Analysis of Deoxyribonucleic Acid Structure by Partition Chromatography*

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ABSTRACT: A partition column has been developed for the fraction of high molecular weight nucleic acids, using a solvent mixture containing *t*-amyl alcohol, alkoxyethanols, tripentylamine acetate, and aqueous trilithium citrate. The column employs an immobile organic phase retained on Sephadex LH-20 and an aqueous phase elution gradient of increasing lithium ion concentration. Adsorption partition chromatography of native deoxyribonucleic acid using this column distinguishes populations of deoxyribonucleic acid molecules containing different but small proportions of single-stranded regions.

Denatured deoxyribonucleic acid can be completely separated from double-stranded deoxyribonucleic acid and can be quantitatively recovered.

Local alterations in secondary structure may be important in the biological functioning of DNA double helices. Such fluctuations may influence the sites of shearing of DNA during extraction and so may be reflected in the structure of the isolated DNA molecules. Of the various available methods that have been applied to the investigation of structural variations of this kind, countercurrent distribution in alkoxyethanol–trilithium citrate solvent systems (Kidson and Kirby, 1963, 1964) appears to be one of the more sensitive. In these solvent systems DNA molecules containing single-stranded regions travel further in the organic (upper) phase while more fully base-paired molecules tend to remain in the aqueous (lower) phase. Separation of populations of DNA molecules on this basis has provided a means of examining structural alterations such as those occurring in the vicinity of the growing point of the bacterial chromosome (Kidson, 1966).

While countercurrent distribution remains an excellent tool for this purpose, its limited availability, rather high solvent volume requirements, and the relatively long time involved in each run make an alternative column method desirable for achieving the same purpose. We have tried unsuccessfully to transpose the above countercurrent procedure, with an immobile aqueous phase, directly to a variety of columns. However, the use of Sephadex LH-20 with an immobile organic phase and a salt gradient of low lithium ion concentration has proved successful for analogous examination of DNA structure.

Materials and Methods

Labeling and Isolation of DNA from Escherichia coli. An aliquot of an overnight culture of *E. coli* B in Bacto nutrient broth (Difco) containing 0.5% yeast extract (Difco) was transferred to fresh medium and grown exponentially at 37°. Cells were labeled with [^{3}H]thymidine (2 Ci/mmole, 50 μCi/100 ml of cells) for at least three generations, then harvested by centrifugation at 10,000*g* at 4°. DNA was isolated by the aminosalicylate–dodecyl sulfate–phenol method (Kidson, 1966).

Labeling and Isolation of Mouse Myeloma DNA. A non-γ-globulin producing mutant, XC1, derived from the C1 culture line of the C3H mouse ascites myeloma, X5563, was obtained initially from Drs. K. Horibata and M. Cohn of the Salk Institute for Biological Studies, LaJolla, Calif. The XC1 line was maintained in ascites form in C3H mice or in suspension tissue culture in a modified Eagle's medium. Cells were grown exponentially at 37° in 10-ml batches, subcultured at 1–2 $\times$ 10^5 cells/ml, and labeled with [^{3}H]thymidine (2 Ci/mmole, 25–50 μCi/plate) for one to two generations. Cells were harvested by centrifugation at 5000*g* at 4°, and DNA was isolated by a phenol procedure using sodium triisopropylnaphthalenesulfonate as detergent (Parish and Kirby, 1966). Following four phenol extractions, DNA and RNA were precipitated twice from the aqueous phase with an equal volume of 2-ethoxyethanol, redissolved in 0.2 M sodium acetate (pH 7.1), and treated overnight at 4° with RNase (10 μg/ml). RNase was removed by phenol extraction, residual phenol extracted with diethyl ether, and the DNA solution dialyzed against 1 M sodium chloride–0.01 M sodium acetate, pH 7.1 at 4°, to remove ribonucleotides.

Characterization of DNA Preparations. The above preparative procedures give DNA of about 10^7 average molecular weight (Kidson and Kirby, 1964). Protein content (Lowry *et al.*, 1951) was <1% and hyperchromicities, measured by thermal denaturation in 0.015 M NaCl–0.0015 M trisodium citrate, were >33%.

DNA Preparations with High Protein Content. DNA preparations having 5% or more protein were prepared in one of two ways: (a) a single extraction with 5% 4-aminosalicylate and an equal volume of phenol–water (90:10), followed by 2-ethoxyethanol precipitation, ribonuclease treatment, reprecipitation and dialysis of redissolved DNA–protein as above; (b) extraction with 5% 4-aminosalicylate–1% dodecyl sulfate without phenol, centrifugation at 10,000*g* for 60 min, followed by resuspension of the pellet and dialysis as above.

