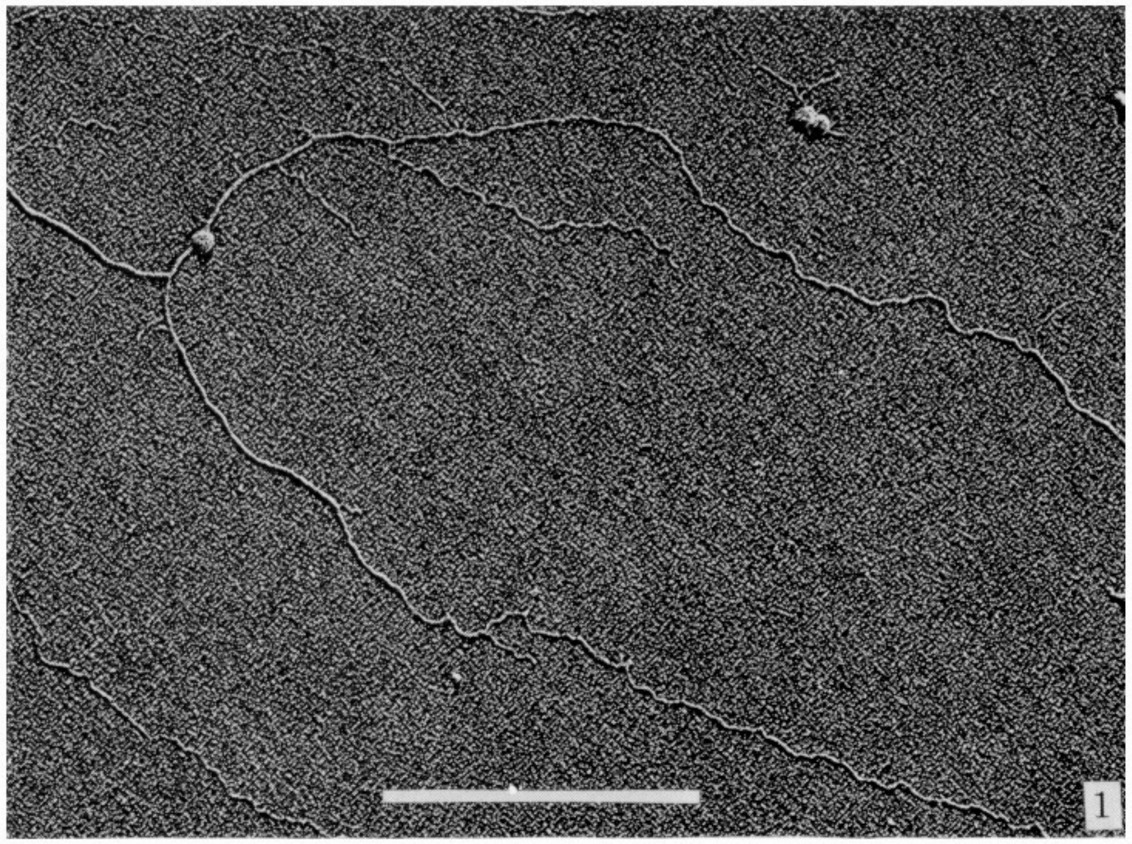
REFERENCES (Lang)

