[3] Chromatography of Nucleic Acids on Hydroxyapatite Columns

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I. Introduction

Hydroxyapatite[1] (HA)[1a] columns, originally developed by Tiselius, Hjertén, and Levin[1b–3] for protein chromatography, were first used with nucleic acids by Semenza,[4] working in Tiselius' laboratory, and by Main *et al.*[5–7] In this early work, it was seen that native DNA could be adsorbed on, and eluted from, the columns, that DNA degraded by pancreatic DNase or by acid had a lower affinity for HA than undegraded DNA, and that protein, TMV RNA, and poly(A) could be partially separated from DNA.

Work begun in 1959 in the author's laboratory,[8,9] as a development of previous investigations on the chromatography of phosphoproteins on HA columns,[10] led to the recognition[11] that HA could discriminate nucleic acids endowed with different secondary structures, rigid, ordered structures having more affinity for HA than flexible, disordered ones,[12–17] a general

[1] Hydroxyapatite, not hydroxylapatite, is the name recommended by Wyckoff,[106] since "hydroxyl" implies the derivatives being named after the substituted ion, a usage which is not observed in the corresponding fluorine and chlorine derivatives (e.g. "fluoroapatite," "chloroapatite," not "fluoridapatite," "chloridapatite").

[1a] Abbreviations: HA, hydroxyapatite; NaP, KP, equimolar mixtures of NaH_2PO_4 and Na_2HPO_4, and of KH_2PO_4 and K_2HPO_4, respectively (pH is close to 6.8; ionic strength is equal to about twice the molarity). The abbreviation PB (phosphate buffer) used by some authors does not indicate the cation; since the eluting power of phosphate is quite different for different salts, it is advisable not to use the abbreviation PB.

[1b] A. Tiselius, S. Hjertén, and Ö. Levin, *Arch. Biochem. Biophys.* **65,** 132 (1956).

[2] S. Hjertén, *Biochim. Biophys. Acta* **31,** 216 (1959).

[3] Ö. Levin, this series, Vol. V, p. 27.

[4] G. Semenza, *Ark. Kemi* **11,** 89 (1957).

[5] R. K. Main and L. J. Cole, *Arch. Biochem. Biophys.* **68,** 186 (1957).

[6] R. K. Main, M. J. Wilkins, and L. Cole, *Science* **129,** 331 (1959).

[7] R. K. Main, M. J. Wilkins, and L. Cole, *J. Amer. Chem. Soc.* **81,** 6490 (1959).

[8] G. Bernardi, *Biochem. Biophys. Res. Commun.* **6,** 54 (1961).

[9] G. Bernardi and S. N. Timasheff, *Biochem. Biophys. Res. Commun.* **6,** 58 (1961).

[10] G. Bernardi and W. H. Cook, *Biochim. Biophys. Acta* **44,** 96 (1960).

[11] G. Bernardi, *Biochem. J.* **83,** 32 P (1962).

[12] G. Bernardi, *Nature (London)* **206,** 779 (1965).

[13] M. R. Chevallier and G. Bernardi, *J. Mol. Biol.* **11,** 658 (1965).

[14] M. R. Chevallier and G. Bernardi, *J. Mol. Biol.* **32,** 437 (1968).

[15] G. Bernardi, *Biochim. Biophys. Acta* **174,** 423 (1969).

[16] G. Bernardi, *Biochim. Biophys. Acta* **174,** 435 (1969).

rule also holding for proteins.[18] Although most fractionations of nucleic acids reported so far are based on this property of HA, subtler differences in secondary and tertiary structures can also be discriminated by HA columns,[12,17,19,20] since they originate different distributions of groups available for the interaction with the adsorbing sites of HA (see Section IX).

This article reviews the known facts and the current ideas on the chromatography of nucleic acids on HA. This is a rather difficult task for two main reasons: (i) chromatography of biopolymers on HA is in a stage of fast development, both experimental and theoretical; (ii) most of the experiments discussed here were performed using conditions chosen in an empirical way; a systematic exploration of the parameters involved in the chromatography of nucleic acids (like that done by Kawasaki and Bernardi,[21,22] for proteins) is not yet available. It should be mentioned here that a theory of the chromatography of rigid macromolecules has been developed[23,24] on the basis of the experimental results and general conclusions arrived at in Tiselius' as well as in the author's laboratories; it is likely that this theory is valid for nucleic acids having a rigid structure.

II. Methods

A. Preparation of Hydroxyapatite

1. *Procedure of Tiselius et al.*[1b]

This procedure is described as used in our laboratory, taking into account minor modifications suggested by Levin,[3] Miyazawa and Thomas,[25] and Bernardi.[15]

a. Materials. In the author's laboratory the following analytical grade reagents (Merck, Darmstadt, Germany) are routinely used: $CaCl_2 \cdot 2H_2O$ (Merck catalog No. 2382), $Na_2HPO_4 \cdot 2H_2O$ (No. 6580), $NaH_2PO_4 \cdot H_2O$ (No. 6346), $K_2HPO_4 \cdot 3H_2O$ (No. 5099), KH_2PO_4 (No. 4873).

b. Preparation of Brushite, $CaHPO_4 \cdot 2H_2O$. Two liters each of 0.5 *M* $CaCl_2$ and 0.5 *M* Na_2HPO_4 are fed at a flow rate of 250 ml/hour (using a multichannel peristaltic pump; two separatory funnels with Pasteur pipettes as outlets may also be used) into a 5-liter beaker containing 200 ml of 1 *M* NaCl; the addition is done under stirring just strong enough to avoid sedimentation of the brushite precipitate. At the end of this step, brushite is allowed to settle; the supernatant is decanted, and the precipitate is washed with two 4-liter volumes of distilled water.

c. Conversion of Brushite into Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$. Brushite is suspended in 4 liters of distilled water and stirred; 100 ml of 40% (w/w) NaOH is added; the mixture is then heated to boiling (40–50 minutes), and boiled for 1 hour with simultaneous stirring. The precipitate is then allowed to settle completely, and the supernatant is siphoned off. The precipitate is then washed with 4 liters of water. The supernatant is siphoned off when a 2-cm layer of precipitate is formed on the bottom of the beaker. This is the only time during the whole procedure when a complete settling of the precipitate is not allowed in order to eliminate the "fines." The precipitate is then washed twice more, allowing complete settling. At this point, the precipitates from two preparations are pooled and suspended in 4 liters of 10 m*M* sodium phosphate buffer, pH 6.8 (NaP)[1a] and brought just to boiling; boiling at this point should be avoided. The precipitate is then suspended in 4 liters of 10 m*M* NaP and boiled for 5 minutes. This operation is repeated once more using 10 m*M* NaP and then again using 1 m*M* NaP; in both cases boiling is done for 15 minutes; 400–500 ml of packed precipitate is obtained from two pooled preparations.

d. Storage of Hydroxyapatite. The final precipitate, in the form of blade-like crystals, can be stored in 1 m*M* NaP for several months at 4° without any change in chromatographic properties. The addition of chloroform as a preservative is not necessary. While HA crystals are being resuspended, strong agitation should be avoided, since this breaks down the crystals and their aggregates, thus rendering the material unsuitable for column chromatography.

2. *Alternative Preparation Procedures*

Other methods for preparing hydroxyapatite have been described by Main, Wilkins, and Cole,[7] Anacker and Stoy,[26] Jenkins,[27] and Siegelman *et al.*[28,29] Results reported with these preparations are rather sparse, and it is therefore difficult to judge their relative merits.

[17] G. Bernardi, *Biochim. Biophys. Acta* **174,** 449 (1969).
[18] G. Bernardi and T. Kawasaki, *Biochim. Biophys. Acta* **160,** 301 (1968).
[19] G. Bernardi, F. Carnevali, A. Nicolaieff, G. Piperno, and G. Tecce, *J. Mol. Biol.* **37,** 493 (1968).
[20] G. Bernardi, M. Faurès, G. Piperno, and P. Slonimski, *J. Mol. Biol.* **48,** 23 (1970).
[21] T. Kawasaki and G. Bernardi, *Biopolymers.* **9,** 257 (1970).
[22] T. Kawasaki and G. Bernardi, *Biopolymers.* **9,** 269 (1970).
[23] T. Kawasaki, *Biopolymers.* **9,** 277 (1970).
[24] T. Kawasaki, *Biopolymers.* **9,** 291 (1970).
[25] Y. Miyazawa and C. A. Thomas, Jr., *J. Mol. Biol.* **11,** 223 (1965).
[26] W. F. Anacker and V. Stoy, *Biochem. Z.* **33,** 141 (1958).
[27] W. T. Jenkins, *Biochem. Prep.* **9,** 83 (1962).
[28] H. W. Siegelman, G. A. Wieczorek, and B. C. Turner, *Anal. Biochem.* **13,** 402 (1965).
[29] H. W. Siegelman and E. F. Firer, *Biochemistry* **3,** 418 (1964).

3. Commercial Hydroxyapatite Preparations

A preparation obtained according to the procedure of Tiselius *et al.*[1b] is sold by Bio-Rad Laboratories (Richmond, California), either as a suspension in 1 m*M* NaP, or as a dry powder. Another preparation is sold by Clarkson Chemical Co. (Williamsport, Pennsylvania). Commercial HA preparations met with criticisms from several laboratories when they were first made available. Comments on the preparations sold during the past two years have been generally favorable.

B. Experimental Techniques with Columns

For general instructions on column chromatography the reader is referred to Determann[30] and Fischer.[31] Some features that are more specific to HA columns will be briefly recalled here.

1. Packing of the Columns

The columns are packed by adding a suspension of HA crystals in Na or K phosphate buffers, pH 6.8 (NaP or KP),[1a] to columns partially filled with the same buffer; the column outlet is progressively open only after a 1-cm layer of HA is settled. Further additions of the HA suspensions are then made to fill the column. The filling operation may be facilitated by the extension of the column with a glass tube of the same diameter. Alternatively, columns may be prepared by adding the HA suspension to a funnel mounted on the top of the column, the whole system being full of starting solvent; the HA suspension in the funnel is stirred during the preparation of the column. This procedure, suggested by Flodin[32] for Sephadex, allows very homogeneous packing.

2. Adsorption and Elution

As a rule, the sample is loaded in the solvent with which the column was previously equilibrated, generally a low-molarity NaP or KP.

As a rule, NaP or KP of increasing molarities are used to elute nucleic acids. NaP cannot be used at 4° at molarities higher than 0.5 *M* because of the limited solubility of Na_2HPO_4 at this temperature. Columns are normally operated under a slight pressure (30–50 cm of water). If controlled by a pump, flow rate should not be kept higher than that of a column flowing under a slight hydrostatic pressure. The phosphate molarity in the

[30] H. Determann, "Gel Chromatography." Springer, New York, 1968.

[31] L. Fischer, *in* "Laboratory Techniques in Biochemistry and Molecular Biology" (T. S. Work and E. Work, eds.), Vol. I, p. 151. North Holland Publ., Amsterdam, 1969.

[32] P. J. Flodin, *J. Chromatogr.* **5**, 103 (1961).

column effluent may be checked by refractive index measurement, phosphorus analysis, or conductimetry.

3. Column Regeneration

If elution of adsorbed material is complete, the column may be simply reequilibrated with the starting buffer and reused, preferably after removal of the top layer. The same column can be reused 3–4 times.

4. Recovery of Irreversibly Adsorbed Materials.

The HA bed may be extruded from the column and treated in one of the following ways: (a) put in dialysis bags and dissolved by dialysis vs. 1 *M* EDTA, pH 8.0; (b) eluted with 0.1 *M* NaOH; (c) dissolved in 1 *N* HCl.

C. The Adsorption-Elution Process

A systematic exploration of the parameters involved in the chromatography of nucleic acids on HA columns is yet to be done. Under these circumstances, it seems useful at least to review briefly the basic features of the adsorption-elution process and to present the limited information on this subject obtained so far in our laboratory.

1. Adsorption

This may be done in batch or on a column. Four sets of parameters should be considered: (a) the HA bed, (b) the material to be adsorbed, (c) the solvent, (d) the temperature at which adsorption takes place, and (e) the time of contact of the nucleic acid solution with HA, respectively.

a. HA Bed. The total volume of packed HA crystals, V_t (total volume), is equal to the sum of three terms: the volume of the "dry crystals," V_c (crystal volume); the volume of the solvent bound to the HA crystals and inaccessible to the material to be adsorbed, V_i (inner volume); and the volume of the solvent between the HA crystals and accessible to the material to be adsorbed, V_o (outer volume):

$$V_t = V_c + V_i + V_o \tag{1}$$

(i) The total volume of the packed HA bed, V_t, can be easily determined by measuring its dimensions. (ii) The outer volume, V_o, can be determined by measuring the elution volume of a nonadsorbed substance, like methyl orange, eosin, fuchsin, methyl red,[3] i.e., the volume of the solvent which leaves an HA column between loading and appearance of this substance in the effluent. (iii) The inner volume, V_i, can be calculated from the difference $(V_o + V_i) - V_o$, the term $(V_o + V_i)$ being determined by measuring the loss in weight, at 110°, of a known amount of packed HA crystals. (iv) The crystal volume, V_c, may be calculated from the difference $V_t - (V_o + V_i)$.

HA preparations obtained by the Tiselius procedure described above, packed under stirring, and equilibrated with 1 mM KP exhibit linear flow rate *vs.* pressure drop diagrams, a pressure drop (hydrostatic pressure divided by the length of the column) of 10 causing a flow rate of ~100 ml/cm²/hour. For these preparations, $V_o = 0.82$, $V_i = 0.10$, and $V_e = 0.08$ ml per milliliter of HA bed. The density of the packed HA crystals (wet) is equal to 1.17 g/ml. The value found for V_o is quite reproducible for preparations obtained according to the method described above and definitely higher than that (0.60–0.75) reported by Levin.[3] Obviously, HA preparations obtained according to different procedures, or preparations in which crystals were broken down, may have different properties. Since HA crystals are in the form of lamellae, it is likely that mechanical breakdown does not cause a very large increase in the surface available for adsorption.

b. The Material to Be Adsorbed. Two parameters are of interest: (i) the amount of material to be adsorbed; this should be established knowing the capacity of HA; as an indication on this point it can be mentioned that the amount of native DNA which can be adsorbed per milliliter of packed HA crystals equilibrated with 1 mM KP is about 10 A_{260} units; (ii) its concentration. The coexistence of different materials to be adsorbed should also be considered, since this will lead to competition for the adsorbing sites and cause displacement effects.

c. The Solvent. The concentration of eluting ions (phosphate ions, as a rule) at the adsorption step is obviously a critical parameter in determining the capacity of HA for a given material to be adsorbed. The presence in the solvent of substances having a stronger affinity for calcium than phosphate, e.g. EDTA and citrate, may decrease the capacity of HA to zero. On the other hand, an increase in ionic strength of the solvent due to ions having an affinity for calcium lower than phosphate, like chlorides, decrease the eluting power of phosphate, probably by diminishing the concentration of ionized phosphate (see also Sections III, A, 4 and IX). KP is remarkably more effective than NaP as an eluting agent.

d. Temperature. Temperature affects: (i) the adsorption phenomenon itself (adsorption isotherm); (ii) the ionization of phosphate ions; (iii) the secondary structure of the nucleic acids to be adsorbed. The effect of temperature on adsorption and on phosphate ionization is not important, yet deserves to be investigated in detail; the effect on the nucleic acid structure may cause serious changes in their affinity for HA (see Sections V, F and VI, D).

e. Time of Contact. Duration of contact between nucleic acids and HA necessary to reach adsorption equilibrium is of the order of half an hour if a solution of native DNA ($A_{260} = 0.4$) in 1 mM KP is put in contact with a large excess of HA. If adsorption is done on a column rather than in batch, one should consider the flow rate while loading the nucleic acid solution.