Preparation of DNA for Column Chromatography. Before being applied to columns, suitable aliquots of each DNA

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preparation were dialyzed against 2.5 mM trilithium citrate. The lithium salts of DNA have been found to give more satisfactory partition in many solvent systems than the sodium or potassium salts (Kidson and Kirby, 1963). Denatured DNA was prepared by heating the required preparations in 2.5 mM trilithium citrate at 100° for 10 min, then cooling rapidly on ice. DNA was applied to columns in aqueous solutions without preequilibration with organic solvents. Application volumes were 1–2 ml for 20 × 1 cm columns; with larger columns, volumes up to 5 ml were applied. With double-stranded DNA containing essentially no protein, the columns could be washed subsequently with the lower ionic strength starting buffer preequilibrated with organic phase without elution of DNA, indicating that DNA was completely adsorbed onto the column from the aqueous solution.

Solvent Systems. The solvent systems employed have been adapted from those used previously for countercurrent partition of DNA (Kidson and Kirby, 1963; Kidson, 1966). In the countercurrent system a stationary aqueous phase was used with a mobile organic phase. The present partition chromatographic system employs a stationary organic phase and a mobile aqueous phase containing an ionic gradient. The following solvents were found to be suitable for use without further purification: *t*-amyl alcohol (Mallinckrodt), 2-methoxyethanol and 2-butoxyethanol (Matheson Coleman), and tripentylamine (Eastman). The solvent system comprised: (a) *t*-amyl alcohol–2-butoxyethanol–2-methoxyethanol (5:4:1, 28 volumes), (b) solution a–tripentylamine–glacial acetic acid (100:6:1.08, 21 volumes), and (c) water–0.033 M trilithium citrate (52 volumes). The amounts of water and trilithium citrate were varied to give a range of trilithium citrate concentration of 2.5 mM to 0.7 M in these 52 volumes (solution c) of the total 92 volumes of upper plus lower phases.

Solvent mixtures were shaken thoroughly at ambient temperature before separating the phases. All solvent mixtures were stored in the dark to prevent oxidation of the amine; fresh batches were prepared weekly. The pH of the aqueous phase was 7.6.

Column Preparation and Procedure. For DNA samples up to 200 μg, a 20 × 1 cm column was used; for greater quantities of DNA, larger columns were used. Sephadex LH-20 was first equilibrated with the organic phase of the solvent mixture containing trilithium citrate at the required initial ionic strength, and degassed under vacuum. The column was packed under atmospheric pressure using this organic phase, then further washed with a volume of the same organic phase roughly equal to that of the bed volume. The organic phase in the external volume of the column was then replaced with the corresponding aqueous phase and the column further equilibrated with about one bed volume of aqueous phase. Monitoring of the washes at 260 and 280 mμ showed that little or no organic phase was removed from the beads during equilibration with aqueous phase. The dialyzed DNA solution was applied to the column, followed by 5–10 ml of the initial aqueous phase and then by a gradient of aqueous phase of increasing ionic strength. The standard procedure adopted for 5–100 μg of DNA on a 20 × 1 cm column was as follows: DNA was applied in 1 ml of 2.5 mM trilithium citrate and washed with 5 ml of aqueous phase containing 2.5 mM trilithium citrate followed by a linear gradient from 2.5 to 10 mM trilithium citrate, total volume 130 ml. About 70 fractions each 1.9 ml were collected and radioactivity either was measured directly in 0.5-ml aliquots in a dioxane-containing phosphor or 1 ml of ethanol added, DNA was precipitated with trichloroacetic acid 10%, washed with trichloroacetic acid 5%, collected on Millipore filters and dried, and radioactivity was measured in a toluene phosphor. Suitable variations in gradient slope and elution volume were employed with larger columns in which greater quantities of DNA were fractionated. Columns up to 60 × 3 cm have been used with either upward or downward flow elution for the fractionation of as much as 3 mg of DNA, and there appears to be no reason why much larger columns cannot be employed. Sephadex LH-20 could be recovered from the columns and reutilized after thorough washing and equilibration with the starting organic phase before repacking the column as described above. This procedure effectively removed any residual nucleic acid from the gel.

Recovery of Fractions. When it was required to recover fractions, the contents of a number of tubes were pooled, organic solvents were extracted with diethyl ether, and the DNA solution was dialyzed against the required buffer. Where necessary, volume reduction was achieved by dialysis against polyethylene glycol (Mallinckrodt Carbowax 20 M).

Melting Curves. Fractions were pooled and recovered as above and dialyzed against 0.015 M NaCl–0.0015 M trisodium citrate; thermal denaturation was followed at 260 mμ in a Zeiss PMQ II spectrophotometer.

Reaction with Formaldehyde. Recovered fractions were treated with 1% formaldehyde containing 2 μCi/ml of [^{14}C]-formaldehyde (New England Nuclear) for 2 hr at 20° according to the method of Boedtker (1967).