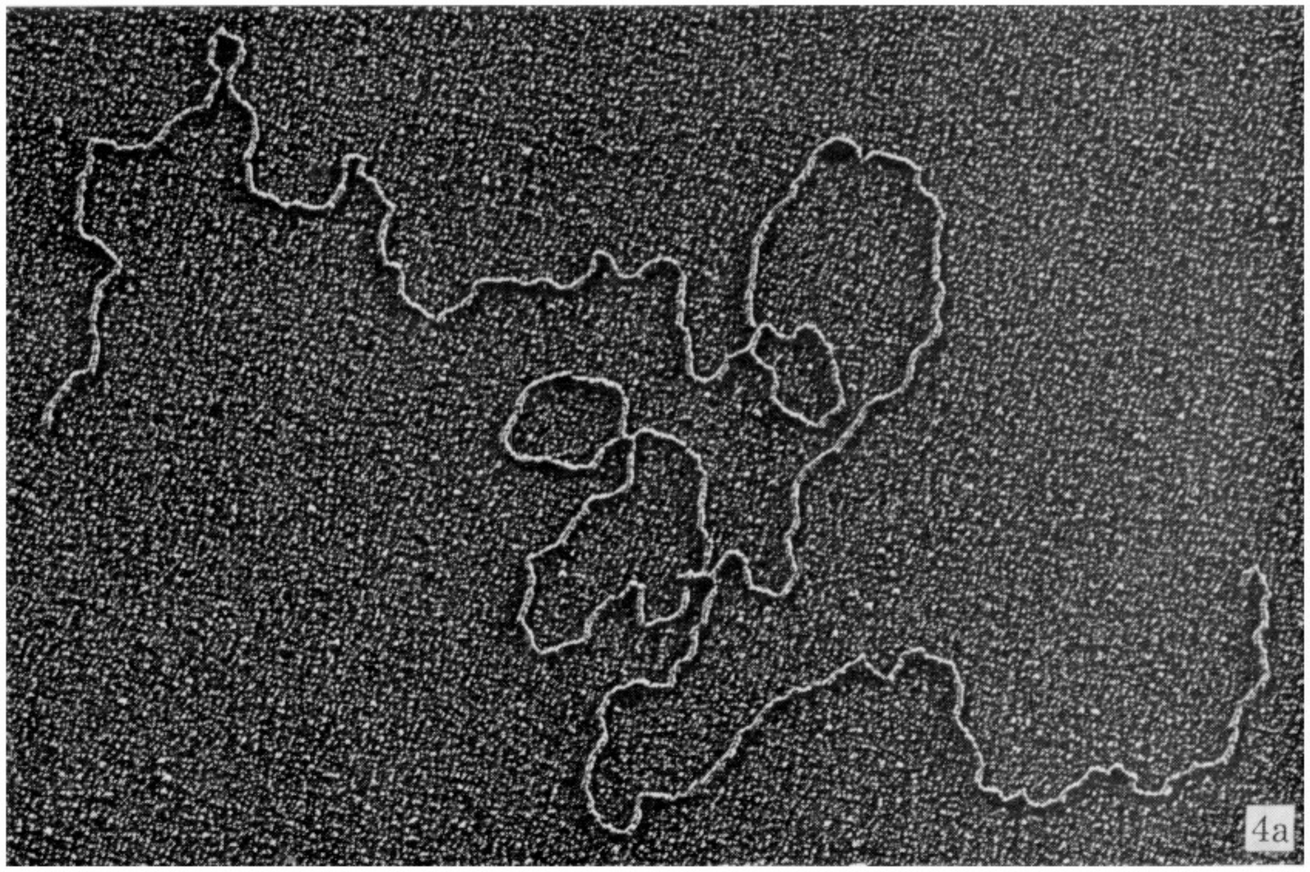
Abelson, J. & Thomas, Jr, C. A. 1966 *J. molec. Biol.* **18**, 262.
Bujard, H. 1969*a* *Proc. natn. Acad. Sci., U.S.A.* **62**, 1167.
Bujard, H. 1969*b* *J. molec. Biol.* **49**, 125.
Burgi, E., Hershey, A. D. & Ingraham, L. 1966 *Virology* **28**, 11.
Crawford, L. V. & Waring, M. J. 1967 *J. molec. Biol.* **25**, 23.