2. Elution

Elution may be performed by increasing the concentration of eluting ions (usually phosphate) either stepwise or continuously. Stepwise elution may be used with both batches and columns; molarity gradient elution can be used with columns only. In both cases, the flow rate of the eluent should be kept within certain limits to avoid a deformation of the chromatographic peaks; flow rates of 5 to 50 ml/hr/cm² were used in most experiments presented here. A third way of eluting nucleic acids, which has been applied to rigid, ordered structures, is to increase the temperature of the column to the point where a helix-coil transition occurs with concomitant decrease in affinity for HA; if the phosphate molarity at which melting takes place is high enough to elute disordered structures, elution occurs[25]; this procedure has nothing to do with a chromatographic elution and will be discussed later (Section V, F).

a. Stepwise Elution. This procedure is very useful in the separation of two (or more) adsorbed substances that have known, different, elution molarities. It has two main disadvantages, when used with columns: (i) tailing of the peaks: substances with strongly curved adsorption isotherms, and therefore extended elution ranges, cannot be eluted by a solvent of constant composition without tailing, unless elution is so strong that the R_f is close to 1.0[1b]; (ii) "false peaks": single substances with strongly curved isotherms may give rise to several peaks, each new molarity step of the eluent releasing an additional amount of substance.[1b]

b. Gradient Elution. Two parameters are very important in determining the resolving power of the columns: (i) the length of the column; (ii) the slope of the gradient in the column (grad). In the usual case of linear molarity gradients, grad may be calculated as follows:

$$\text{grad} = \frac{\Delta M}{V} \frac{S}{V_o/V_t} \tag{2}$$

where ΔM is the difference in phosphate molarity between the initial and the final buffer; V the total volume of the buffer; S the cross-sectional area of the column; V_o and V_t, the outer volume and the total volume of the column already defined. By the above definition, grad represents the increase in phosphate molarity per centimeter of column, if S and V are expressed in cm² and cm³, respectively.

It is important to stress that most experiments described in the following pages were done under conditions of low resolution, i.e., using columns shorter than 10 cm and grad values of the order of 1 mM/cm.

When elution is done by a linear phosphate molarity gradient, the chromatographic behavior of a nucleic acid is characterized by two parameters[21]: (i) the elution molarity, m_{elu}, which is defined as the phosphate molarity

at which the center of gravity of the nucleic acid peak is eluted; the center of gravity of the peak is given by:

$$\bar{V} = \int V f \, dV / \int f \, dV \tag{3}$$

where f is the distribution function of the peak, and V the volume of the solvent; (ii) the width of the peak; this can be calculated as its standard deviation and should be normalized by dividing it by S:

$$\sigma = [\int (V - \bar{V})^2 f \, dV / \int f \, dV]^{1/2} \frac{1}{S} \tag{4}$$

Both chromatographic parameters, m_{elu} and σ, depend upon several factors, e.g., column length, slope of the gradient, presence of other chromatographic components.

III. Chromatography of Native DNA

A. Chromatography of Native DNA

1. Properties of Native DNA after Chromatography

No significant changes in the physical, chemical, and biological properties of native DNA take place upon the adsorption-elution process, as indicated by the following results.[15]

DNA samples from calf thymus, chicken erythrocytes, and *Escherichia coli*, displaying molecular weights (as determined by light scattering) in the 4 to 6 $\times$ 10^6 molecular weight range, did not show, after the adsorption-elution process, any significant difference with respect to the original samples in any of the following properties: light-scattering envelope (and therefore weight-average molecular weight and radius of gyration), sedimentation coefficient, ultraviolet spectrum, and ultraviolet melting curve.

DNA samples from the same sources, but showing a molecular weight higher than 6 $\times$ 10^6 often showed a lower molecular weight after the adsorption-elution process. This phenomenon was apparently due, in some cases, to the removal of a small amount of large aggregates from DNA sample; in other cases, in which aggregated samples had been prepared by using steps leading to an aggregation of DNA (e.g., alcohol precipitation at an early stage of the deproteinization procedure), the adsorption-elution process caused a disaggregation of the intermolecular complexes possibly linked through protein material.

Results obtained with DNA samples from phages T1, T2, T4, T5, and λ showed that the sedimentation constants of the loaded and the eluted samples were identical. In the case of T2 DNA, preparations of "whole" molecules (MW = 1.3 $\times$ 10^8), as obtained by chromatography on methylated serum albumin-kieselguhr (MAK) columns, were used. It appears, therefore, that breakage by shearing does not occur during the elution of very high molecular weight DNA from HA.

Transforming *Haemophilus influenzae* DNA was adsorbed on, and eluted from, HA without any modification in the biological activity of 3 different genetic markers (the ultraviolet monitoring system was not used in these experiments).

2. Recovery of Native DNA from the Columns

In the large majority of cases a complete recovery was obtained, as judged from A_{260} measurements. Incomplete recoveries from HA may be obtained with DNA preparations containing aggregated material (see above).

3. Stepwise Elution

When elution was carried out stepwise according to the scheme shown in Fig. 1, DNA samples from calf thymus and chicken erythrocytes (MW = 4 to 6 $\times$ 10^6) were eluted at 0.20 *M* and 0.25 *M* KP (Fig. 1A). Occasionally, minor additional fractions were eluted when the KP molarity was further raised to 0.30 *M* and 0.50 *M*. Upon rechromatography each one of the two fractions (as well as the occasional minor peaks) was eluted again in two peaks, at 0.20 *M* and 0.25 *M* KP, respectively (Figs. 1B and 1C), indicating that these peaks may be considered as "false peaks"[1b,2] (see Section II, C, 2, a).

4. Gradient Elution

When elution was performed with a linear molarity gradient of KP, the chromatogram obtained with DNA's from higher organisms or bacteria (MW = 4 to 6 $\times$ 10^6) showed only one peak centered at 0.20–0.22 *M* (Figs. 2A and 2B) whereas samples from T5 and T2 phages were eluted in single peaks centered at 0.27 *M* (Figs. 2C and 2D). It has been reported[33] that the DNA from a mouse lymphoma was only partially eluted by 0.26 *M* NaP, the rest being removed from the column by 1.0 *M* NaP under conditions where mouse thymus DNA was eluted in 94% yield by 0.26 *M* NaP. The meaning of this finding is not clear, however, since the starting DNA and the fractions were not characterized.

Interestingly enough, gradient elution can also be done at a practically constant ionic strength, using gradients formed by 1 m*M* KP + 1 *M* KCl as the starting buffer and 0.5 *M* KP as the final buffer; in both these sol-

[33] A. S. Patel and K. S. Korgaonkar, *Experientia* **25**, 25 (1969).

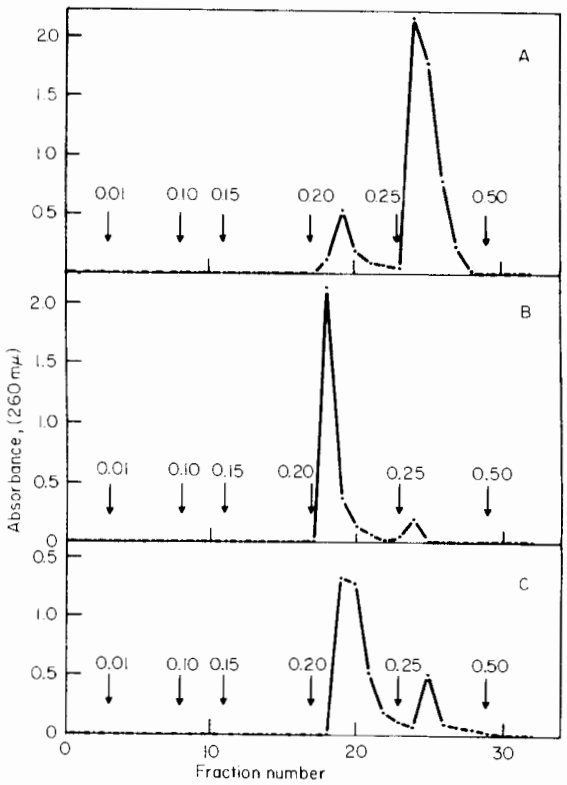

FIG. 1. Chromatography of native calf thymus DNA (preparation A1). (A) Chromatography of 1.28 mg of DNA on a 1.3 cm × 5 cm column. (B) Rechromatography of the 0.25 *M* fraction; 0.78 mg of DNA as loaded on a 1.3 × 3 cm column. In all cases, 3-ml fractions were collected. Rechromatography experiments were done on pooled 0.20 and 0.25 *M* fractions from two chromatographic experiments. DNA loading took place at fraction 0; the stepwise increases in KP molarity are indicated by the vertical arrows. Reproduced from G. Bernardi [*Biochem. Biophys. Res. Commun.* **6**, 54 (1961)].

vents the ionic strength is approximately 1.0, since the ionic strength of NaP and KP is equal to about twice their molarities.[1a] In this case, DNA is eluted at the same phosphate molarity as in the absence of KCl. If a still higher ionic strength is used, the two buffers being, for instance, 1 m*M* KP *plus* 2 *M* KCl and 0.5 *M* KP *plus* 1 *M* KCl, the phosphate eluting molarity was higher and equal to 0.27 *M*. This increase in phosphate eluting molarity is already observed at a 1 *M* level when using a Na^+ system; for instance, using 1 m*M* NaP + 1 *M* NaCl and 0.5 *M* NaP as the limiting buffers, DNA was eluted at a NaP of 0.31.[15]

B. Fractionation of Native DNA

1. Fractionation According to Molecular Weight

HA columns have a very low degree of discrimination toward molecular weight. For instance, under experimental conditions similar to those of Fig. 2, calf thymus DNA samples ranging in molecular weight from 6×10^6 to 1×10^5, obtained by limited degradation by spleen acid DNase (an enzyme known to break both DNA strands at the same level and to cause no significant changes in the secondary structure of DNA in the molecular weight range under consideration[34,35]) are eluted at the same KP molarity (0.20–0.22). Similar results were obtained with DNA samples sheared in a high-speed VirTis homogenizer in the presence of chloroform and isoamyl alcohol (E. G. Richards, unpublished experiments, 1962). In contrast to this behavior, DNA samples from bacteria and higher animals displaying molecular weights in excess of 10^7 were eluted at a slightly higher KP molarity (0.22–0.25), and the large DNA's from T2 and T5 phages were eluted at about 0.27 *M* KP, as already mentioned.

Moderate degrees of fractionation were obtained when running artificial mixtures of degraded and undegraded DNA. For instance, T2 phage DNA "whole" molecules (MW = 1.3×10^8) could be separated to a fair extent from sonicated T2 DNA (MW $\cong 5 \times 10^5$), but not at all from T2 DNA "half" molecules.[15] Similarly, artificial mixtures of intact DNA and of DNA partially degraded by spleen acid DNase (see above) could be fractionated to some extent by stepwise elution.[15] In these cases it is possible that fractionation depends: (i) upon the displacement of the shorter molecules by the large ones, and (ii) upon physical fractionation according to the hydrodynamic volume of DNA molecules during the flow through the HA column.

2. Fractionation according to Secondary or Tertiary Structures, Glucosylation, and Base Composition

When DNA samples from calf thymus and chicken erythrocytes or *H. influenzae* are chromatographed under experimental conditions similar to those shown in Fig. 2, i.e., conditions of low resolution, no fractionation with respect to base composition or to genetic markers can be detected.

In a few cases, native DNA's have a chromatographic behavior different from that just described because of their particular secondary or tertiary structures, glucosylation, and base composition. These DNA's can therefore be separated from those exibiting the "usual" behavior.

[34] G. Bernardi and C. Sadron, *Biochemistry* **3**, 1411 (1964).
[35] E. T. Young II and R. L. Sinsheimer, *J. Biol. Chem.* **240**, 1274 (1965).

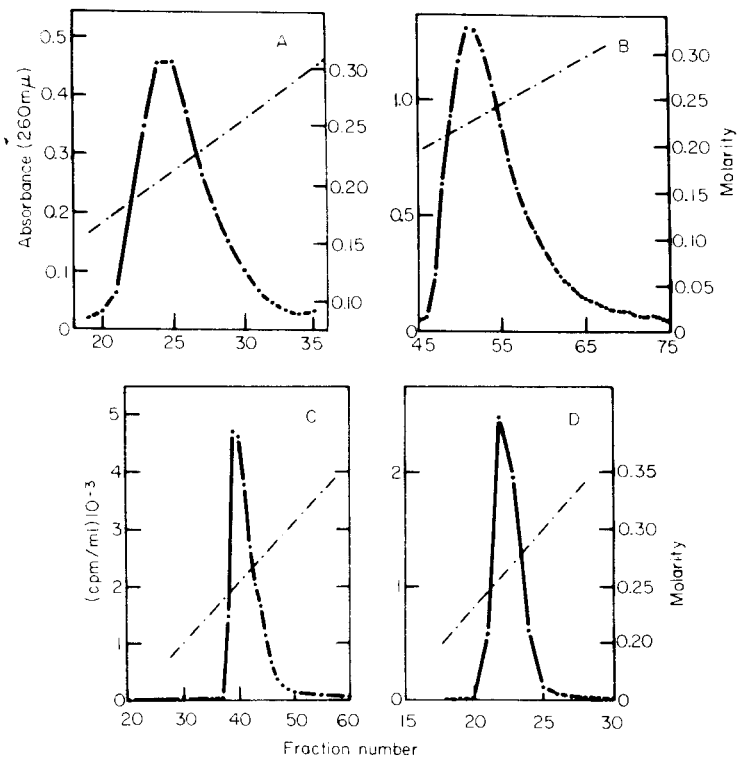

FIG. 2. (A) Chromatography of native calf thymus DNA (preparation B15). Two milliliters of a solution having an $A_{260} = 5.0$ was loaded on a 1 × 4.5 cm column. This experiment was carried out at 4°. Fractions of about 3.8 ml were collected. Elution was carried out with 100 + 100 ml of 0.001 and 0.5 *M* KP. (B) Chromatography of *H. influenzae* DNA. Four milliliters of a solution having an $A_{260\ m\mu} = 5.01$ was loaded on a 1 cm × 10 cm column. Fractions of 3 ml were collected. Elution was carried out with a molarity gradient (150 + 150 ml) of KP (0.001–0.5 *M*). Flow rate, 36 ml/hour. Recovery was 92%. (C) Chromatography of ^{32}P-labeled T5 DNA on a 1 × 6 cm column. Elution was carried out with a molarity gradient of KP, 2.6-ml fractions were collected, 1.2 × 10^5 cpm were loaded and recovered from the column. (D) Chromatography of ^{32}P-labeled T2 DNA on a 1 × 10 cm column. Elution was carried out with a molarity gradient of KP containing 1% formaldehyde, 5.2-ml fractions were collected. Recovery was 92%. Flow rate, 40 ml/hour. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **174**, 423 (1969)].

a. The Single-Stranded DNA from φX174 Phage. The DNA is eluted, using the stepwise elution, at 0.10 *M* and 0.15 *M* KP (Fig. 3A), therefore at molarities much lower than double-stranded DNA molecules (see also Sections V and IX).

b. The Twisted Circular DNA from Polyoma Virus. This is eluted at a lower molarity than the open linear and circular forms of the same DNA (Fig. 3B); the latter forms being eluted at the same molarity. This interesting observation[36,37] will be discussed in Section IX.

c. Glucosylated DNA from T-even Phages. This DNA is eluted at a higher molarity than nonglucosylated *E. coli* or T5[15,38] (see Fig. 3C). DNA from a nonglucosylated mutant strain of T4 bacteriophage (which, however, contains 5-hydroxymethylcytosine, like T4 DNA) is also separated, although to a lesser extent, from *E. coli* DNA (Fig. 3D). It is also interesting that the separation of T4 DNA and *E. coli* DNA is not observed anymore when these DNA's are chromatographed in a denatured state.[38]

d. Mitochondrial Yeast DNA's. These DNA's are eluted at a higher molarity than nuclear yeast DNA's[19,20] (Fig. 4). This separation is not related to a difference in molecular weight, since both DNA's practically had the same molecular weight, nor to a difference in the tertiary structure, since both DNA's were formed by open, linear molecules (a very small percentage of open circular molecules present in mitochondrial DNA showed the same chromatographic behavior as the open, linear ones,[19] a finding in agreement with the similar observation on the polyoma DNA[36,37]).