Sucrose Density Gradient Analysis. In some experiments, DNA fractions from columns were recovered and volumes reduced as described above, then analyzed by sedimentation on neutral or alkaline sucrose gradients. Fractions of 0.4 ml were layered on 4.5-ml neutral linear gradients of 20–5% sucrose in 0.2 M NaCl–0.01 M sodium acetate, pH 7, or on 4.5-ml alkaline linear gradients of 20–5% sucrose in 0.2 M NaCl–0.1 N NaOH. Gradients were centrifuged in the SW50 head of the Spinco L2 centrifuge at 45,000 rpm for 4 hr. Fractions of 0.17 ml were collected from the bottom of the tubes, and DNA was precipitated with 10% trichloroacetic acid, collected on Millipore filters, washed with 5% trichloroacetic acid, dried, and counted in toluene phosphor.

Results

Partition Behavior of Native DNA. The partition behavior of small quantities of *E. coli* DNA on a 20 × 1 cm Sephadex LH-20 column is illustrated in Figure 1. The fractionation profiles of bacterial and mammalian DNAs were roughly reproducible both for several runs of the same DNA preparation (Figure 1a) and for different DNA preparations made under identical conditions from the same source but differentially labeled and cofractionated on the same column (Figure 1b). Mammalian DNA behaved similarly. With a 20 × 1 cm column the void volume was about 12 ml (~first seven fractions); elution of DNA began after a further 30–40 ml, at 4 mM trilithium citrate, and was complete in the range 7–8 mM trilithium citrate. At the lower ionic strength a major peak was eluted from the column; with increasing ionic strength a continuum of minor peaks was eluted. The general profiles of native DNA obtained by partition in this solvent

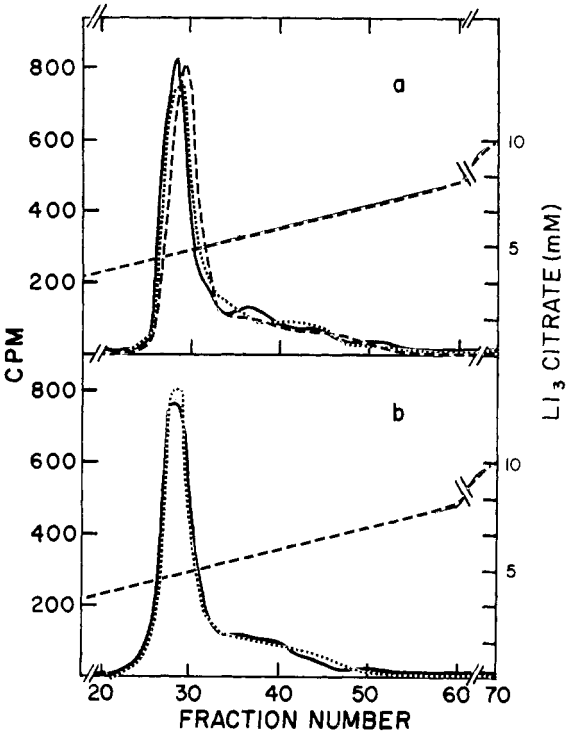

FIGURE 1: Fractionation of native *E. coli* B DNA on a standard partition column as described in the text. (a) Superimposed profiles of three separate runs of the same DNA sample. (b) ^{3}H-labeled (—) and ^{14}C-labeled (- - - - -) *E. coli* B DNA samples fractionated together on the same column.

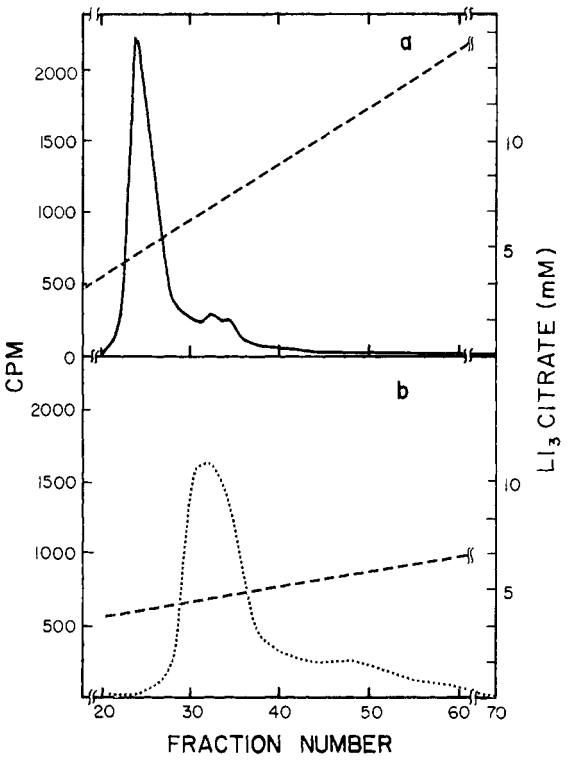

FIGURE 2: Effect of ionic strength on the fractionation of ^{3}H-labeled native *E. coli* B DNA. (a) Gradient from 2.5 to 17 mM trilithium citrate, total elution volume 130 ml. (b) Gradient from 2.5 to 8 mM trilithium citrate, total elution volume 130 ml.