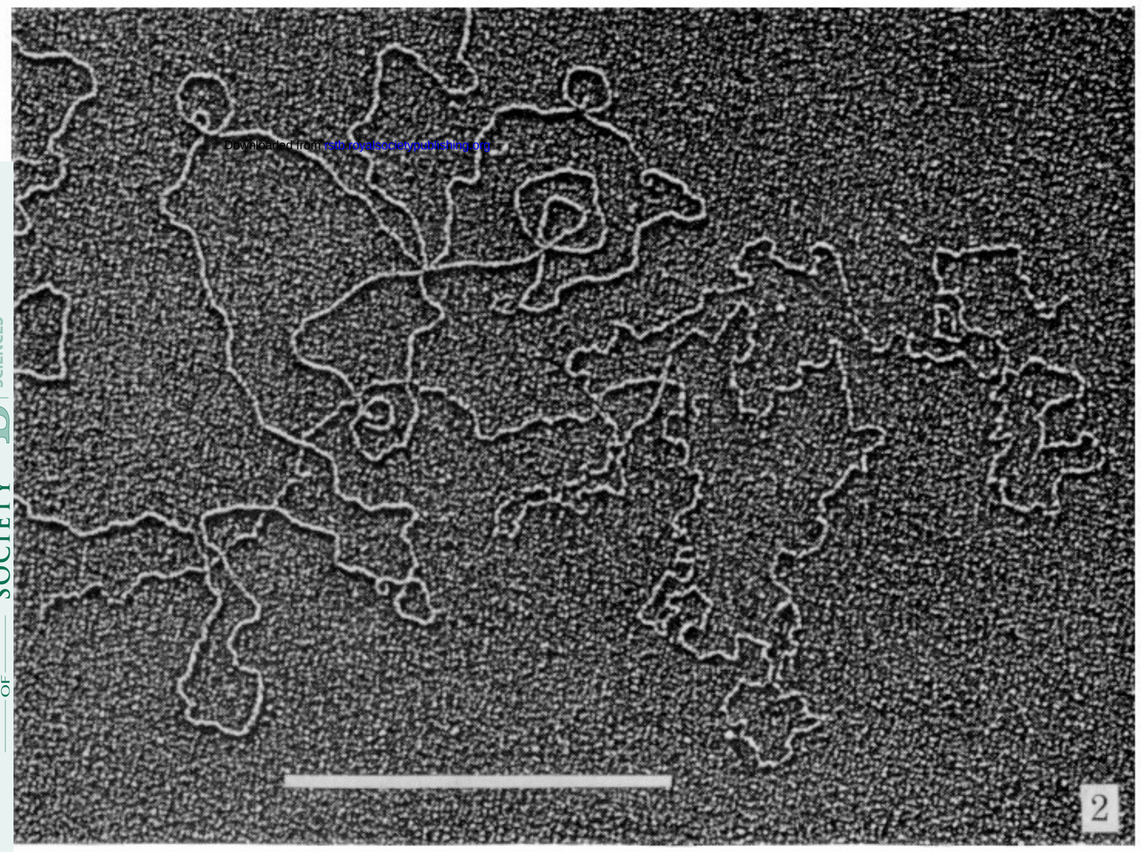
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

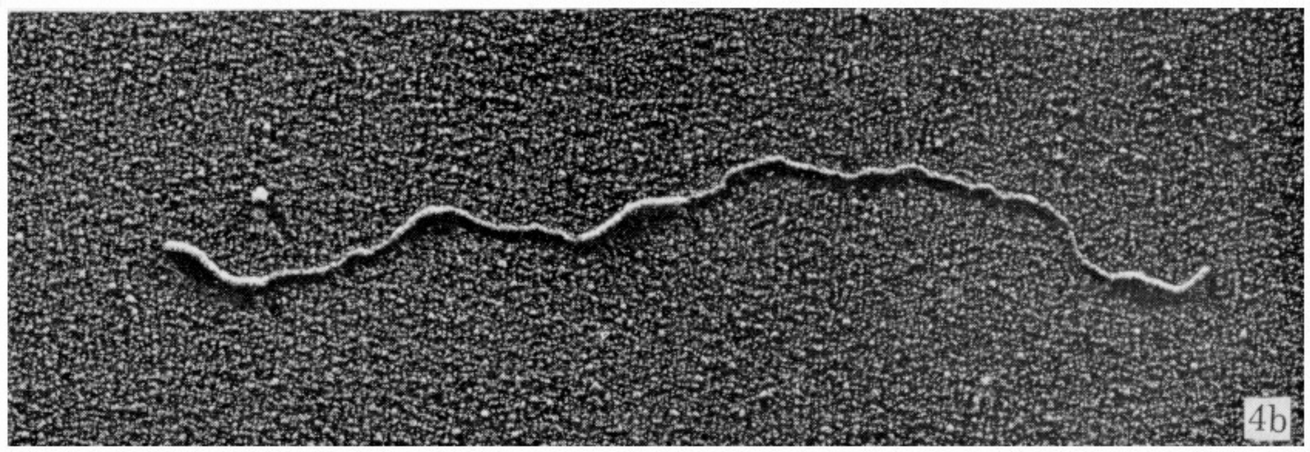
Davis, R. W. & Davidson, N. 1968 *Proc. natn. Acad. Sci., U.S.A.* **60**, 243.
Fuller, W. & Waring, M. J. 1964 *Ber. Bunsenges. phys. Chem.* **68**, 805.
Harm, H. 1970 *Molec. gen. Genetics* **107**, 71.
Hearst, J. E., Schmid, C. W. & Rinehart, F. P. 1968 *Macromolecules* **1**, 491.
Inman, R. B. 1966 *J. molec. Biol.* **18**, 464.
Inman, R. B. 1967 *J. molec. Biol.* **25**, 209.
Kleinschmidt, A. K., Lang, D., Jacherts, D. & Zahn, R. K. 1962 *Biochim. biophys. Acta* **61**, 857.
Kleinschmidt, A. K. & Zahn, R. K. 1959 *Z. Naturf.* **14***b*, 770.
Lang, D. 1969 *J. molec. Biol.* **46**, 209.
Lang, D., Bujard, H., Wolff, B. & Russell, D. 1967 *J. molec. Biol.* **23**, 163.
Lang, D. & Coates, P. 1968 *J. molec. Biol.* **36**, 137.
Lang, D. & Mitani, M. 1970 *Biopolymers* **9**, 373.
Lanni, Y. T. 1968 *Bact. Rev.* **32**, 227.
Miller, Jr, O. L. & Beatty, B. R. 1969 *Science, N.Y.* **164**, 955.
Nomura, M. & Benzer, S. 1961 *J. molec. Biol.* **3**, 684.
Rubenstein, I. 1968 *Virology* **36**, 356.
Westmoreland, B. C., Szybalski, W. & Ris, H. 1969 *Science, N.Y.* **163**, 1343.