Yeast mitochondrial DNA's are rather exceptional in their base composition. In fact, DNA's from wild-type cells have an AT contents of 83%, and DNA's from different cytoplasmic "petite" mutants have AT contents ranging from 85% to 96% according to the mutants.[20] Furthermore, yeast DNA's contain not only alternating dAT:dAT stretches, but also nonalternating dA:dT ones.[19,20,39] It is probable that the presence of the latter causes mitochondrial DNA to be eluted at a higher molarity. In fact biosynthetic nonalternating poly(dA:dT) has a high elution molarity, whereas the alternating poly(dAT:dAT) has an elution molarity close to that of nuclear DNA. These results are very interesting in that they show that HA can discriminate slightly different native DNA structures.

IV. Chromatography of Nucleohistones

As just reported in Section III, A, native DNA can be eluted from HA columns by phosphate buffers in the presence of chlorides without any major change in its chromatographic behavior. Since nucleohistones can be progressively dissociated into their DNA and histone components by exposure to increasing salt concentrations, and since histones are less strongly adsorbed by HA than DNA in the presence of salt,[40] it should be

[36] P. Bourgaux and D. Bourgaux-Ramoisy, *J. Gen. Virol.* **1**, 323 (1967).
[37] D. Bourgaux-Ramoisy, N. Van Tieghem, and P. Bourgaux, *J. Gen. Virol.* **1**, 589 (1967).
[38] M. Oishi, *J. Bacteriol.* **98**, 104 (1969).
[39] G. Bernardi and S. N. Timasheff, *J. Mol. Biol.* **48**, 43 (1970).
[40] G. Bernardi, experiments to be published.

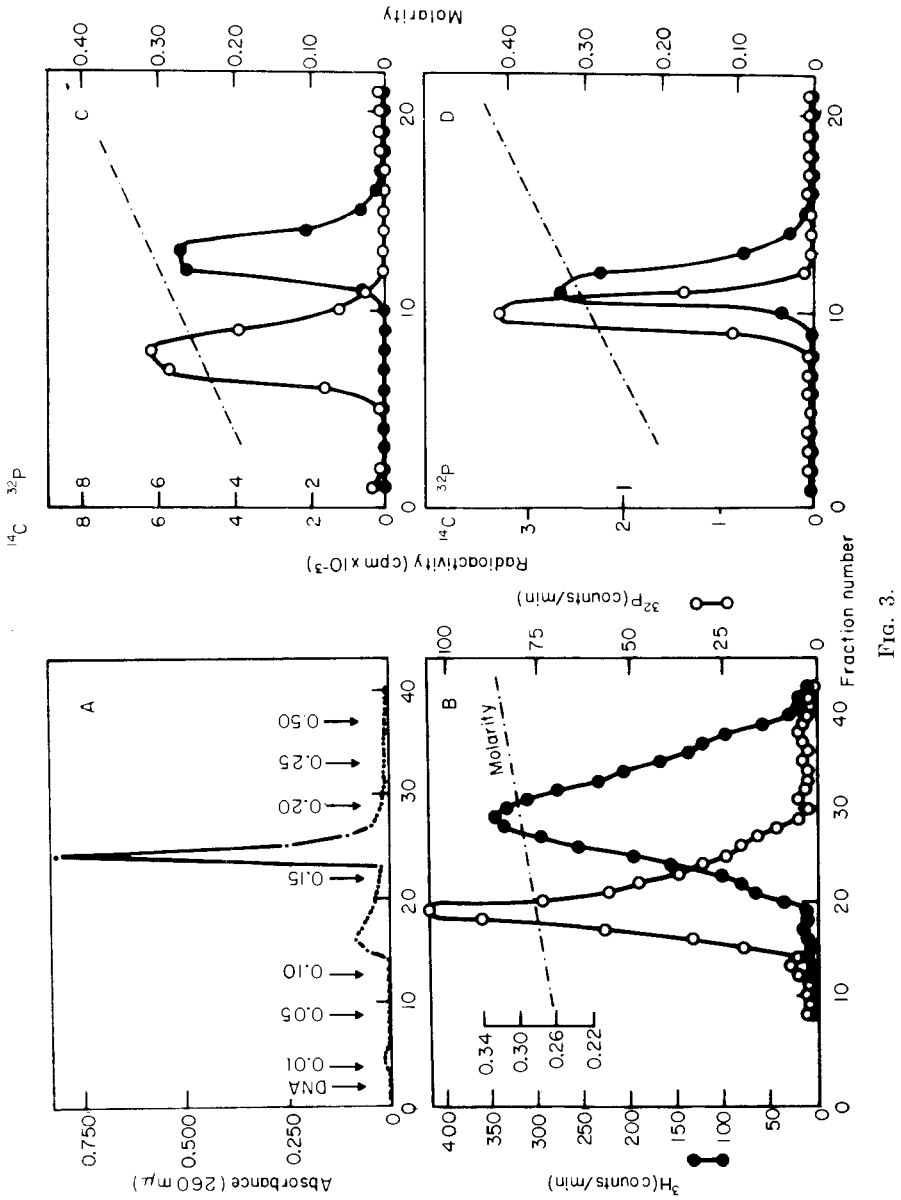

FIG. 3.

feasible to separate on HA histone fractions, released by salt, from the residual partial nucleoproteins. This is indeed what happens, as shown in Fig. 5.[41] In these experiments, calf thymus nucleohistone solutions in 0.7 m*M* KP and 0–3 *M* KCl were loaded on HA columns equilibrated with the same solvents. After washing the columns with the equilibration solvent, elution was performed with a linear molarity gradient of KP, the KCl concentration being kept constant and equal to that used in the dissociation step. The elution pattern of nucleohistone run in the absence of KCl resembles that of native DNA (Fig. 5A); it is very likely that a partial dissociation of nucleohistone takes place at its elution molarity, since the A_{260} tracing trails both the A_{235} and the A_{260} patterns. Nucleohistone solutions in increasing KCl concentrations show increasing amounts of a protein component eluting at 0.07–0.10 *M* KP; this protein peak had an A_{280}/A_{260} ratio equal to 1.2–1.3; its lysine-arginine molar ratio was higher in fractions dissociated at 0.75 *M* and 1 *M* KCl than in fractions dissociated by 2.0 *M* and 3.0 *M* KCl, in agreement with the known easier dissociability of lysine-rich histones. The main component, formed by partially or totally dissociated nucleohistone, is eluted at a molarity of 0.2–0.3 *M* KP, the eluting KP molarity being higher when the KCl concentration was higher (see Section III, A, 4.). The nucleohistone peak showed A_{280}/A_{260} and A_{235}/A_{260} ratios which approached the values obtained with pure DNA as increasing KCl concentrations were used, the ratios obtained with the material dissociated by 3 *M* KCl being the same as for DNA obtained from nucleohistone by the detergent procedure.[34]

The original work of Faulhaber and Bernardi[41] was performed on the "soluble" fraction of calf thymus nucleohistone preparations obtained ac-

[41] I. Faulhaber and G. Bernardi, *Biochim. Biophys. Acta* **140,** 561 (1967).

FIG. 3. (A) Chromatography of φX174 DNA on a 1.3 × 3 cm; 5 ml of DNA solution having an $A_{260\ m\mu} = 1.68$ were loaded; 3.3-ml fractions were collected. Recovery was 96%. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **174,** 423 (1969)]. (B) Chromatography of native polyoma virus DNA. A sample of tritium-labeled component II (16 S, untwisted, circular), mixed with component I (20 S, twisted, circular), labeled with ^{32}P, was loaded on a column and eluted with a linear concentration gradient (0.23 *M* to 0.32 *M*) of NaP; 0.5-ml fractions were collected. Reproduced from P. Bourgaux and D. Bourgaux-Ramoisy [*J. Gen. Virol.* **1,** 323 (1967)]. (C) Chromatography of a mixture (1.0 ml) of ^{32}P-labeled T4 DNA (0.3 μg) and ^{14}C-labeled *E. coli* DNA (1.6 μg). The sample in 50 m*M* phosphate, pH 7.0, was applied to a 1 cm × 3 cm column and was eluted by a linear molarity gradient of phosphate, pH 7.0 (0.18–0.40). Two-milliliter fractions were collected. Recovery of the DNA was 85% for T4 DNA and 87% for *E. coli* DNA. Reproduced from M. Oishi [*J. Bacteriol.* **98,** 104 (1969)]. (D) Chromatography of a mixture (1.0 ml) of ^{32}P-labeled T4 gt DNA (0.4 μg) and ^{14}C-labeled *E. coli* DNA (1.6 μg). Experimental conditions as under C. Recovery of the DNA was 99% for T4 gt DNA and 96% for *E. coli* DNA. Reproduced from M. Oishi [*J. Bacteriol.* **98,** 104 (1969)].

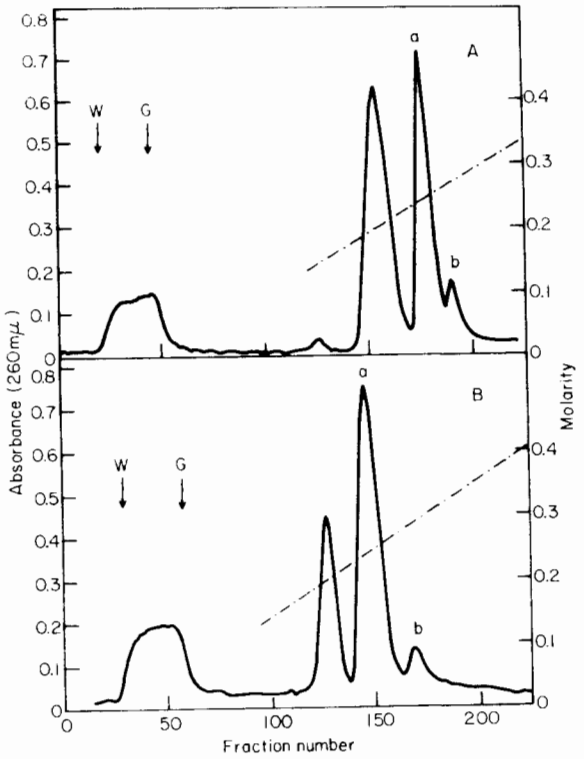

FIG. 4. (A) Chromatography of a DNA preparation from a wild-type yeast. One hundred milliliters of DNA solution in 0.1 *M* NaP, A_{260} = 0.820, as loaded on a 2 × 40 cm HA column; the column was then washed with 100 ml of 0.1 *M* NaP; elution was carried out with a linear gradient (450 ml + 450 ml) of NaP (0.1–0.5 *M*). Loading was started at fraction 0, washing at fraction marked by arrow W, gradient at fraction marked by arrow G; 3.8-ml fractions were collected. Flow rate was close to 55 ml/hour. A_{260} recovery was 98%. (B) Chromatography of a DNA preparation from a "petite" cytoplasmic mutant. One hundred milliliters of DNA solution in 0.1 *M* NaP, A_{260} = 0.800, were loaded on a 2 × 34 cm HA column; 3.5-ml fractions were collected. Flow rate was about 50 ml/hour. A_{260} recovery was 100%. All other indications are as above. Reproduced from G. Bernardi, F. Carnevali, A. Nicolaieff, G. Piperno, and G. Tecce [*J. Mol. Biol.* **37**, 493 (1968)].

cording to Zubay and Doty.[42] More recent work[43] has shown that total ("soluble" + "gel" fractions) nucleohistone can also be chromatographed on HA using 3 *M* KCl as the dissociating salt concentration.

[42] G. Zubay and P. Doty, *J. Mol. Biol.* **1**, 1 (1959).
[43] M. André and G. Bernardi, in preparation.

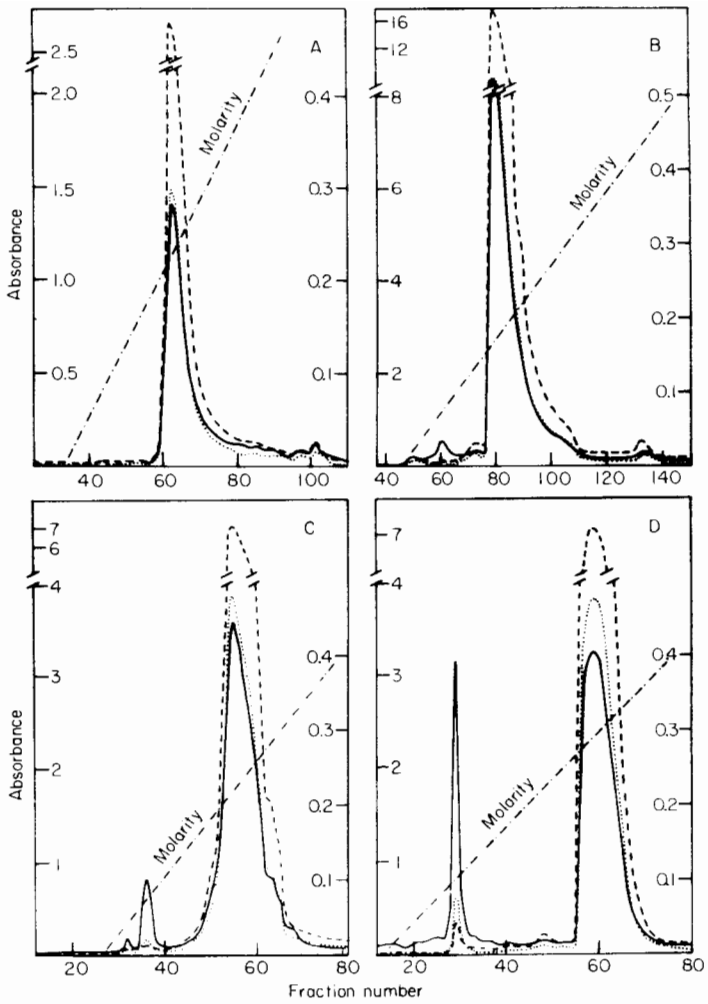

FIG. 5. Chromatography of calf thymus nucleohistone on 2.3 × 19 cm columns. Elution was performed with a linear KP molarity gradient (450 ml; 0.001–0.5 *M*). The concentration of KCl in the eluting buffer varied from 0 to 3 *M* in different experiments. Curves indicated absorbances at 260 mμ (broken line), at 280 mμ (dotted line), and at 235 mμ (continuous line). (A) Chromatography in the absence of KCl. Total load was 160 A_{260} units; fraction volume 6.7 ml; recovery 93%. Absorbance ratios of eluted material were A_{235}/A_{260} = 0.532; A_{280}/A_{260} = 0.553. (B) Chromatography in the presence of 0.75 *M* KCl. Total load was 900 A_{260} units; fraction volume 4.4 ml; recovery 97%. Absorbance ratios of the main peak were A_{235}/A_{260} = 0.525; A_{280}/A_{260} = 0.555. (C) Chromatography in the presence of 1 *M* KCl. Total load was 450 A_{260} units; fraction volume 6.8 ml; recovery 96%. Absorbance ratios of the main peak were A_{235}/A_{260} = 0.500; A_{280}/A_{260} = 0.548. (D) Chromatography in the presence of 3 *M* KCl. Total load was 450 A_{260} units; fraction volume 6.8 ml; recovery 95%. Absorbance ratios of the main peak were A_{235}/A_{260} = 0.455; A_{280}/A_{260} = 0.532. Reproduced from I. Faulhaber and G. Bernardi [*Biochim. Biophys. Acta* **140**, 561 (1967)].