system on Sephadex LH-20 were very similar to those obtained for carefully characterized bacterial DNA preparations fractionated by countercurrent distribution in analogous solvent systems (Kidson, 1966). This immediately suggests that the partition column can substitute for the countercurrent method. In our initial studies on the countercurrent distribution of double-stranded DNA (Kidson and Kirby, 1963) a number of discrete peaks were observed for DNA from *E. coli*, rat liver, and *Drosophila melanogaster;* many of these additional peaks appear to have resulted from less stringent preparative methods, such as drying of the DNA. In the present experiments, as previously (Kidson, 1966), DNA preparations were stored in solution in 1 M NaCl–0.1 M sodium acetate at 4°. So stored, these preparations gave identical fractionation profiles on countercurrent distribution or Sephadex LH-20 partition chromatography when tested at various times over a 4-month period.

Effect of Ionic Strength and Elution Volume. By altering the slope and extent of the ionic gradient in the eluting aqueous phase the fractionation profile of native *E. coli* DNA could be changed (Figure 2). A slightly steeper gradient of trilithium citrate resulted in less spreading of the latter (Figure 2a). A slightly shallower gradient resulted in broadening of the major peak and greater spreading of minor peaks (Figure 2b). Initiation of the gradient at higher ionic strength (10 mM trilithium citrate) resulted in elution of most of the DNA in the void volume of the column. When the whole gradient was run in a lower range than the optimum, most of the DNA remained on the column or was eluted late, depending upon the ionic strength. The Li^+ concentration in the eluting aqueous phase, at a constant concentration of amine in the immobile organic phase, is thus the determining factor in the elution behavior of double-stranded DNA. The concentration of amine in the organic phase was chosen in accord with the ionic gradient employed; a substantial reduction of the amine concentration resulted in elution of DNA in the void volume even at very low ionic strengths. This dependence upon ionic strength is reminiscent of the effect of altering the Li^+ concentration in the aqueous phase on the countercurrent distribution of native DNA (Kidson and Kirby, 1964; Kidson, 1966). Changes in the Li^+ concentration in the aqueous phase in the countercurrent system led to alterations in the extent of spreading of the DNA but did not alter the general fractionation behavior. The trilithium citrate concentration, found to be optimal for the countercurrent distribution of native DNA (7 mM), lies in the range of the gradient found to be optimal for partition in the present studies. The columns are much more flexible than the countercurrent system, since the ionic gradient can be altered during elution. At the same time, with both methods of fractionating native DNA, comparative studies require strict adherence to standardized conditions.

That ionic strength is the main determinant of the position of elution of different DNA molecules is supported by experiments in which the elution volume was varied but the gradient limits were maintained. The elution positions of the major and minor peaks were constant with respect to ionic strength and independent of elution volume, although greater spreading of all peaks was observed with larger elution volumes.

Effect of DNA Concentration. The fractionation behavior

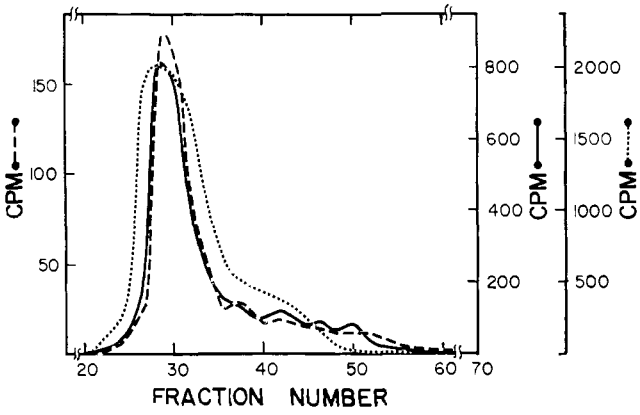

FIGURE 3: Effect of concentration on the fractionation behavior of ^{3}H-labeled, native XC1 DNA. Columns (20 × 1 cm) were loaded with different quantities of the same DNA preparation and eluted under identical conditions as described in the text. Superimposed profiles are shown for three columns loaded with 10 μg of DNA (- - -), 50 μg of DNA (———), and 150 μg of DNA (- - - - -). Gradient conditions as in Figure 1.

of native DNA on the partition column is affected by DNA concentration. Figure 3 shows the profiles of 10, 50, and 150 μg of [^{3}H]thymidine-labeled XC1 myeloma DNA fractionated on a 20 × 1 cm column. At the highest concentration a slightly greater proportion of the input DNA was eluted in the region of the major peak, suggesting that aggregation occurs of some DNA molecules that at lower concentrations would be eluted at a higher ionic strength.When much larger amounts (in the milligram range) of DNA were applied to the same size column, a part of the input was eluted in the void volume, indicating definite limits to the column capacity. A 20 × 1 cm column will handle satisfactorily 50–100 μg of DNA. Larger columns can be used very successfully for the fractionation of milligram quantities of DNA.

Fraction Recovery and Recycling. Experiments designed to check the behavior of column fractions on repeated partition combined fractions eluted from different positions in the salt gradient of successive runs of DNA preparations with different radioactive labels. An XC1 cell culture in logarithmic growth phase was divided into two halves, one labeled for 20 hr with [^{14}C]thymidine, the second labeled for the same period with [^{3}H]thymidine. Each DNA preparation was fractionated on a 20 × 1 cm column, then tube fractions under the initial peak (fraction I) and under the combined minor peaks (fraction II) were recovered in each case as described under Methods (Figure 4a). ^{14}C-Labeled fraction I and ^{3}H-labeled fraction II were combined, as were ^{14}C-labeled fraction II and ^{3}H-labeled fraction I. Each combination of DNA fractions was then rechromatographed on 20 × 1 cm columns. Within reasonable limits each fraction behaved similarly with respect to its elution position in the salt gradient (Figure 4b,c). The reproducibility of partition behavior on these columns is similar to that noted on repeat countercurrent distribution of recovered DNA fractions (Kidson and Kirby, 1964).

Sucrose Density Gradient Analysis of Recovered Fractions. [^{3}H]Thymidine-labeled *E. coli* B DNA from logarithmic phase cells was fractionated on a 20 × 1 cm column and fractions recovered and analyzed by neutral and alkaline

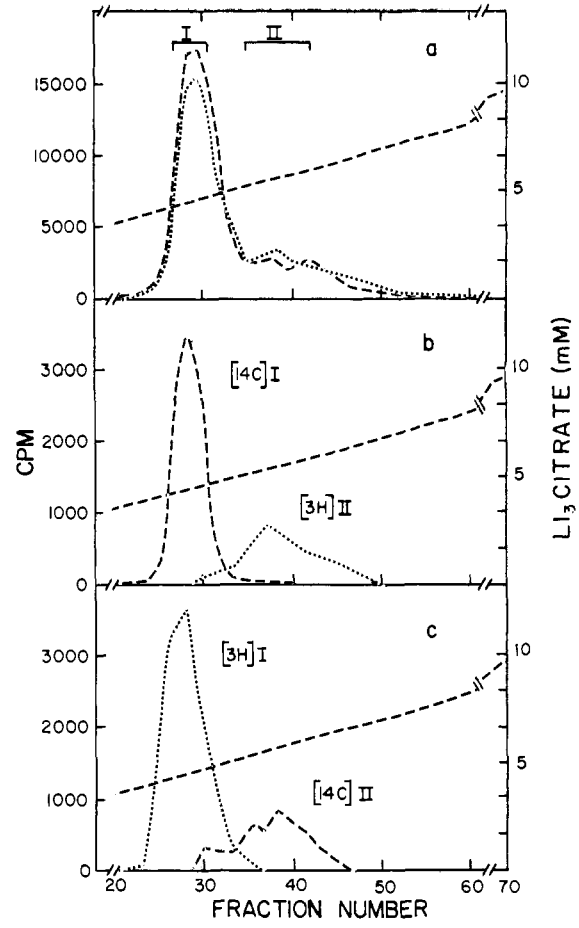

FIGURE 4: Recovery and recycling of partition column fractions of native XC1 DNA labeled with ^{3}H (- - - - -) or ^{14}C (— — —). (a) Superimposed profiles of differently labeled DNA preparations fractionated on parallel columns, showing the tube fractions pooled and recovered from each column as described in the text, to give ^{3}H-labeled fractions I and II, and ^{14}C-labeled fractions I and II, respectively. (b) Cofractionation of ^{14}C-labeled fraction I and ^{3}H-labeled fraction II. (c) Cofractionation of ^{3}H-labeled fraction I and ^{14}C-labeled action II.

sucrose density gradient sedimentation. No gross variation in sedimentation behavior was observed, suggesting that partition behavior on Sephadex LH-20 in these solvents is not dependent to any measurable extent on differences in net size of double-stranded DNA molecules. This finding correlates well with ultracentrifugal analysis of sedimentation coefficients of recovered fractions from countercurrent distribution in the same solvents (Kidson and Kirby, 1964).

Behavior of DNA–Protein and Degraded DNA. DNA preparations containing large amounts of protein prepared as described in Methods behaved differently from purified DNA preparations. Much of the DNA passed through the column in the void volume as a DNA–protein complex, indicating that such complexes are rather insoluble in the organic solvents employed. Radioactively labeled DNA degraded with pancreatic DNase (10 μg/ml, 4 hr at 20°) behaved similarly, more than 95% of the input counts being recovered in the void volume of the column. DNA that had been denatured but not degraded behaved quite differently, as discussed below.

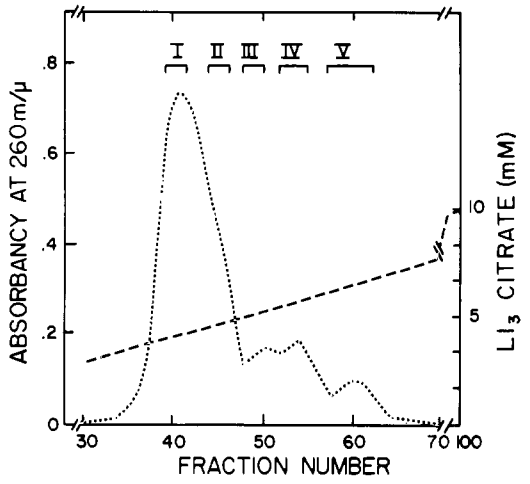

FIGURE 5: Fractionation and recovery of native bovine spleen DNA (isolated by the method described for XC1 DNA) for thermal denaturation studies. DNA (2.5 ml) was fractionated on a 60 × 3 cm column, using a gradient from 2.5 to 10 mM trilithium citrate and an elution volume of 250 ml. Fractions I–V were recovered, dialyzed as described in the text, and used to determine hyperchromicities (Table I).

TABLE I: Hyperchromicities of Recovered Column Fractions.[a]

Fraction	Hyperchromicity (%)
I	39
II	36
III	34
IV	34
V	32

[a] These fractions were obtained from the column illustrated in Figure 5.

Thermal Denaturation of Recovered DNA Fractions. In experiments designed to test the relation between partition behavior and secondary structure of column-separated DNA molecules, the hyperchromicity of DNA fractions was examined by thermal denaturation. Fractions were recovered, volumes suitably reduced as described above and dialyzed against 0.015 M NaCl–0.0015 M sodium citrate, and hyperchromicities determined from the melting curves. In a typical experiment, bovine spleen DNA (2.5 mg) was fractionated on a 60 × 3 cm column and fractions were recovered as designated in Figure 5. Hyperchromicities of these fractions are shown in Table I. It is evident that there was a gradient of decreasing hyperchromicity corresponding to the gradient of increasing ionic strength required for elution of DNA molecules. By the criterion of thermal denaturation, then, ionic gradient elution of adsorbed DNA from the column is dependent upon DNA secondary structure: the more denatured the DNA molecule, the higher the ionic strength required to shift its partition coefficient in favor of solution in the aqueous phase. It is important to note, however, that the range of hyperchromicities was not particularly great from fractions I–V. The hyperchromicity of fraction V exceeded 30%, indicating that even those molecules eluted at the highest salt strength were predominantly intact double helices as judged by their average hyperchromic value.

Reaction with [^{14}C]Formaldehyde. The known reactivity at low temperatures of single-stranded, but not intact double-helical DNA with formaldehyde (Fraenkel-Conrat, 1954; Haselkorn and Doty, 1961; Stollar and Grossman, 1962) was used as an additional test of secondary structure of DNA fractions recovered from the partition columns. Following fractionation, individual tube fractions were recovered, treated with [^{14}C]formaldehyde, and counted as described above. In a typical experiment, [^{3}H]thymidine-labeled XC1 DNA was fractionated on a standard 20 × 1 cm column with a gradient of 2.5–10 mM trilithium citrate. Formaldehyde was then reacted with each recovered fraction and the specific reactivity expressed as the ^{14}C/^{3}H ratio (Figure 6). The ^{14}C/^{3}H ratio increased with the increasing Li^+ concentration required to elute the corresponding DNA molecules. Thus, the higher the ionic strength required for elution, the greater the number of free amino groups in these DNA molecules, *i.e.*, the greater the proportion of nucleotide bases not immediately involved in base pairs. This finding confirms the melting curve data and indicates that secondary structure plays an important role in the partition behavior of DNA molecules on the columns, as it does in countercurrent distribution in similar solvent systems (Kidson and Kirby, 1964). However, it is interesting to note that even the initial major peak (fractions 28–34) showed some reaction with formaldehyde, the ^{14}C/^{3}H ratio increasing greatly toward the right-hand edge of the peak. This observation indicates that some molecules in this initial DNA peak have single-stranded regions, although from their high average hyperchromic value (Table I) such regions must represent a very small proportion of the total double-helical chain length.

Partition Behavior of Denatured DNA. In experiments designed to assess the partition behavior of completely denatured DNA, [^{3}H]thymidine-labeled native XC1 DNA and [^{14}C]thymidine-labeled denatured XC1 DNA were mixed and fractionated together on a 20 × 1 cm column. First, an elution gradient from 2.5 to 10 mM trilithium citrate (60 ml each side) was applied. This was followed successively by 20 ml each of 33 and 500 mM trilithium citrate (Figure 7). Each lithium citrate solution was preequilibrated with the organic phase. More than 95% of the ^{3}H-labeled DNA was eluted within the initial Li^+ gradient; none of the ^{14}C-labeled DNA appeared in this region. Very small fractions of ^{14}C- and ^{3}H-labeled DNAs were eluted by 33 mM trilithium citrate. More than 98% of the ^{14}C-labeled DNA was eluted with 500 mM trilithium citrate, as was the remainder (<5%) of the ^{3}H-labeled DNA. Single-stranded DNA is thus eluted at ionic strengths very much higher than those required to elute double-stranded DNA and DNA containing limited proportions of non-base-paired regions. In these experiments, nearly 100% of input counts were recovered from the column, indicating that both native and denatured DNAs can be eluted. During elution with high salt aqueous phase considerable contraction in volume of the Sephadex LH-20 bed occurred, but this did not appear to affect the quantitative recovery of single-stranded DNA.

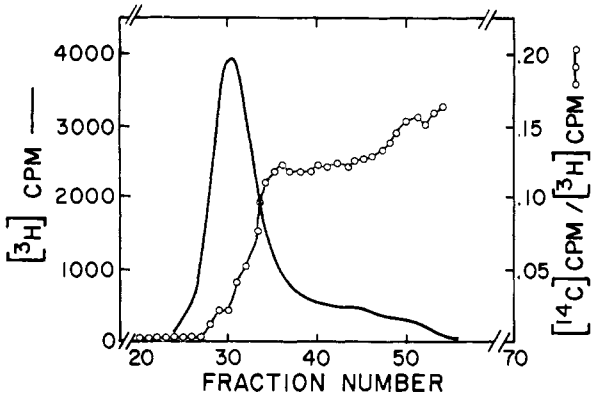

FIGURE 6: Reaction of [^{14}C]formaldehyde with recovered column fractions. Native ^{3}H-labeled XC1 DNA was fractionated on a 20 × 1 cm column. Individual tube fractions were recovered and treated with [^{14}C]formaldehyde as described in the text.

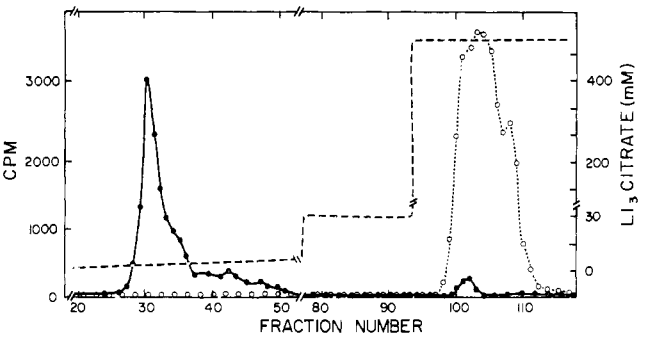

FIGURE 7: Cofractionation of ^{3}H-labeled native XC1 DNA (●——●), and ^{14}C-labeled denatured XC1 DNA (○——○), on a 20 × 1 cm partition column. A gradient from 2.5 to 10 mM trilithium citrate was followed by stepwise elution with 33 mM and 0.5 M trilithium citrate as described in the text.

Discussion

Comparison of the present data with information derived previously about the secondary structure of DNA molecules in the same solvent systems (Kidson and Kirby, 1964; Kidson, 1966) demonstrates similarity of the behavior of DNA on adsorption partition chromatography on Sephadex LH-20 and on countercurrent distribution. In both systems, the partition coefficient of a given DNA molecule is influenced by the presence of non-base-paired regions. On this basis, it is possible to use the partition properties to separate molecules having different net proportions of open regions. Adsorption partition chromatography on Sephadex LH-20 offers a readily available, rapid analytical system for this purpose. It utilizes small solvent volumes and can handle very small quantities of DNA.

Although most DNA preparations are fractionated on these partition columns into a major peak and a series of minor peaks, the reaction of recovered fractions with [^{14}C]-formaldehyde indicates that even molecules situated in the trailing (right-hand) edge of the major peak have some open regions. The inference is that in this solvent system DNA molecules fall into populations having (a) zero, one, two, or more non-base-paired regions or having (b) zero, short, or long open regions and that the majority have few or no such regions. The technique cannot, of course, distinguish between situations a and b. The hyperchromic values derived from melting profiles of recovered fractions (Table I) indicate that the average proportion of non-base-paired regions *vs.* double-helical regions even in the most "open" molecules is not large, suggesting that this solvent system is sensitive to small variations in secondary structure. It has been observed in countercurrent distribution studies (Kidson and Kirby, 1964) that the sum of hyperchromicities of recovered fractions was usually slightly less than the average hyperchromicity of input DNA, suggesting that the organic solvents may somewhat exaggerate preexisting regional denaturation. In those studies and in the present ones, however, recycling of fractions resulted in similar partition behavior, suggesting that new regions of denaturation are not initiated during fractionation.

Adsorption partition chromatography on Sephadex LH-20 differs from countercurrent distribution in similar solvent systems with respect to the behavior of denatured DNA. In the countercurrent system it was observed that denatured fragments (occurring in the cells of origin or resulting from the isolation process) were much more soluble in these organic solvents than native DNA, and at the ionic strength suitable for fractionation of the latter, denatured DNA traveled almost entirely with the organic phase (Kidson and Kirby, 1964). However, a small increase in trilithium citrate concentration (from 7 mM for native DNA to 8 mM for denatured DNA) permitted fractionation of denatured DNA fragments, 4 S in size (Kidson and Kirby, 1963). A slightly higher trilithium citrate concentration (9 mM) has been used for countercurrent fractionation of nonfragmented, heat-denatured DNA (Baldwin and Kidson, 1968). On the partition columns, nonfragmented, heat-denatured DNA was eluted only at very high trilithium citrate concentrations (10 mM to 0.5 M). From a practical viewpoint, this is advantageous, since it permits ready discrimination between partially denatured DNA and single DNA strands. At the same time, the single strands can be recovered more easily, for example, than from methylated albumin columns.

The adsorption of DNA on the column and the ionic strength at which various molecules are eluted would appear to depend principally on two factors. First, even DNA molecules with intact double helices (leading or left-hand edge of the major peak) are adsorbed onto the Sephadex LH-20 beads containing the organic solvent phase. Adsorption of these molecules depends upon the interaction of amine with DNA phosphate groups in exchange for lithium ions. This exchange is reversible at slightly increased Li^{+} concentration, with consequent elution of these molecules. Secondly, in regions where there are unpaired bases, the free bases can react more fully with the organic solvent phase in which they are soluble when this phase is in equilibrium with low Li^{+} concentrations. There may be, of course, a further differential partition depending upon the identity of the free bases, since adenine-rich regions are more soluble in these solvents than are guanine-rich regions (Kidson and Kirby, 1963). Because of the enhanced solubility of unpaired bases in the organic phase, higher Li^{+} concentrations are required to alter the net partition coefficients of these molecules in favor of the aqueous phase and so cause their elution from the column.

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Multiple Molecular Forms of α-Amylase from the Rabbit*

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ABSTRACT: Electrophoretic variants of α-amylase were observed in various vertebrate species (lungfish, chicken, beef, guinea pig, rat, rabbit, and human). Three variants of the enzyme were isolated from the rabbit pancreas and another single amylase from the parotid gland. Some of their catalytic and molecular properties have been defined. The amino acid compositions, molecular weights (approximately 54,000), and catalytic properties of the enzymes were very similar. The number of peptides observed in tryptic maps closely corresponds to the predicted number of trypsin-sensitive bonds. This finding and the results of ultracentrifugal studies in 6 M guanidine hydrochloride suggest the molecule is composed of a single subunit.

Reproducible differences in the tryptic maps of each of the enzyme preparations were also detected. These results are compatible with the postulate that the molecules have unique sequences, and are thus the products of separate genes. However, phenotypic modifications of a single gene product are not ruled out, especially since the enzymes appeared somewhat heterodisperse on ultracentrifugation in 6 M guanidine hydrochloride (possible degradation?). The difficulties in resolving differences in primary structure from modifications of a single structure in multiple closely related (homologous) molecules are emphasized.

The enzyme α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) is a prominent constituent of the mammalian pancreas and, in some species, the parotid glands. Preliminary evidence has suggested that a number of molecular forms may contribute to the amylase activity measured in pancreatic and salivary extracts. For example, Marchis-Mouren and Pasero (1967) demonstrated two forms of amylase in the hog pancreas, and Sick and Nielsen (1964) detected two amylase isozymes in the mouse pancreas, while Lamberts *et al.* (1965) reported the existence of four electrophoretically distinct amylases in human saliva. In no instance, however, has the molecular basis of these variants been defined. These variants might possess a unique primary structure and hence be products of different genes. On the other hand, if the enzyme were composed of multiple subunits, then the biosynthesis of more than one subunit could result in both heteromeric and homomeric combinations yielding hybrid molecules which possess, as in the case for lactic dehydrogenase (Appella and Markert, 1961) and aldolase (Penhoet *et al.*, 1967), different electrophoretic mobilities. Alternatively, phenotypic alterations such as binding of carbohydrate residues or limited proteolysis could generate variants with differing electrophoretic mobilities from a single molecular species.

The pancreas and parotid glands share a similar physiological function and are largely composed of similar cell types. It is not known, however, whether the pancreatic and parotid amylase molecules are identical and hence the products of the same gene(s) in these glands. A comparative study of crystalline human salivary and pancreatic amylase made by Bernfeld *et al.* (1950) suggested that the proteins were closely related or identical. More recently, McGeachin *et al.* (1966) found the rabbit pancreatic and salivary amylases to be immunologically identical. These experimental results, though they certainly indicate a close similarity in the molecules, do not prove identity.

The present investigation was undertaken with the purpose of isolating the amylases from the pancreas and parotid glands of the rabbit and determining the basis of the molecular variation in amylases from these tissues. A characterization of the amylases, including molecular weight determinations and amino acid and peptide compositions, were carried out.

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