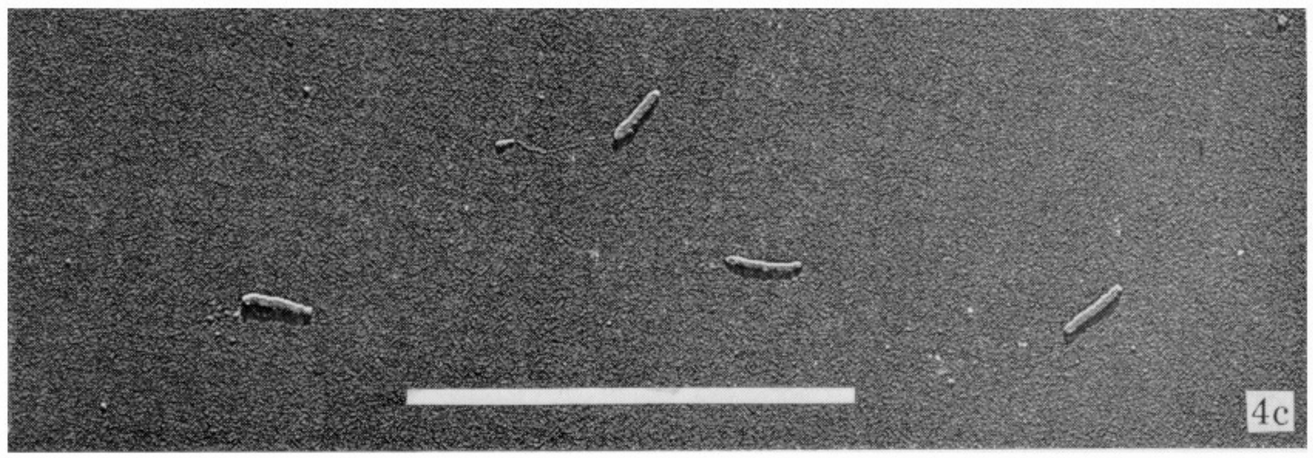
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES









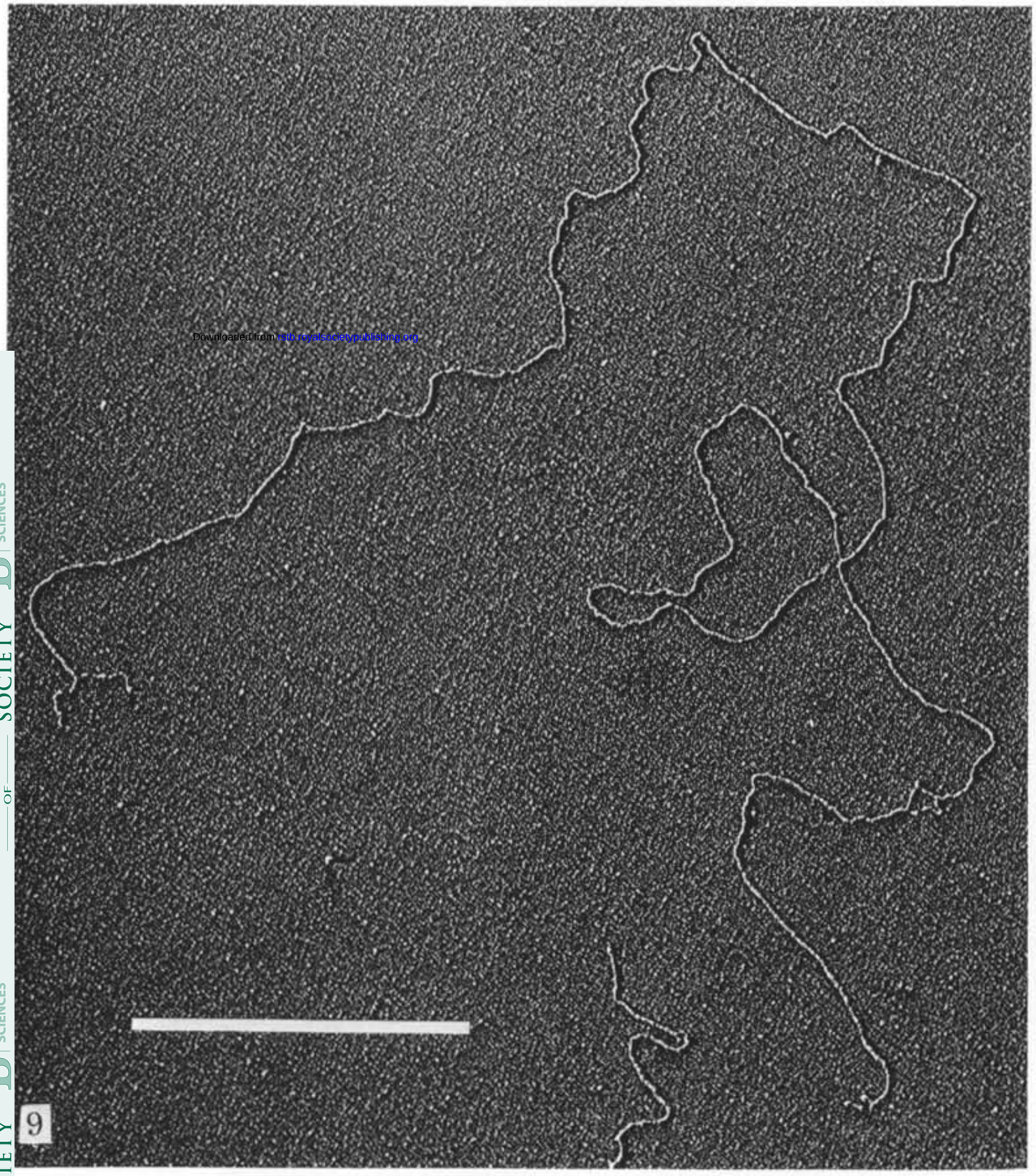


PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

FIGURE 1. DNA dried onto a specimen grid and shadowed with platinum. Lateral aggregation and preferential orientation in one direction are typical drying artefacts. The bar represents 1 μm. (Magn. $\times$ 26 000.)

FIGURE 2. Double- and single-stranded DNA prepared on the same grid by the diffusion method. Single-strand DNA has less contrast and is more kinked. (Magn. $\times$ 32 000.)

FIGURE 4. DNA appears as a loose coil when prepared from aqueous solution (*a*, diffusion method). In ethanol, a DNA molecule collapses upon itself into a compact, asymmetric rod structure (*c*) if the DNA concentration is sufficiently low to prevent formation of a macroscopic precipitate. The middle illustration (*b*) shows an intermediate state. The average volume of a rod is equal to the calculated volume of a T3 DNA molecule (3.8×10^{-17} cm^3). The DNA in the top and middle figures appear much thicker as they really are because they were prepared with cytochrome *c* and shadowed under 7°, whereas the rods were obtained on the grid without cytochrome *c* and shadowed under 20°. (Magn. $\times$ 37 000.)