V. Chromatography of Denatured DNA

A. Chromatography of Denatured DNA

1. Stepwise Elution

This method was investigated[11,12,16] using DNA samples from calf thymus and chicken erythrocytes.

a. DNA Partially or Totally Denatured by Heat. DNA denatured by heating for 15 minutes at 100°, at a concentration of 50–100 μg/ml in 0.13 *M* NaCl plus 0.01 *M* KP, and fast cooled, showed a chromatographic behavior quite different from that of native DNA (shown in Fig. 6A), since it was eluted in three fractions at 0.15 *M*, 0.20 *M*, and 0.25 *M* KP (Fig. 6C); minor fractions were occasionally eluted at 0.10 and 0.50 *M* KP. When DNA was heated up to temperatures between 85° and 100°, the elution patterns were intermediate between those of native and fully denatured (100°) DNA. As denaturing temperatures were increased, increasing

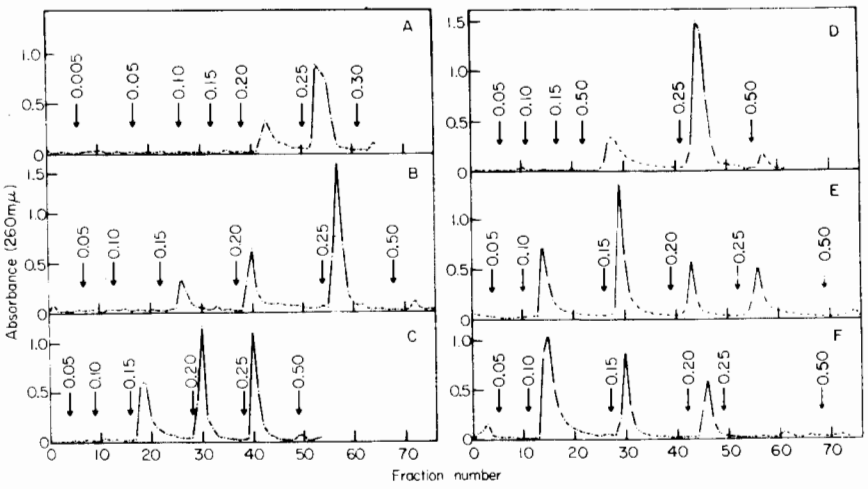

FIG. 6. Chromatography of calf thymus DNA (preparation B3) on 1.3 × 7 cm hydroxyapatite columns. DNA solutions, 10–20 ml, having an A_{260} in the 1–2.5 range, were loaded at fraction number zero; 3.8-ml fractions were collected. Recoveries were 100% except where otherwise stated. Stepwise elution of (A) native DNA; (B) DNA heated up 90° and then fast-cooled; (C) DNA heated up to 100° and then fast-cooled; in this case the recovery was 95%. Stepwise elution in the presence of 1% formaldehyde of (D) native DNA; (E) DNA heated up to 100°, fast-cooled and then reacted with formaldehyde, (F) DNA heated up to 100° in the presence of formaldehyde; in this case, the recovery was 93%. Reproduced from G. Bernardi [*Nature* (*London*) **206**, 779 (1965)].

amounts of material were eluted at lower molarities; in other words, a gradual shift to the left of the elution pattern was obtained when running DNA samples which had been heated up to increasing temperatures in the range 85°–100°. As an example, Fig. 6B shows a chromatogram obtained with a DNA sample heated up to 90°.

b. Heat-denatured DNA Reacted with Formaldehyde. The behavior of heat-denatured, fast-cooled DNA reacted for 24 hours at 25° with 1% (final concentration) neutralized formaldehyde, was studied using KP containing 1% formaldehyde as the eluent. The chromatographic pattern was slightly different from that just described for heat-denatured DNA, since most of the material was eluted by 0.15 *M* KP and smaller fractions were eluted at 0.10 *M*, 0.20 *M*, and 0.25 *M* KP; occasionally, a minor fraction was eluted by 0.50 *M* KP (Fig. 6E). The elution profile appeared therefore shifted to the left when compared with that obtained when using heat-denatured DNA which had not been reacted with formaldehyde. In contrast, native DNA treated with formaldehyde and eluted with formaldehyde-containing KP showed the same elution pattern as native DNA run in the usual conditions (Fig. 6D; compare this figure with Fig. 6A).

Rechromatography experiments performed on the fractions obtained from heat-denatured, fast-cooled, formaldehyde-reacted DNA showed the following results: (i) the 0.15 *M* and 0.10 *M* fractions contained, respectively, very little and no material eluting at molarities higher than 0.15 *M* (Figs. 7B and 7A); (ii) the 0.20 *M* fraction showed two main fractions eluting at 0.15 *M* and 0.20 *M*, and a minor one eluting at 0.10 *M* (Fig. 7C); (iii) the 0.25 *M* and 0.50 *M* fractions contained, respectively, very little and no material eluting at molarities lower than 0.25 *M* (Figs. 7D and 7F); upon a third chromatography, the 0.25 *M* fraction did not show any material eluting at a lower molarity (Fig. 7E).

These rechromatography experiments suggest the existence of two distinct fractions in heat-denatured DNA: a large one eluting at molarities lower than 0.20 *M*, and a small one eluting at molarities higher than 0.20 *M*.

c. DNA Denatured by Heat in the Presence of Formaldehyde. DNA which had been heated up to 100° in the presence of 1% formaldehyde (under which conditions the melting temperature is lowered by 10°–15°[44]) showed, upon chromatography with formaldehyde-containing KP, an elution pattern further shifted to the left as compared with that of heat-denatured, formaldehyde-treated DNA (Fig. 6F; compare this figure with Fig. 6B). An important feature of the elution profile obtained under these conditions is the absence of fractions eluting at molarities higher than 0.20 *M* KP.

[44] L. Grossman, S. S. Levine, and W. S. Allison, *J. Mol. Biol.* **3**, 47 (1961).

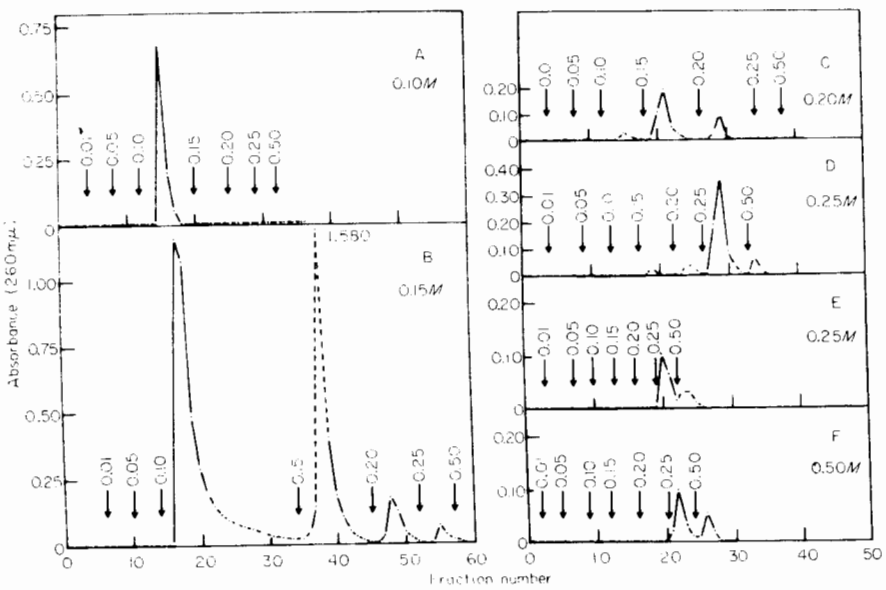

FIG. 7. Rechromatography experiments performed on the fraction obtained from heat-denatured, fast-cooled, and formaldehyde-reacted DNA. Stepwise elution of (A) 0.10 *M* fraction; (B) 0.15 *M* fraction; (C) 0.20 *M* fraction; (D) 0.25 *M* fraction; (E) 0.25 *M* fraction from B (third chromatography); (F) 0.50 *M* fraction. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **174,** 423 (1969)].

2. Gradient Elution

Using the gradient elution procedure, denatured DNA from animal tissues or bacteria is eluted in one main fraction at 0.12–0.14 *M* KP, followed by a smaller fraction at about 0.20–0.22 *M* (Figs. 8A and 10A). The chromatographic validity of these fractions can be demonstrated by rechromatography experiments (see, for example, Fig. 8B). The two fractions shown by gradient elution are equivalent to those eluting below and above 0.20 *M*, respectively, in the stepwise chromatography.

If denatured DNA from animal tissues or bacteria is reacted with formaldehyde and then eluted from HA columns by molarity gradients of KP containing formaldehyde, the elution pattern obtained is very similar to that just described, with the only difference that the amount of material eluting at the molarity of native DNA is now reduced in amount (Fig. 9B; compare, also, Fig. 10A), a result not unexpected in view of the similar findings obtained by stepwise elution. Also in agreement with the stepwise elution results, is the finding that the chromatographic behavior of native

DNA treated with formaldehyde and eluted by a molarity gradient of KP containing formaldehyde (Fig. 8A) does not differ from that of native DNA run in the absence of formaldehyde.

3. Recovery of Denatured DNA

Recovery from the columns is often found to be incomplete, and yields of only 50–80% are not rare. Several findings suggest that low recoveries may be due to aggregations of denatured DNA molecules, mediated by residual protein and/or intermolecular base pairing: (a) Repeated deproteinization treatments of native DNA samples from bacteria or higher organisms with chloroform plus isoamyl alcohol improves the recovery from the columns of these samples after they have been denatured; since this treatment shears at the same time as it deproteinizes DNA, it is impossible to decide whether a decrease in molecular weight or deproteinization, or both, are responsible for the better yields obtained in this case. (b) Treatment of denatured DNA with formaldehyde improves the recovery. (c) Chromatography of DNA denatured by heat in the presence of formaldehyde gives, as a rule, complete recoveries. (d) Raising the ionic strength of the DNA solutions up to 2–3 just before cooling (or neutralizing if alkali denaturation was used) raises the recovery of denatured DNA to over 90%.[43] (e) Low recoveries predominantly affect the first large fraction of single-stranded molecules (see below).

B. The Nativelike Fraction of Denatured DNA

Both stepwise and gradient elution results show that the bulk of denatured DNA from either bacteria or animal tissues is eluted at a lower phosphate molarity than native DNA. The properties of the main fraction of denatured DNA are those of single-stranded DNA: (1) its melting curve shows a slow continuous increase of A_{260} and a low hyperchromicity, 10–15%[16]; (2) its reaction with formaldehyde at room temperature is complete and no further increase is obtained upon heating[16]; (3) its buoyant density is 15 mg/cm^3 higher than that of native DNA[43]; (4) its electron-microscopic appearance is that of single-stranded DNA[14]; (5) its chromatographic behavior on HA is similar to that of single-stranded DNA from ϕX174 phage; (6) in the case of transforming DNA, its biological activity is extremely low.[13,14]

In contrast, a small fraction of denatured DNA is eluted at the same phosphate molarity as native DNA. This fraction, first recognized several years ago,[11] has been called "nativelike" since its properties are similar to those of native DNA.[12–14,16] The existence of a nativelike fraction in denatured DNA's has been confirmed by independent work from Doty's lab-

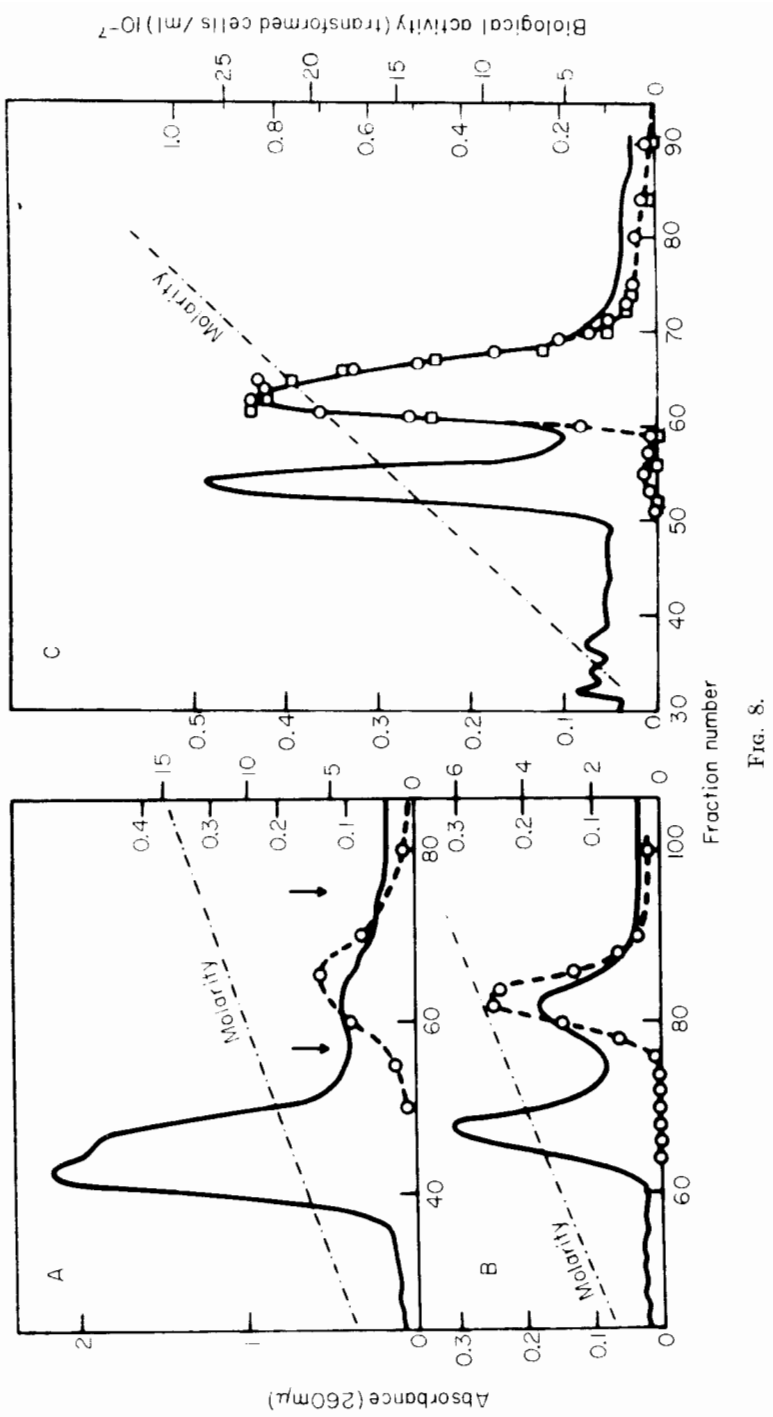

FIG. 8.

oratory,[45–48] where it was isolated from both bacterial and animal tissues using the aqueous dextran-polyethylene glycol two-phase system of Albertsson,[49] and also by Walker and McLaren,[50] who prepared it according to the procedure described from sonicated mouse DNA. As just mentioned, the properties of the nativelike DNA fraction are similar to those of native DNA: (1) its melting curve shows a sharp increase in A_{260} in the 80°–100° range; yet some increase takes already place in the 50°–80° range, and the total hyperchromicity is only 25%[14,16]; (2) its reaction with formaldehyde at room temperature is very slight, whereas heating the DNA solution in the presence of formaldehyde elicits a hyperchromic shift of about 25%[16]; (3) its chromatographic behavior on HA is that of double-stranded DNA, from which it cannot be separated, at least at the level of resolution used (Fig. 8C); (4) in the case of *H. influenzae* DNA, its microscopic appearance is that of double-stranded DNA with, however, frequent single-stranded ends[14]; (5) in the case of *H. influenzae* DNA, the nativelike fraction is the

[45] R. Rownd, D. M. Green, R. Sternglanz, and P. Doty, *J. Mol. Biol.* **32,** 369 (1968).
[46] B. M. Alberts and P. Doty, *J. Mol. Biol.* **32,** 379 (1968).
[47] B. M. Alberts, *J. Mol. Biol.* **32,** 405 (1968).
[48] C. Mulder and P. Doty, *J. Mol. Biol.* **32,** 423 (1968).
[49] P. A. Albertsson, *Arch. Biochem. Biophys.*, Suppl. 1, 264 (1962).
[50] P. M. B. Walker and A. McLaren, *Nature (London)* **208,** 1175 (1965).

FIG. 8. (A) Chromatography of alkali-denatured *Haemophilus influenzae* DNA; 200 ml of a DNA solution (sample N_2A; 37 μg/ml in 0.15 *M* NaCl plus 0.01 *M* phosphate, pH 7.0) was adjusted to pH 12.8 with 5 *N* NaOH at room temperature. After about 10 minutes at this pH, the solution was neutralized with 2 *M* KH_2PO_4, diluted with 0.15 *M* NaCl plus 0.01 *M* phosphate, pH 7.0, to 200 ml and loaded on a 1 × 20 cm column. Elution was carried out with a linear molarity gradient (150 + 150 ml) of KP (0.001–0.5 *M*, inner scale); 2.4-ml fractions were collected. Recovery of both A_{260} and biological activity (cathomycin marker, shown as circles) was 51%. (B) Rechromatography of fractions 57–75 from previous chromatogram (pooled and brought to 400 ml with 0.15 *M* NaCl plus 0.01 *M* phosphate, pH 7.0) on a 1 × 10 cm column. Elution was carried out with a linear molarity gradient (100 + 100 ml, inner scale) of KP; 2.4-ml fractions were collected. Recovery of A_{260} was 60%, of biological activity 49%. (C) Chromatography of a mixture of native (streptomycin marker) and heat-denatured (cathomycin marker) *H. influenzae* DNA; 31 ml of a DNA solution (sample N_2; 25 μg/ml in 0.15 *M* NaCl–0.01 *M* phosphate, pH 7.0) were heat-denatured, added to 5 ml of a native DNA solution (sample S; 75 μg/ml in 0.15 *M* NaCl–0.01 *M* phosphate, pH 7.0), and loaded on a 2 × 15 cm column. Elution was carried out with a linear molarity gradient (100 + 100 ml) of KP (0.001 to 0.5 *M*) 2.7-ml fractions were collected. Circles indicate the cathomycin activity (right-hand, inner scale); squares indicate the streptomycin activity (right-hand, outer scale). The elution molarity of the first peak was 0.14 *M*; that of the second peak, 0.21 *M*. Recovery of A_{260} was 76%; recovery of streptomycin activity 76%; recovery of cathomycin activity 62%. Biological activity was tested at a DNA concentration of 0.05 μg/ml. Reproduced from M. R. Chevallier and G. Bernardi [*J. Mol. Biol.* **32,** 437 (1968)].

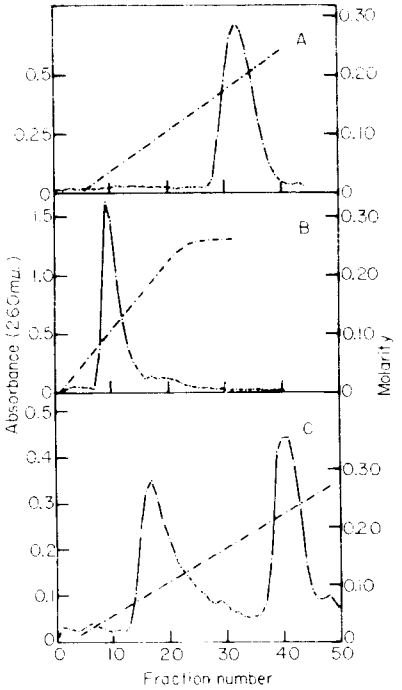

FIG. 9. Chromatography of calf thymus DNA (preparation B3) on 1.3 cm × 7 cm columns. DNA solution, 10–20 ml (A_{260} in the 1–2.5 range) was loaded at fraction number zero; 3.8 ml fractions were collected. Recoveries were 100%, except where otherwise stated. Gradient elution in the presence of 1% formaldehyde of (A) native DNA; (B) heat-denatured DNA; recovery, 95%; (C) a 1:1 mixture of native and heat-denatured DNA. Reproduced from G. Bernardi [*Nature (London)* **206,** 779 (1965)].

carrier of the residual transforming activity, surviving denaturation, exhibited by all tested genetic markers.[13,14]

The different properties of the nativelike fraction compared to native DNA are due, in part, to the presence of contaminating single stranded molecules from the main fraction, and in part to real structural differences.[14,16] It should be mentioned here that, if DNA is denatured in the presence of formaldehyde, no nativelike fraction appears in the chromatogram.

Even if the properties of the nativelike fraction from bacterial and animal sources are similar enough to be described together, their origin is different. In the case of bacterial DNA's, the available evidence[13,14,15,48] suggests that the nativelike fraction is formed by fragments of the bacterial genome whose strands never came apart completely because of interstrand cross-links of unknown origin. Two explanations have been suggested for the origin of the cross-links[47]: (a) a biological origin within the cell and (b)

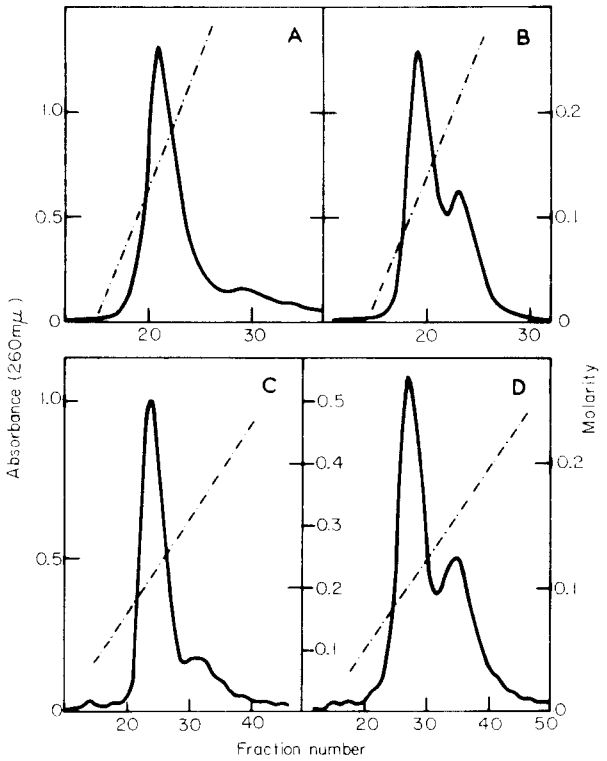

FIG. 10. Chromatography of denatured salmon sperm DNA. (A) Intact DNA. (B) DNA treated with mustard gas (230 moles/20,000 nucleotides). Both samples were dissolved in 5 × 10^{-4} KP at a concentration of 2 mg/ml; after 48 hours at room temperature, the samples were diluted to 50 ml with double-distilled water and kept 30 minutes at 37°. After cooling to room temperature, samples were made 0.15 *M* in NaCl by addition of 1 *M* NaCl. The addition of 1 *M* NaCl caused a decrease in the $A_{260\ m\mu}$ of the samples of 19%. Both samples were then treated with 1% formaldehyde and chromatographed in the presence of formaldehyde. In both runs, 27-ml solutions of DNA ($A_{260\ m\mu}$ = 0.910 for A; $A_{260\ m\mu}$ = 0.725 for B) were loaded on 1 cm × 10 cm columns. One hundred milliliters of each 10 m*M* KP was used for the gradient elution. The experiments shown in C and D also concern intact and alkylated salmon sperm DNA, respectively; the only difference with those reported in A and B is that formaldehyde treatment was omitted. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **174,** 435 (1969)].

the induction by shearing during the preparation of DNA. These explanations are not mutually exclusive. The cross-links present in bacterial DNA's might have indeed a biological origin and exist prior to the DNA extraction (see Section V, E for one possible explanation). In addition, some cross-links might be the result of chemical reactions caused by shearing occurring during DNA preparation.[47] This latter possibility is supported by the fact

that this type of artifact has been also found in mammalian DNA's[47] (see also below) and in sheared DNA from SP82[47]. As to the chromatographic behavior of DNA which has been cross-linked *in vitro* and then denatured, see Section V, C.

In the case of DNA's from animal cells, the nature and the origin of the nativelike fraction is much more complex. In calf-thymus DNA, for instance, the fast-renaturing satellite DNA's are present in the nativelike fraction[16,43]; these molecules undergo strand separation during the renaturation process, but this is followed by a rapid reassociation, very probably due to the repetitive nucleotide sequences[51] they contain. Similarly, the presence of satellite DNA has been reported in the nativelike fraction of denatured mouse DNA.[52] Other DNA molecules which renature rapidly, like mitochondrial DNA's, might be present as well in the nativelike fraction. Two other species of DNA molecules appear to be present in the nativelike fraction: (i) DNA molecules that have been cross-linked *in vivo* or during the extraction procedure[43,46,53] and (ii) single-stranded molecules having a base composition, and consequently a secondary structure, such that their elution molarity is particularly high[43] (see also Section V, D).

C. Chromatography of Denatured, Cross-linked DNA and Renatured DNA

1. Denatured, Cross-linked DNA

The results obtained by running salmon sperm DNA, cross-linked by mustard gas, and then denatured are shown in Fig. 10. A much larger amount of DNA was eluted at the molarity of native DNA in the cross-linked samples compared to the untreated samples. In both cases, reaction with formaldehyde reduced the amount of material eluting like native DNA.

In another series of experiments, *Micrococcus lysodeikticus* (*M. luteus*) DNA was cross-linked by treatment with nitrous acid at pH 4.2 for various periods of time and then heat-denatured. In this case the amount of material eluted at the molarity of native DNA increases with increasing contact time with the cross-linking agent.[16]

2. Renatured DNA

As expected, bacterial renatured DNA showed the chromatographic behavior of native DNA, except for the presence of some residual denatured material eluting at a lower molarity.[16]

[51] M. Waring and R. J. Britten, *Science* **154,** 791 (1966).
[52] P. M. B. Walker and A. McLaren, *Nature (London)* **221,** 771 (1969).
[53] A. McLaren, *Mol. Gen. Genet.* **104,** 104 (1969).

D. Fractionation of Denatured DNA

Denatured DNA molecules are fractionated by HA according to their average base composition.[12,16] Experiments carried out on formaldehyde-reacted, denatured calf thymus and chicken erythrocytes DNA's by stepwise elution have shown higher levels of A and T in the low-eluting fractions and lower levels of A and T in the high-eluting fractions. A and T, and G and C, respectively, are not present in equimolar amounts in the fractions eluting below 0.20 *M*. The results obtained with formaldehyde-reacted polyribonucleotides (see Section VI, D) suggest that this fractionation of single-stranded DNA molecules is due to the fact that they have slightly different structures as a consequence of their different base sequences (see also Section IX). It should be remarked that the nativelike fraction of calf thymus DNA fits by chance the trend of increasing GC contents with increasing elution molarity shown by single-stranded DNA, since it is mainly formed by satellite DNA's having a higher GC contents than the main DNA. This coincidence and the fact that our early analytical results on the fractions indicated equimolar amounts of A and T, and G and C, respectively, led us to suggest that the fractionation of denatured DNA was a fractionation of double-stranded molecules containing different amounts of disordered A-T rich regions.[12]

E. Separation of Native and Denatured DNA

1. Separation at Room Temperature

Because of their widely different elution molarities, native and denatured DNA can be easily separated by either stepwise or gradient elution, in the absence or in the presence of formaldehyde. Examples of separations of artificial mixtures of native and denatured DNA's are shown in Figs. 8C and 9C. Obviously, in these cases, the nativelike fractions of denatured DNA are eluted together with native DNA, at least under the low-resolution conditions used in the experiments shown.

Separations of native and denatured DNA at room temperature, using the gradient elution technique, have been performed in several laboratories, particularly in connection with problems related to DNA replication. Okazaki *et al.*[54–56] and Oishi[57–59] have reported that newly synthesized DNA

[54] R. Okazaki, T. Okazaki, K. Sakabe, K. Sugimoto, and A. Sugino, *Proc. Nat. Acad. Sci. U.S.* **59,** 598 (1968).
[55] K. Sugimoto, T. Okazaki, and R. Okazaki, *Proc. Nat. Acad. Sci. U.S.* **60,** 1356 (1968).
[56] K. Sugimoto, T. Okazaki, Y. Imae, and R. Okazaki, *Proc. Nat. Acad. Sci. U.S.* **63,** 1343 (1969).
[57] M. Oishi, *Proc. Nat. Acad. Sci. U.S.* **60,** 329 (1968).
[58] M. Oishi, *Proc. Nat. Acad. Sci. U.S.* **60,** 691 (1968).
[59] M. Oishi, *Proc. Nat. Acad. Sci. U.S.* **60,** 1000 (1968).

from *E. coli* and *B. subtilis* is in the form of small pieces about 1000 nucleotides long and that at least some of this DNA is single-stranded rather than double-stranded. Similar results have been reported by Painter and Schaefer[60] and Habener *et al.*[61] for newly replicated HeLa cell DNA. Pauling and Hamm[62] have provided evidence that newly replicated *E. coli* DNA exists temporarily in a form that rapidly renatures following heat-denaturation and perhaps represents the forked molecule postulated as an intermediate in DNA replication. It would be interesting to know whether any relationship exists between this fast-renaturing material and the nativelike fraction of bacterial DNA (see Section V, B). HA chromatography has also been used by Lark[63] and Schandl and Taylor[64] to investigate the chromosome replication in *E. coli* and Chinese hamster cells, respectively. Other examples of separations of native from denatured DNA can be found in investigations on phage DNA's by Thomas *et al.*[65-67] Along a different line of research, Zimmermann *et al.*[68] have developed an assay method for DNA ligase based on the separation of denatured λDNA from denatured λDNA which has become covalently bound to cross-linked λDNA.

2. *Separation at High Temperature (50°–70°)*

As to be expected from the results just mentioned, the separation of denatured and native DNA on HA columns can take place at any temperature below the melting range of DNA.[25] Fractionation at 50°-60° has been intensively used[69-71] to investigate the reassociation kinetics of DNA's from different sources. In a typical experiment (Fig. 11A) DNA is sheared (and simultaneously denatured) by passing it twice through a needle with a pressure drop of 3.4 kilobars, and incubated at concentrations ranging from 2 to 8600 μg/ml at 60° in 0.12 *M* phosphate; at various times, samples are passed over a HA column kept at 60° and equilibrated with 0.12 *M* NaP to separate nonretained single-stranded fragments from adsorbed fragments which contain double-stranded regions. The reassociation kinetics measured

[60] R. B. Painter and A. Schaefer, *Nature* **221,** 1215 (1969).
[61] J. F. Habener, B. S. Bynum, and J. Shack, *Biochim. Biophys. Acta* **186,** 412 (1969).
[62] C. Pauling and L. Hamm, *Biochem. Biophys. Res. Commun.* **37,** 1015 (1969).
[63] K. G. Lark, *J. Mol. Biol.* **44,** 217 (1969).
[64] E. K. Schandl and J. H. Taylor, *Biochem. Biophys. Res. Commun.* **34,** 291 (1969).
[65] M. Rhoades, L. A. MacHattie, and C. A. Thomas, Jr., *J. Mol. Biol.* **37,** 21 (1968).
[66] M. Rhoades and C. A. Thomas, Jr., *J. Mol. Biol.* **37,** 41 (1968).
[67] M. S. Reznikoff and C. A. Thomas, Jr., *Biology* **37,** 309 (1969).
[68] S. B. Zimmerman, J. W. Little, C. K. Oshinsky, and M. Gellert, *Proc. Nat. Acad. Sci. U.S.* **57,** 1841 (1967).
[69] R. J. Britten and D. E. Kohne, *Carnegie Inst. Wash. Yearb.* **65,** 73 (1966).
[70] R. J. Britten and D. E. Kohne, *Carnegie Inst. Wash. Yearb.* **66,** 73 (1967).
[71] R. J. Britten and D. E. Kohne, *Science* **161,** 529 (1968).

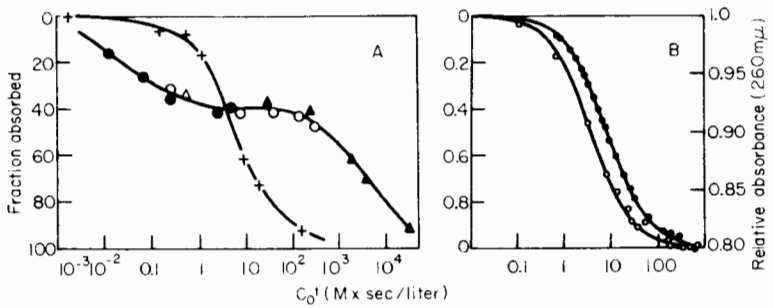

FIG. 11. (A) The kinetics of reassociation of calf thymus DNA measured with hydroxyapatite. The DNA was sheared at 50,000 psi and incubated at 60° in 0.12 *M* PB. At various times, samples were diluted, if necessary (in 0.12 *M* PB at 60°), and passed over a hydroxyapatite column at 60°. The DNA concentrations during the reaction were △, 2; ●, 10; ○, 600; ▲, 8600 μg/ml; +, radioactively labeled *E. coli* DNA at 43 μg/ml present in the reaction containing calf thymus DNA at 8600 μg/ml. Reproduced from R. J. Britten and D. E. Kohne [*Carnegie Inst. Wash. Yearb.* **66,** 73 (1967)]. (B) The time course of reassociation of sheared (50,000 psi) *E. coli* DNA. Solid circles and right scale decrease in optical density at 260 mμ; open circles and left scale binding to hydroxyapatite. Reproduced from R. J. Britten and D. E. Kohne [*Carnegie Inst. Wash. Yearb.* **65,** 73 (1966)].

in the way just described parallels the optical reassociation kinetics (Fig. 11B), yet appears to progress more rapidly, the half-reaction time being higher by a factor of 2 to 3. The discrepancy has been explained as due to the fact that the optical method measures the fraction of the length of the DNA that is actually paired, whereas HA measures the fraction of the total number of DNA fragments that have some portion of their length reassociated. Reaction with formaldehyde at room temperature[16] of the reassociated fraction would probably permit to check whether the rate overestimation obtained from the HA values is due only to this factor. A batch procedure for the thermal elution of DNA from HA has been described by Brenner *et al.*[72]

Contrary to the opinion expressed by McCallum and Walker,[73] there is no essential difference between the results obtained at 50°–70° and those obtained at room temperature, particularly if chromatography is performed after reaction with formaldehyde, using formaldehyde-containing buffers.

Szala and Chorazy[74] have fractionated calf thymus DNA treated at different temperatures within the melting range on HA columns thermostated at 3°–5° lower than the temperature used in the partial denaturation. Ex-

[72] D. J. Brenner, C. R. Fanning, A. V. Rake, and K. E. Johnson, *Anal. Biochem.* **28,** 447 (1969).
[73] M. McCallum and P. M. B. Walker, *Biochem. J.* **105,** 163 (1967).
[74] S. Szala and M. Chorazy, *Bull. Acad. Polon. Sci.* **17,** 277 (1969).

cept for the fact that columns were thermostated at relatively high temperature, the approach is that used in previous experiments by Bernardi[12]; (see Fig. 6). Accordingly, the results are the same: i.e., as the denaturation temperature is increased, the amount of DNA eluting at the position of denatured DNA increases. As expected, the fraction eluted as native DNA, being enriched in the higher melting molecules, shows an increase in its T_m as the fractionation is performed at increasingly higher temperatures.

F. Thermal Chromatography

A very interesting application of the ability of HA to discriminate native and denatured DNA is the "thermal chromatography".[25] In a typical experiment, sonicated native DNA (MW = 200,000) is adsorbed on a HA column; this is then percolated by a phosphate buffer (80 m*M* NaP) able to elute denatured, but not native DNA; at the same time the temperature of the column is increased in steps to slightly over 100°. At each temperature within the melting range, a certain fraction of the double-stranded segments is denatured and washed through the column. As expected, fractions eluted at lower temperatures are richer in A and T, whereas those eluted at higher temperatures are richer in G and C; all fractions display a molar equivalence of A and T, and G and C, respectively. The resulting chromatogram as a function of temperature (Fig. 12A) proves to be almost exactly the derivative of the optical melting curve (Fig. 12B). The slightly higher T_m shown by the thermal chromatogram has been ascribed to the higher effective ionic strength prevailing in the neighborhood of the highly charged HA crystal. Figure 12C shows that the mole fraction G + C of the fractions is generally proportional to temperature, showing a variation of 2.0% per degree, a value which is to be compared with 2.4% per degree obtained by optical melting of a large number of DNA's from different species.[75] In view of the significant differences in solvent and molecular weight between the two experiments, the apparent agreement between these two numbers may be fortuitous. Several reservations have been made by Miyazawa and Thomas[25] to the general conclusion that thermal chromatograms are direct reflections of the heterogeneity of composition existing among the various segments: (1) The form of the thermal chromatograms depends on the length of the duplex segments; this is not surprising since this is in the range of strong dependence of T_m on fragment size. (2) When nucleotide analysis is made on the fractions in the leading edge of the thermal chromatogram which contain less than 1% per degree, the mole fraction G + C departs from the line drawn in Fig. 12C; this probably represents a nonspecific leakage of material which was damaged by sonic

[75] J. Marmur and P. Doty, *J. Mol. Biol.* **5**, 109 (1962).

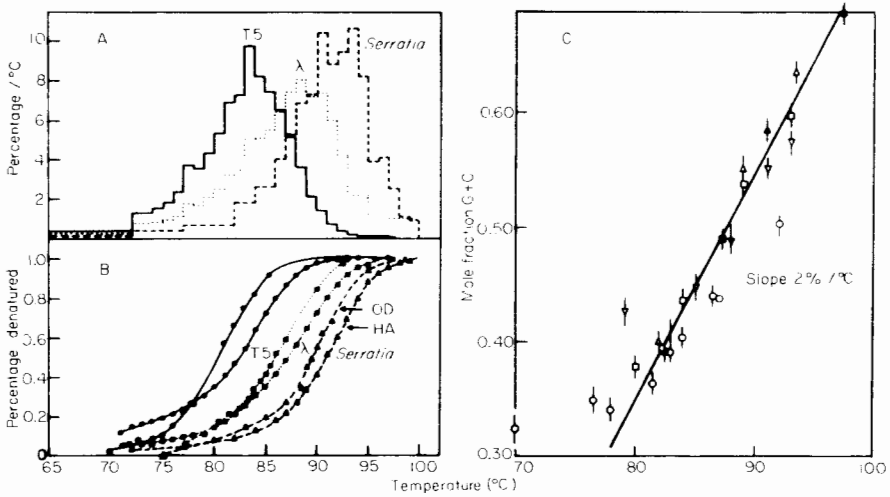

FIG. 12. (A) Thermal chromatograms of three different DNA's. The results with T2 DNA are superimposable with those of T5. Those with *Haemophilus* DNA are very similar to T5 and T2 DNA (B) Optical melting curves plotted with integral form of thermal chromatograms. Notice that the optical melting curves and the integral form of the thermal chromatograms shown in A are very similar. (C) Results of nucleotide analysis. The observed mole fraction of G + C is plotted against the mean value of the temperature over which they were collected. The solid points represent results obtained on sonic fragments which were not subjected to thermal chromatography. Nucleotide analysis made of fractions coming from the low-temperature edges of the chromatogram which contain less than 1% per degree centigrade, give G + C values which fall above the line. ○, T5 fractions; ●, T5 unfractionated; □, λ fractions; ■, λ unfractionated; △, *Serratia* fractions; ▲, *Serratia* unfractionated; ▽, *Escherichia coli* fractions; ▼, *E. coli* unfractionated. Reproduced from Y. Miyazawa and C. A. Thomas, Jr. [*J. Mol. Biol.* **11**, 223 (1965)].

treatment, or exceptionally short fragments which would melt at a lower temperature. (3) Repeated thermal chromatography and nucleotide analysis of T5 DNA segments generate points which fall on a line of lower slope; these observations are statistically significant and may indicate that there are still other effects not known which affect the temperature-composition relation as seen in these experiments. In spite of these reservations, Fig. 12C shows that it is possible to fractionate and identify segments having compositions which are quite far removed from the mean value for a given DNA; for instance, T5 DNA produces segments which have G + C contents ranging from 32 to 50%, λDNA from 38 to 60%, *Serratia* DNA from 40 to 69% and *E. coli* from 42 to 57%. T2 DNA and *H. influenzae* DNA show thermal chromatograms identical to that of T5 DNA.

The most interesting result obtained by thermal chromatography obvi-

ously is the demonstration of the compositional heterogeneity of relatively long DNA segments. The technique has been also used, however, to obtain other information.

The specific association of ribooligonucleotides of known chain length synthesized by RNA polymerase on T4 or T7 DNA with denatured T4 or T7 DNA's has been investigated by thermal chromatography.[76] The results obtained indicate that in experiments testing interspecies homology by RNA-DNA hybridization about 12 perfectly complementary nucleotides must be involved in the complex. Thermal chromatography has also been used to investigate the stability of oligoadenylate plus polyuridylate complexes.[77] The temperature of release from HA was found to be a function of the chain length of the oligonucleotides, the presence of Mg^{2+} and the presence or absence of terminal phosphates on the oligonucleotides; unfortunately it was impossible to decide whether 1A:1U or 1A:2U structures predominated at the surface of the HA crystals. Other recent applications of thermal chromatography have concerned the preparation of poly-(dAT:dAT) from crab DNA,[78] the thermal stability of reassociated DNA[71,79] and the characterization of poliovirus-specific double-stranded RNA.[80]

VI. Chromatography of RNA's and Synthetic Polyribonucleotides

Investigations on the chromatography of RNA's and synthetic polyribonucleotides have been much less extensive than those on DNA's. This is particularly regrettable if one considers the multiplicity of secondary structures that exist among natural and synthetic polyribonucleotides and the potential usefulness of HA columns in investigating those structures.

A. Chromatography of Ribosomal and Viral RNA's

Using the stepwise elution technique, the ribosomal RNA's from yeast and from Ehrlich ascites tumor cells, and the RNA's from tobacco mosaic virus, turnip yellow mosaic virus, and alfalfa mosaic virus are all eluted in two peaks at 0.15 *M* and 0.20 *M* KP (Fig. 13A). When elution is carried out with a molarity gradient, high-molecular-weight RNA's are all eluted as single peaks (Fig. 13B), at a molarity close to 0.15 *M* KP. These results suggest that the two peaks obtained with high-molecular-weight RNA's are "false" peaks.

No detailed investigation has been done, so far, on the dependence of

[76] S. N. Niyogi and C. A. Thomas, Jr., *Biochem. Biophys. Res. Commun.* **26,** 51 (1967).
[77] S. N. Niyogi and C. A. Thomas, Jr., *J. Biol. Chem.* **243,** 1220 (1968).
[78] A. Brzezinski, P. Szafranski, P. H. Johnson, and M. Laskowski, *Biochemistry* **8,** 1228 (1969).
[79] B. L. McConaughy, C. D. Laird, and B. J. McCarthy, *Biochemistry* **8,** 3289 (1969).
[80] J. M Bishop, G. Koch, and L. Levintow, *in* "The Molecular Biology of Viruses" (J. S. Colter and W. Paranchych, eds.), p. 355. Academic Press, New York, 1967.

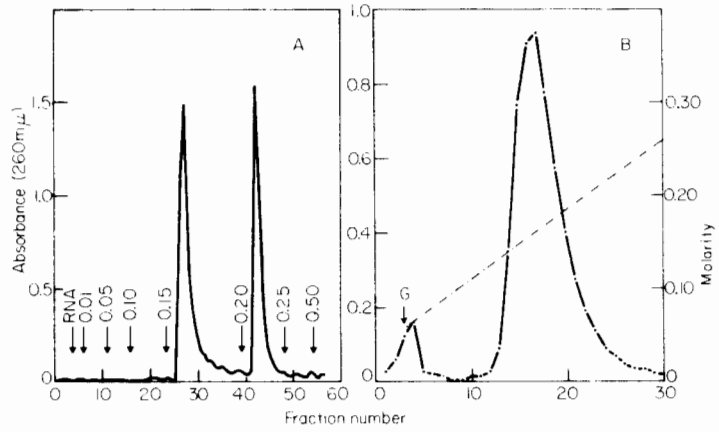

FIG. 13. (A) Chromatography of ribosomal RNA from Ehrlich ascites tumor on a 1.3 × 6 cm column. Eight milliliters of an RNA solution having an $A_{260\,m\mu} = 3.12$ were loaded on the column at fraction No. 4; 3.2-ml fractions were collected, using the stepwise elution technique. Recovery was 100%. (B) Chromatography of 20.8 $A_{260\,m\mu}$ units of ribosomal RNA from yeast on a 1 cm × 10 cm column. Loading took place at fraction number zero. A molarity gradient of KP was started at the fraction marked *G*; 2.9-ml fractions were collected. Recovery was 91%. This experiment was performed at 4°. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **174,** 449 (1969)].

the elution molarity of high-molecular-weight RNA's upon temperature or molecular weight. No supporting evidence was obtained in our laboratory in favor of the claim[81,82] that HA columns fractionate high-molecular-weight RNA's on the basis of molecular weight.

It should be pointed out that in several cases the sedimentation coefficient of high-molecular-weight RNA's eluted from HA were found to be lower than those of the starting materials. This decrease in sedimentation coefficients seems to be due to the fact that HA "disaggregates" the large polynucleotide fragments forming RNA "molecules" which contain "hidden breaks." That HA columns do not cause by themselves any breakage of large RNA molecules is shown by the good recovery of infectivity obtained when viral RNA's are chromatographed on HA.[83,84]

B. Chromatography of Double-Stranded RNA

In contrast with the claim that the replicative intermediate of viral RNA's are not separated from ribosomal RNA on HA columns,[85] both the

[81] A. D. Vizoso and A. T. H. Burness, *Biochem. Biophys. Res. Commun.* **2,** 102 (1960).
[82] A. T. H. Burness and A. D. Vizoso, *Biochim. Biophys. Acta* **49,** 225 (1961).
[83] L. Pinck, Ph.D. Thesis, Univ. of Strasbourg, 1969.
[84] F. Brown, J. F. E. Newmann, and D. L. Stewarts, *Nature (London)* **197,** 590 (1963).
[85] R. M. Franklin, *Proc. Nat. Acad. Sci. U.S.* **55,** 1504 (1966).

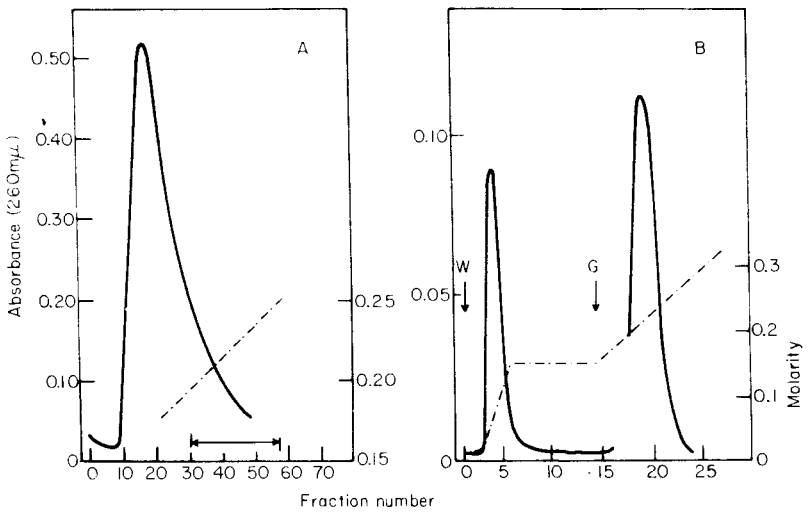

FIG. 14. (A) Chromatography of total RNA from tobacco leaves harvested 11 days after infection with alfalfa mosaic virus. Twenty milligrams was loaded on a 2 × 15 cm column equilibrated with 1 m*M* KP. The column was then washed with 0.15 *M* KP until the ultraviolet absorption of the eluent was negligible. A molarity gradient (0.15–0.30 *M* KP) was applied to the column at fraction zero; 5-ml fractions were collected. Fractions indicated by the arrow were rechromatographed. (B) Rechromatography of fractions eluted by 0.20–0.25 *M* KP of total RNA from tobacco leaves harvested 8 days after infection; 1.4 A_{260} units were loaded on a 1 × 10 cm column, equilibrated with 1 m*M* KP. The column was then washed with 0.15 *M* KP (arrow *W*) until the ultraviolet absorption of the effluent was negligible. A molarity gradient (0.15–0.50 *M* KP) was then applied to the column (arrow *G*); 2-ml fractions were collected. Reproduced from L. Pinck, L. Hirth, and G. Bernardi [*Biochem. Biophys. Res. Commun.* **31,** 481 (1968)].

replicative form of turnip yellow mosaic virus RNA[86] and the replicative intermediate of alfalfa mosaic virus RNA[87] can be easily separated from single-stranded RNA on HA columns, since these double-stranded RNA's are eluted only by 0.20–0.22 *M* KP, like native DNA (Fig. 14). This separation is therefore analogous to that of denatured and native DNA (see Section V, E).

C. Chromatography of Transfer RNA's

Transfer RNA's were first fractionated on HA columns using the stepwise elution procedure by Hartmann and Coy.[88] Subsequent work by several groups of investigators[89–94] led to remarkable separations (Fig. 15), that give an excellent idea of the discriminating power of HA columns with respect to the secondary and tertiary structures of rather similar molecules. It may be interesting, in this connection, to recall that, under conditions of low resolution (similar to those of Fig. 2), tRNA is eluted as a single, sharp peak.[12] Muench and Berg[91] and Dirheimer[92] used as the eluent

[86] L. E. Bokstahler, *Mol. Gen. Genet.* **100,** 337 (1967).
[87] L. Pinck, L. Hirth, and G. Bernardi, *Biochem. Biophys. Res. Commun.* **31,** 481 (1968).
[88] G. Hartmann and U. Coy, *Biochim. Biophys. Acta* **47,** 612 (1961).

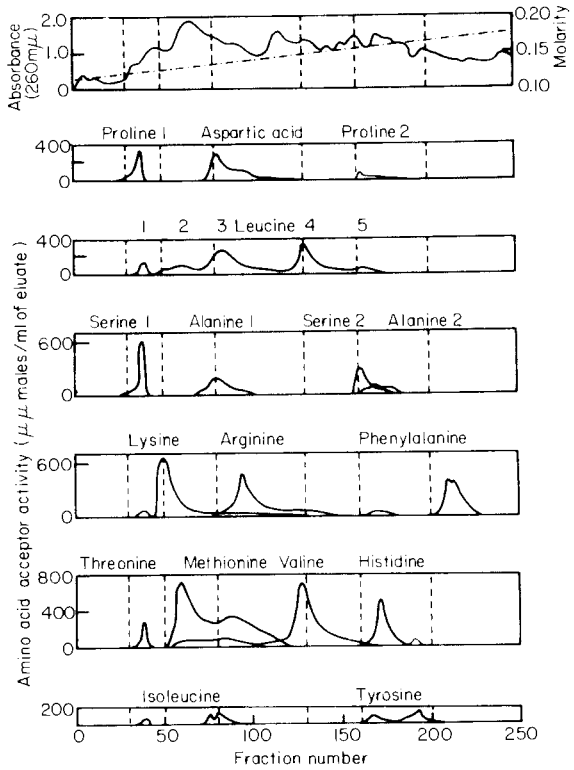

FIG. 15. Chromatography of *Escherichia coli* B tRNA (100 mg in 2 ml of 0.1 *M* NaP) on a 2 × 68 cm column at room temperature. Two liters of a NaP molarity gradient (0.1–0.2) was used at a flow rate of 12 ml/hr; 4-ml fractions were collected. Reproduced from R. L. Pearson and A. D. Kelmers [*J. Biol. Chem.* **241,** 767 (1966)].

[89] R. L. Pearson and A. D. Kelmers, *J. Biol. Chem.* **241,** 767 (1966).
[90] U. Harding, H. Schauer, and G. Hartmann, *Biochem. Z.* **346,** 212 (1966).
[91] K. H. Muench and P. Berg, *Biochemistry* **5,** 982 (1966).
[92] G. Dirheimer, *Bull. Soc. Chim. Biol.* **50,** 7 (1968).
[93] A. B. Legocki and J. Pawelkiewcz, *Bull. Acad. Polon. Sci.* **15,** 517 (1967).
[94] R. Giege, J. Heinrich, J. H. Weil, and J. P. Ebel, *Biochim. Biophys. Acta* **174,** 43 (1967).

phosphate buffer having a pH of 5.8 and 5.4, respectively; this allows isotopically labeled aminoacyl-tRNA's to be chromatographed as such. It is evident that, in combination with other available methods, HA chromatography is very useful in the separation and purification of transfer RNA's.

D. Chromatography of Synthetic Polyribonucleotides

Poly(U) is eluted at 0.10 *M* KP at 25° (Fig. 16A) under conditions where it is completely devoid of any secondary structure[95]; at 4°, where it has some

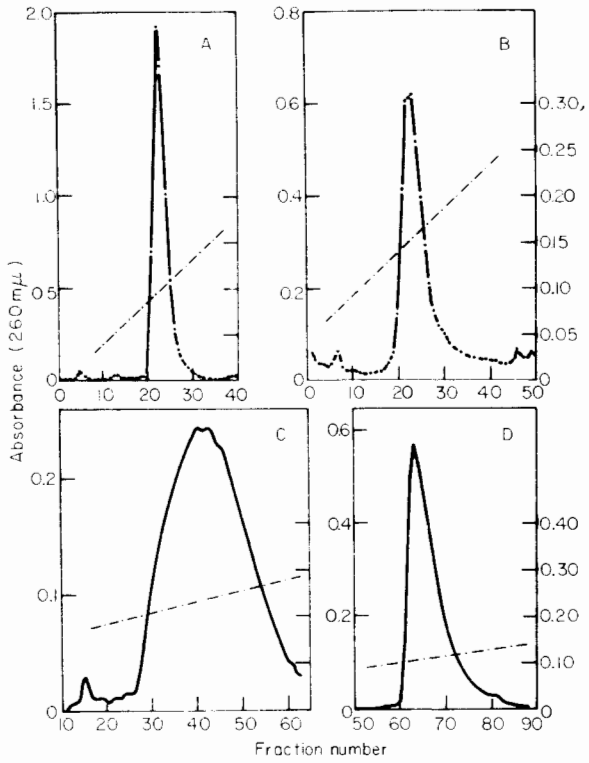

FIG. 16. Chromatography of (A) polyuridylic acid; this experiment was performed at 25°; 20 $A_{260\ m\mu}$ units were loaded; recovery was 98%; 2.9-ml fractions were collected; (B) polyuridylic acid; this experiment was performed at 4°; 6.5 $A_{260\ m\mu}$ units were loaded and recovered; (C) polyadenylic acid; experiment at room temperature; 20.9 $A_{260\ m\mu}$ units were loaded; recovery was 81%; 3-ml fractions were collected; (D) polycytidylic acid; 9.9 $A_{260\ m\mu}$ units were loaded and recovered; 2.9-ml fractions were collected. In all cases 1 × 10 cm columns were used and loading took place at fraction zero. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **174,** 449 (1969)].

[95] E. G. Richards, C. P. Flessel, and J. R. Fresco, *Biopolymers* **1,** 431 (1965).

sort of helical secondary structure (as shown by its hypochromism and increase in positive optical rotation[95,96]), poly(U) acid is only eluted by 0.15 *M* KP (Fig. 16B). Likewise, poly(C) is eluted at room temperature by 0.12 *M* KP (Fig. 16D), and by a slightly higher molarity at 4°. Recovery of poly(U) and poly(C) from the columns has always been complete.

In contrast with the two polypyrimidinic acids just mentioned, poly(I) gave very irregular results and poor recoveries and poly(A) was eluted in a broad peak around 0.2 *M* KP with recoveries of only 60–85% (Fig. 16C). It is known that, at room temperature and neutrality, poly(A) has a structure which is random with respect to total conformation, but ordered in

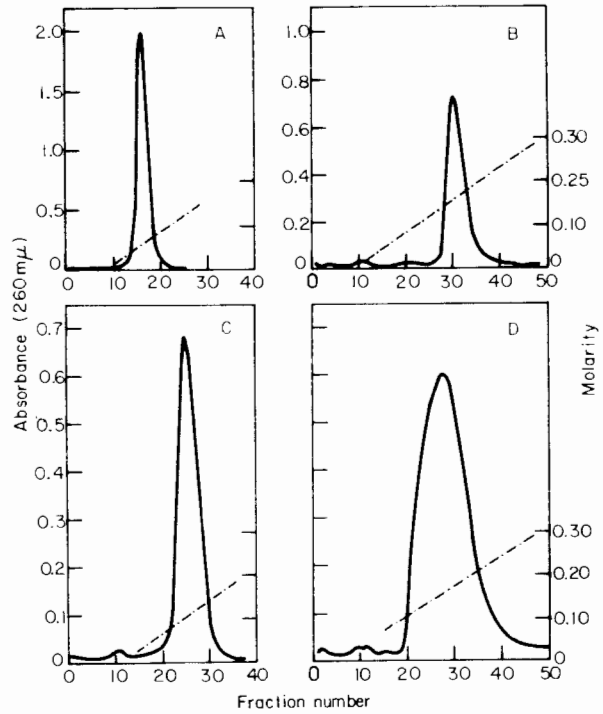

FIG. 17. Chromatography of (A) 11 $A_{260\ m\mu}$ of polycytidilic acid; (B) 23.3 $A_{260\ m\mu}$ units of polyadenylic acid; (C) 19.5 $A_{260\ m\mu}$ units of polyurydilic acid; (D) 11.7 units of polyinosinic acid. Polyribonucleotides were heated up to 100° for 5 minutes and fast-cooled in 10 m*M* KP containing 1% neutralized formaldehyde; this was also present in the eluting buffers. Columns of 1 cm × 20 cm were used; 3-ml fractions were collected. Recovery was 100% in all cases. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **174,** 449 (1969)].

[96] M. N. Lipsett, *Proc. Nat. Acad. Sci. U.S.* **46,** 445 (1960).

terms of short-range interactions, the ordered regions having a single-stranded, stacked, helical structure.[97–100]

Experiments intended to show how HA columns can discriminate among different single-stranded structures and also to help understanding the fractionation of heat-denatured and formaldehyde-reacted DNA according to its base composition, were done with synthetic polyribonucleotides heated up to 100° for 5 minutes in 10 m*M* KP containing 1% formaldehyde. Recoveries were complete in all cases, and the elution molarities were 0.05 *M* KP for poly(U), 0.12 *M* for poly(C), 0.16 *M* for poly(I) and 0.17 *M* for poly(A) (Fig. 17).

The artificial complexes of poly(U) and poly(A) were also examined under conditions where double-stranded poly(A)·poly(U) or triple-stranded

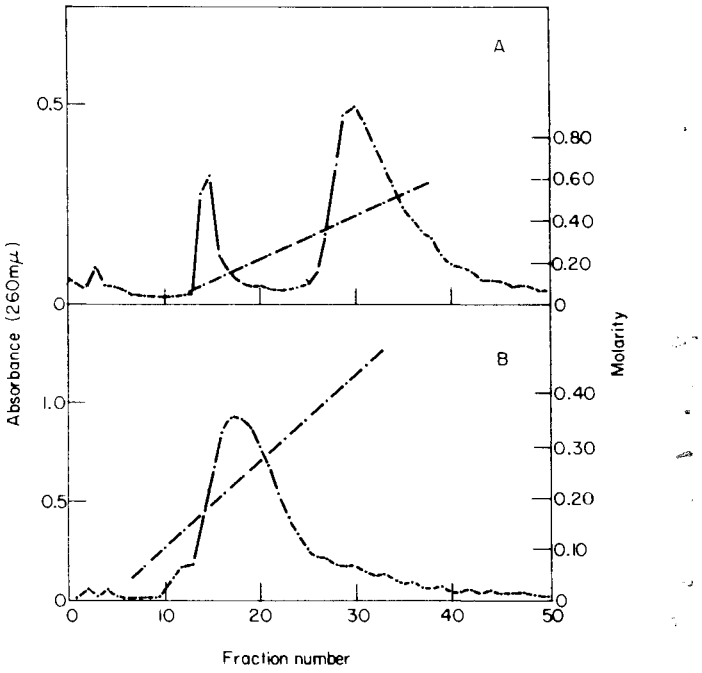

FIG. 18. Chromatography of (A) polyuridylic acid + polyadenylic acid (3:1); (B) polyuridylic acid + polyadenylic acid (1:3) on 1 × 10 cm columns. Three-milliliter fractions were collected. Recoveries were 100 and 95%, respectively. Reproduced from G. Bernardi [*Nature (London)* **206,** 779 (1965)].

[97] V. Luzzati, A. Mathis, F. Masson, and J. Witz, *J. Mol. Biol.* **10,** 28 (1964).
[98] K. E. Van Holde, J. Brahms, and A. M. Michelson, *J. Mol. Biol.* **12,** 726 (1965).
[99] M. Leng and G. Felsenfeld, *J. Mol. Biol.* **15,** 455 (1966).
[100] A. M. Michelson, J. Massoulie, and W. Guschlbauer, *Progr. Nucl. Acid Res.* **6,** 83 (1967).

2 poly(U)·poly(A) were formed. The double-stranded complex was eluted in the molarity region where the native DNA is eluted, whereas the triple-stranded complex was eluted only by 0.45–0.50 *M* KP (Fig. 18). The double-stranded poly(I)·poly(C) complex was also eluted at a molarity close to 0.20 *M* KP, like poly(A)·poly(U).[17]

VII. Chromatography of Oligonucleotides

Only a very limited number of reports have dealt so far with the chromatography of oligonucleotides. Main and Cole[5] were the first to report that the pancreatic DNase digest is eluted at very low phosphate molarities, most of the material being not retained by the column equilibrated with 5 m*M* NaP, whereas a minor peak was eluted at 45 m*M* NaP. RNA "core," the water-undialyzable fraction of the oligonucleotides formed by pancreatic RNase digestion, was mostly eluted at 15 m*M* KP, with a minor peak eluting about at 0.10 *M* KP.[12] The higher eluting molarity of the

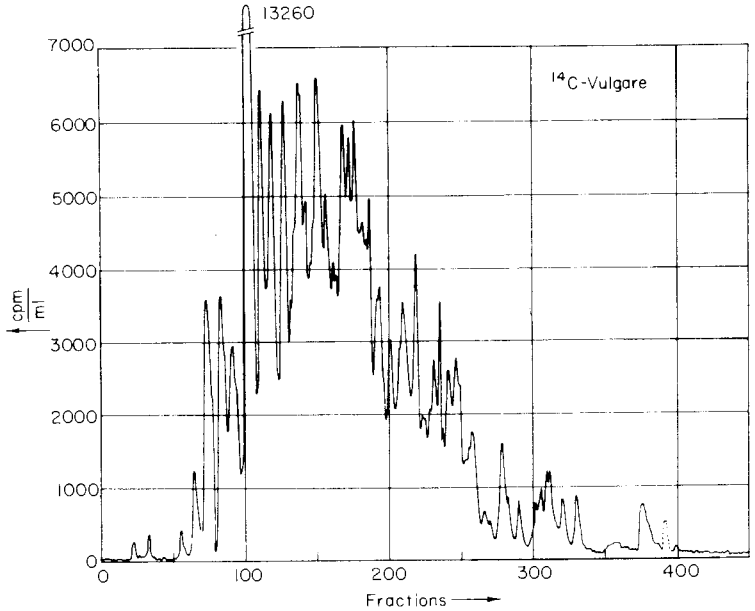

FIG. 19. Elution profile of Vulgare-TMV-RNA, hydrolyzed with T1 ribonuclease, precipitated with 1/9 volume of 20% trichloroacetic acid in absolute ethanol, and dissolved in 1 m*M* NaP plus 7 *M* urea. A 2.2 × 45 cm column was used. Elution was done with a linear molarity gradient (2 + 2 liters) of 1 m*M* NaP plus 0.2 *M* NaP, both buffers containing 7 *M* urea; 5-ml fractions were collected. Flow rate was 30 ml/hour. The dotted peak and the obvious asymmetry of the "last peak" are artifacts due to interruption of the elution by an accident. Reproduced from K. W. Mundry [*Z. Vererbungslehre* **97,** 281 (1965)].

RNA "core" compared to the pancreatic DNase digest might to be due to the larger size of the fragments and/or to their secondary structure (RNA "core" has a high purine contents).

The fractionation of large oligonucleotides on HA columns, both in the presence and in the absence of urea or formaldehyde, seems to be very promising: the results of Mundry[101,102] and ourselves[26] indicate an excellent reproducibility of the complex patterns which are obtained (Fig. 19). It is evident that large oligonucleotides are eluted in a relatively high molarity range.

VIII. Chromatography of Bases, Nucleosides and Nucleoside Mono- and Polyphosphates[103]

Purine and pyrimidine bases, ribo- and deoxyribonucleosides, and coenzyme derivatives like thiamine and riboflavin, were not retained by HA columns equilibrated with 1 m*M* KP.

Nucleoside monophosphates (2′,3′ mixed isomers and 5′ isomers of the ribo series, 3′ and 5′ isomers of the deoxyribo series), thiamine monophosphate and riboflavin 5′-phosphates were also eluted by 1 m*M* KP, but they were slightly retarded. Using long enough columns, they could be separated from nucleoside.

Nucleoside polyphosphates were strongly adsorbed and needed rather high KP molarities to be eluted. As an example, Fig. 20 shows a chromatogram obtained with a mixture of AMP, ADP, ATP, and adenosine tetraphosphate. The complete separation obtained was controlled by rechromatography experiments. The peaks were identified on the basis of their typical elution molarity by running the single compounds separately.

Similar experiments showed that other nucleoside 5′-diphosphates (CDP, UDP) and thiamine pyrophosphate were eluted at the same molarity as UDP and that other nucleoside 5′-triphosphates (deATP, CTP, UTP) were eluted like ATP. An interesting exception is that GDP and GTP, and at a lower extent, IDP and ITP, are eluted at higher phosphate molarities than the other di- and triphosphates, respectively. This finding is probably related to phenomena of intermolecular association, of the type described by Gellert *et al.* for GMP.[104]

Compounds in which the pyrophosphate has no free secondary acid group, like ADP-ribose, NAD, FAD, UDP-glucose, were not retained by the columns equilibrated with 1 m*M* KP. NADP and coenzyme A, which, besides a pyrophosphate with no free secondary acid group, have a terminal

[101] K. W. Mundry, *Z. Vererbungslehre* **97,** 281 (1965).
[102] K. W. Mundry, *Mol. Gen. Genet.* **105,** 361 (1969).
[103] G. Bernardi, *Biochim. Biophys. Acta* **91,** 686 (1964).
[104] M. Gellert, M. N. Lipsett, and D. R. Davies, *Proc. Nat. Acad. Sci. U.S.* **48,** 2013 (1962).

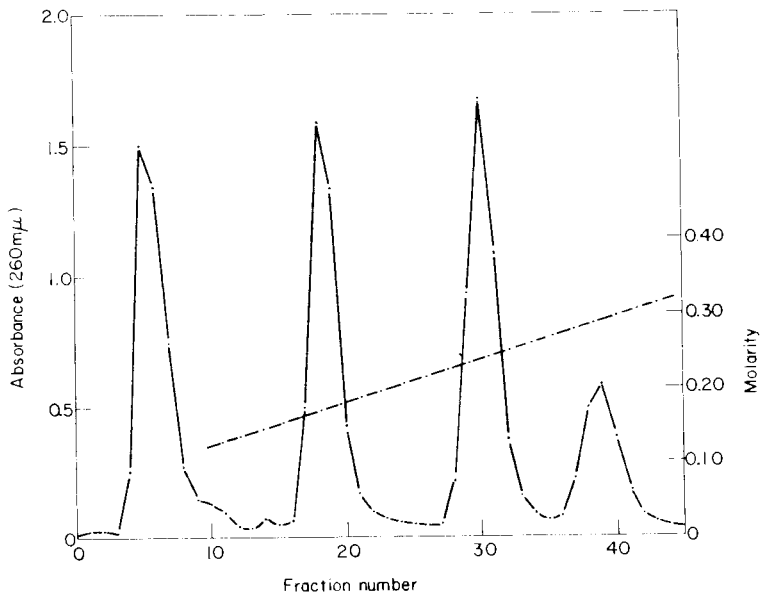

FIG. 20. Chromatography of a mixture of AMP, ADP, ATP, and adenosine tetraphosphate. Recoveries were 133, 113, and 150%, respectively, owing to the fact that ADP, ATP, and, to a much larger extent, adenosine tetraphosphate, were contaminated by the lower phosphates, as indicated by separate experiments. The overall recovery was 101%. Three-milliliter fractions were collected. Elution was carried out with a KP molarity gradient 1 m*M* to 0.5 *M* (100 + 100 ml). A 1 cm × 10 cm column was used. Reproduced from G. Bernardi [*Biochim. Biophys. Acta* **91,** 686 (1964)].

phosphate were retained slightly more than nucleoside monophosphates. Finally, phosphoribose pyrophosphate was eluted at a higher molarity than triphosphates.

These observations, while of great interest for our understanding of the mechanism of adsorption on HA, have also practical usefulness since they indicate the potentialities of HA columns for the separation of nucleic acid derivatives from nucleic acids and proteins.

IX. Mechanism of Adsorption and Elution of Nucleic Acids. Relationships between Elution Molarity and Secondary Structure of Nucleic Acids

The main factor involved in the adsorption of nucleic acids on HA seems to be the interaction between the phosphate groups of nucleic acids and calcium ions on the surface of HA crystals. This is suggested by several facts:

1. Treatment of HA with compounds having a very strong affinity for calcium, like EDTA, citrate, and polyphosphates, decrease their adsorption

capacity for nucleic acids. Compounds having a lower degree of affinity for calcium, like phosphates and several carboxylic compounds, may be used as eluents; ions having a very low affinity for calcium, like chlorides, practically do not interfere with the adsorption of nucleic acids.

2. Electrophoresis of HA prepared according to Tiselius *et al.*[1b] in 1 m*M* KP shows that HA crystals have a net positive charge. The isoelectric point was not determined; it is known from the work of Mattson *et al.*[105] that HA crystals are amphoteric and that the isoelectric point of HA varied, for different preparations examined by those authors, from 6.5 to 10.2.

3. The sedimentation rate of HA crystals in 1 m*M* KP is greatly increased by the addition of DNA; this may be due to a decrease in electrostatic repulsion among the positively charged crystals.

4. Phosphoproteins have a much higher affinity for HA than nonphosphorylated proteins.

5. Chromatography of nucleosides, some coenzymes, and their mono and polyphosphate derivatives shows that the elution molarity of these materials is dependent only upon their phosphate group(s). The nonphosphorylated derivatives are not retained by HA equilibrated with 1 m*M* KP; monophosphates are eluted by 0.001 *M* KP, but they are retarded; di-, tri-, and tetraphosphates are eluted at increasingly higher characteristic phosphate molarities, independently of the organic molecules to which they are bound.

It should be pointed out, however, that if the interaction between phosphates and calcium plays a prominant role in the mechanism of adsorption of nucleic acids, amino groups of nucleotides, when exposed, might also become involved in the interaction with HA. In this connection, it should be mentioned that mononucleotides run in 0.001 *M* NaH_2PO_4, pH 5.2, are fractionated on HA columns,[40] a finding implying that bases may be involved in the interaction with HA when their amino groups are ionized and available. It has also been shown[40] that basic polyamino acids show a strong interaction with HA.

Elution of polynucleotides from HA by phosphate appears to be due to a specific competition between phosphate ions of the eluent and phosphate groups of polynucleotides for adsorbing sites on HA and not simply to an increase in ionic strength. In fact, as mentioned in Section III, A, elution can be performed at a practically constant ionic strength, native DNA being eluted at the same phosphate molarity independently of the ionic strength of the eluting buffer. In some cases, mentioned in Section III, A, addition of chlorides to the eluting buffer causes an increase in the phosphate molarity eluting native DNA. It is likely that this effect is due to the

[105] S. Mattson, E. Kontler-Andersson, R. B. Miller, and K. Vantras, *Kgl. Lantbruks-Hoegsk. Ann.* **18,** 493 (1951), quoted by S. Larsen, *Nature (London)* **212,** 212 (1966).

repression of phosphate dissociation, rather than to a slight change in the structure of native DNA at high ionic strength; the latter phenomenon cannot, however, be ruled out.

Concerning the relationships between the elution molarities of polynucleotides and their secondary (and tertiary) structures, the results discussed so far lead to the following conclusions:

1. In the case of polynucleotides endowed with a rigid structure, it is evident that all those possessing a double-stranded structure [native and renatured DNA, replicative RNA, poly(A)·poly(U) and poly(I)·poly(C)] are eluted in the same molarity range. Differences in the elution molarities exist, however (see, for example, Section III, B), and suggest that conditions of higher resolution may lead to interesting separations. The only triple-stranded polynucleotide examined, 2 poly(U)·poly(A), is eluted at a much higher phosphate molarity (about 0.45 *M* KP). The higher elution molarity of this complex may be possibly explained by the fact that triple-stranded polynucleotides have a higher linear charge density than double-stranded polynucleotides, and therefore a larger number of phosphate groups per unit length is available for interaction with HA, and/or by the fact that the distribution of these groups in 2 poly(U) poly(A) matches the distribution of adsorbing sites on HA better than that of phosphates of double-stranded polynucleotides.

2. As far as polynucleotides endowed with a random-coiled structure are concerned, their chromatographic behavior needs to be investigated under a much wider set of experimental conditions than done so far, since their structure is strongly dependent upon ionic strength, temperature, and presence of organic reagents like formaldehyde and urea. In all cases, however, randomly coiled polynucleotides are eluted from HA columns by lower phosphate molarities than rigid, helical polynucleotides. The property of HA of discriminating these two types of structures seems to be of general validity since in the case of proteins, too, the disruption of their secondary and tertiary structures causes a drastic decrease in their affinity for HA. An explanation for this phenomenon is that the groups which were available for the interaction with adsorbing sites on HA in the rigid, ordered structures, greatly decrease in number on the "outer surface" of the randomly coiled, denatured nucleic acids or proteins. A similar explanation may hold for the decreased affinity for HA observed for the twisted circular form compared to both the linear open and the linear circular forms of polyoma virus DNA (see Section III, B). Furthermore, local concentrations of interacting sites due to the existence of rigid secondary and tertiary structures disappear upon denaturation. An example of the importance of charge distribution is given by the chromatographic behavior of nucleoside triphosphates and trinucleotides: in spite of the fact that these compounds have the same charge, nucleoside triphosphates are eluted by 0.2 *M* KP,

whereas trinucleotides are eluted by 1 m*M* KP. Finally, it should be mentioned that, as in the case of rigid polynucleotides, HA can discriminate among different structures existing in flexible polynucleotides; the different chromatographic behavior of formaldehyde-reacted polyribonucleotides and the fractionation of single-stranded, denatured DNA being typical examples.

In conclusion, HA chromatography is a new, powerful technique in the field of nucleic acids. Among its advantages over other techniques, three are of special importance: (1) the chromatographic material being an inorganic, crystalline, insoluble salt, it is possible to work over a wide range of temperatures, as well as in the presence of salts and organic reagents, like formaldehyde, phenol, chloroform, urea, etc.; (2) the adsorption-elution phenomenon is basically associated with the density and distribution of interacting groups at the surface of polynucleotides; this makes the process understandable in physicochemical terms and permits a theoretical approach[23,24]; (3) the technique is simple, reproducible and does not require any expensive equipment or reagent.

Addendum

Chromatography of nucleic acids on HA columns is enjoying a rapidly increasing popularity, as witnessed by a large number of investigations published after this article was written. Some of these recent papers will be mentioned here since they provide examples of different applications of the technique.

The most widespread use of HA columns remains the separation of denatured from native, or renatured, DNA,[107–117] of RNA from DNA,[118] and of single-stranded from double-stranded RNA.[119] Investigations on naturally[120] or artificially[121] cross-linked DNA's have also been reported.

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Thermal chromatography has been used for the partial purification of sea urchin satellite DNA,[122] though a fractionation involving a polyethylene glycol–dextran system seems to be preferable in this case[123] for determining the genetic relatedness between *Leptospira* strains,[124] for separating polyribo(GC) from template polyribo(IC),[125] and for preparing high GC segments from mycoplasma DNA[126] and high GC satellite DNA's.[127] In this latter case 7.2 *M* sodium perchlorate was used in the eluting buffer to decrease the melting temperature. HA columns have also been used for the separation of DNA–RNA hybrids.[128–133] The separation of yeast mitochondrial DNA from nuclear DNA[19,20] has been used by Fukuhara.[134,135]

Several investigations in the area of polyribonucleotides have been presented in a symposium on hydroxyapatite chromatography held in Strasbourg. They dealt with the separation of ribooligonucleotides and the relationship between elution molarity and chain length,[136] the fractionation of tRNA,[137] the chromatography of nucleic acids from uninfected and virus-infected plants,[138] the separation of three 5 S RNA species from wheat germs, which seem to differ only in their terminal phosphorylation,[139] and the isolation of ribooligonucleotides obtained from yeast RNA by pancreatic RNase digestion; these contain 72% G and are eluted at 0.45 *M* NaP.[140]

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