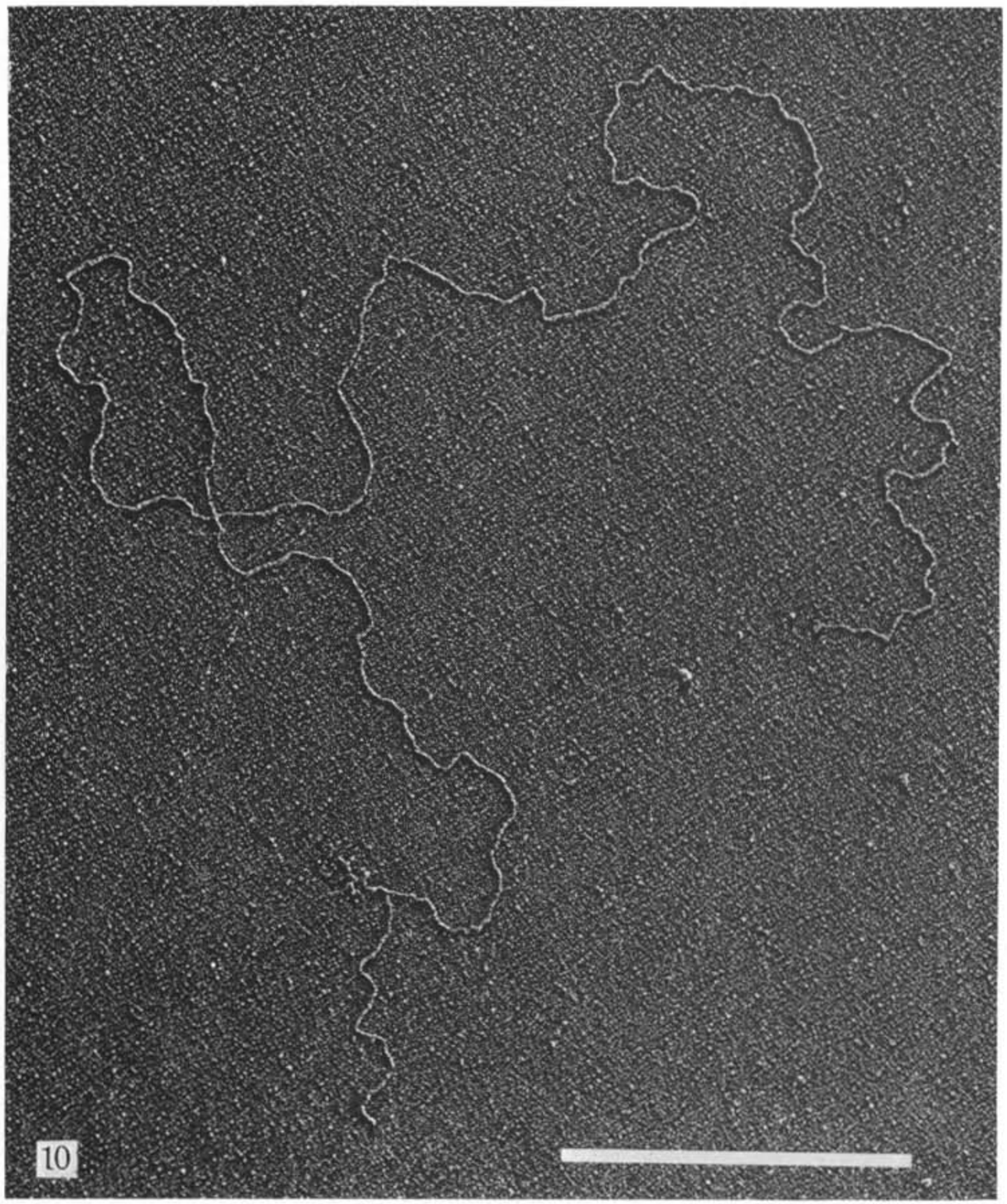


FIGURE 9. The T7 DNA molecule shown has been partially denatured by heating in presence of dimethylsulphoxide and formaldehyde. A collection of such molecules reveals a pattern of adenine-thymine rich regions which melt first. Prepared with the spontaneous-adsorption method. (Magn. × 33 000).

FIGURE 10. *In vitro* synthesis of messenger RNA along the T7 DNA template. The RNA appears as a bush attached to DNA. The DNA was incubated for 5 min with *E. coli* RNA polymerase in buffer containing nucleotides and 0.2 mol l^{-1} KC1 at pH 7.5. (Magn. × 33 000.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES