Hydroxyapatite Fractionation Procedures in the Study of the Mammalian Genome

BY MORA MCCALLUM AND P. M. B. WALKER

Chemical Biology Unit, Department of Zoology, University of Edinburgh

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Three methods of fractionating DNA are described, in which DNA fragments are separated on the basis (1) of their base composition, (2) of the ability of denatured DNA to renature after incubation, and (3) of the presence of intrastrand secondary structure in denatured DNA. The ability of some of these fractions to form specific duplexes with whole denatured DNA isolated from the same and from a different mammal has been investigated. The results are presented to demonstrate how the fractionation procedures described may provide new information about the organization of the mammalian genome.

Hydroxyapatite fractionation of polynucleotides depends on the finding that at certain phosphate buffer concentrations extended single chains of denatured DNA or of RNA do not remain adsorbed on to the column material, whereas duplex molecules are still firmly bound (Miyazawa & Thomas, 1965; Bernardi, 1965). Evidence presented by Walker & McLaren (1965b) strongly suggested that in DNA these duplex molecules could be native, be renatured or merely contain stretches of intramolecular hydrogen-bonding comparable with those found in RNA at low temperatures and high salt concentrations.

In this paper we explore the capacity to distinguish extended single chains from these three states of DNA in order to provide new information about the mammalian genome. Each state can be separated from the single-stranded molecule by fractionation procedures in which the previous treatment as well as the phosphate and temperature conditions during fractionation are varied. The methods are: (1) thermal chromatography of native DNA at a constant phosphate concentration, which distinguishes DNA fragments by their base composition; (2) salt chromatography of denatured DNA at high temperature and after various times of incubation before fractionation, which distinguishes renatured molecules from the remainder; (3) salt chromatography at lower temperatures without prior incubation, in which DNA is separated on its ability to form intramolecular secondary structure stable at a defined temperature.

Each of these procedures has been applied to low-molecular-weight samples of mouse DNA,

produced by ultrasonic treatment, and the significance of some of these fractions in relation to the organization of mammalian DNA is discussed.

METHODS

Preparation of DNA. Our earlier method (Walker & McLaren, 1965a) has been slightly modified; in place of the earlier multiple extractions of the cellular homogenate with phenol, ether and chloroform, we now incubate the homogenate with 0.3 M-sodium trichloroacetate-1% sodium dodecyl sulphate, pH 6.8, for 15 min. at 37° before extracting with phenol, and precipitating with 1 vol. of ethanol. After dissolving the precipitate in $0.01 \times \text{SSC}$ (SSC is 0.15 M-NaCl-0.015 M-sodium citrate) and making up to M-NaCl, we then proceed as in the earlier method. This modification is quicker and gives a higher yield without altering the behaviour of the DNA. DNA was prepared from tissues and tissue-culture cells from *Mus musculus* L. and *Rattus norvegicus* L. of mixed laboratory strains.

The ^{32}P -labelled DNA was prepared from mouse L-strain cells grown as surface tissue-cultures in 101. roller bottles, to which [^{32}P]orthophosphate (approx. 1 mc/50 ml.) (PBS 1; The Radiochemical Centre, Amersham, Bucks.) had been added 3 days previously.

Preparation of the hydroxyapatite. The method used is that of Tiselius, Hjerten & Levin (1956), as slightly modified by Miyazawa & Thomas (1965). The free-running properties of the columns depend on maintaining a high proportion of the large and fragile crystals; we therefore avoid shaking or resuspending our stock material and remove the precipitate with a spoon.

Loading the hydroxyapatite columns. The molecular weight of the DNA must be reduced before it is adsorbed on to hydroxyapatite, both because viscous samples quickly block the column and because shorter molecules will be more homogeneous in the properties on which fractionation depends. We ordinarily use ultrasound under conditions

already described (Walker & McLaren, 1965a), which gives samples of mol.wt. about 500000. After ultrasonic treatment the fragmented DNA is dialysed for at least 15 hr. against 0.03M-phosphate buffer, pH 6.8, to which chloroform has been added at 0.25 ml./l. The dialysed DNA fragments can then, if required, be denatured in a boiling-water bath for 10 min., chilled in an ice bath and diluted with 2 vol. of cold distilled water, before being adsorbed on to the hydroxyapatite. The required amount of hydroxyapatite is added to the solution (the addition of hydroxyapatite is a most effective way of concentrating DNA) and the resulting slurry run on to the column. The quantity of hydroxyapatite is not critical; our smaller water-jacketed columns used for isotopically labelled samples have a 1 cm. bore, and 1–1.5 cm. of packed hydroxyapatite above the coarse-grade sintered disk suffices for up to about 500 μ g. of DNA. Larger columns with a capacity up to 5 mg. have been used. The temperature of the slurry is maintained at less than 0.5° below that of the reservoir in the range 60–95° by a Circotherm II (Shandon) circulating pump.

At high temperatures air bubbles sometimes form in the column, but do not appear to affect the results. Normally 1 ml. and 3 ml. washes are used for the smaller and larger columns respectively. Running the columns is simple and reproducible, and a stepwise elution programme at one temperature can be run simultaneously from four columns in 2 hr. without automatic equipment except a finger pump.

Determination of base ratios. We used enzymic digestion with crystalline pancreatic deoxyribonuclease I (batch no. D1239-42) and *Crotalus adamanteus* venom diesterase (VPH, batch no. 111), both from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

To obtain the 5'-nucleotides, the DNA was dialysed overnight against 10 mM-NaCl–5 mM-tris, pH 9.0. It was then made 2 mM in $MgCl_2$ and 10 μ g./ml. in deoxyribonuclease by using a stock solution containing 3.0 mg. of deoxyribonuclease, 0.2 ml. of 1% bovine serum albumin, 0.2 ml. of M-NaCl and 1.6 ml. of distilled water. After incubation for 1 hr. at 25°, the mixture was made 50 mM in tris and 5 mM in $CaCl_2$, and 0.1 mg. of venom diesterase was added. This was then incubated for 2 hr. at 45° and either deep-frozen or applied directly to the column. Extraction with Norit showed, in preliminary experiments, that less than 1.0% of the radioactivity in the digested sample was in the form of free phosphate.

Columns (1 cm. $\times$ 23 cm. long) of Bio-Rad AG1 (X8; 200–400 mesh; formate form) were used, with formic acid as the eluent. A simple gradient machine similar to the gradient mixing device of Bock & Ling (1954) was made, in which 4N-formic acid was placed in a standard 250 ml. conical flask (height 13 cm., bottom diam. 8 cm.). This was joined at the base to a parallel 250 ml. container (6.5 cm. diam.) filled with water which was stirred and connected to the top of the column. A finger pump was adjusted to deliver the eluent into 70 tubes of an automatic fraction collector in 16–18 hr. With this arrangement not more than five tubes were required for each peak. Typical base ratios for a number of relevant fractions are shown in Table 1.

Isotopes were measured by procedures already described (Walker & McLaren, 1965a).

Incubation of DNA in agar. The method by which one can test the capacity of DNA fragments to form specific duplexes is as follows. Denatured high-molecular-weight DNA is

trapped in an agar gel, into which isotopically labelled DNA fragments of low molecular weight, dissolved in $2 \times SSC$, are free to diffuse. After an overnight incubation at, usually, 60° those fragments that have not found a specific hydrogen-bonding site on the high-molecular-weight DNA are removed by ten washes of 10 ml. of $2 \times SSC$ at 60°. The temperature is then raised to 75° and five more washes with $0.01 \times SSC$ are given, which conditions break any duplexes formed. The ratio of the radioactivity in the last five washes to that in the whole 15 gives a measure of the proportion of fragments bound, which we call the 'binding factor'.

The detailed procedure is essentially that of Walker & McLaren (1965a), except that we have discarded water-jacketed columns in favour of the method outlined by Hoyer, McCarthy & Bolton (1964). We incubate the DNA-agar mixture in test tubes made from standard Quickfit and Quartz capped sockets (SCB 14), which allow the immersion of the tube in the water bath as far as the top of the ground joint, thus eliminating changes in concentration of the incubation mixture due to condensation. After overnight incubation, quantitative transfer of the DNA-agar into a special polypropylene tube with the first wash of 10 ml. of $2 \times SSC$ is easy, provided that the tubes are silicone-treated and a pouring lip is made in the cap. This polypropylene tube (1 cm. internal diam.) has a cone at the top to facilitate transfer, and a 60-mesh stainless-steel gauze, which supports the agar gel, fused to its base.

The incubation mixture is washed into the polypropylene tube, while it is standing in an empty scintillation vial, partially immersed in a water bath at 60°. The solution, but not the DNA-agar, can pass through the gauze into the vial. Then 5 min. later the polypropylene tube and agar are transferred into a second vial containing 10 ml. of $2 \times SSC$. These transfers are repeated serially for a total of 15 vials each containing 10 ml. of the appropriate salt solution at 60° or 75°. This ensures the transfer of the labelled fragments to the appropriate solutions, which are counted in the normal way.

Two trays each holding 60 vials, sufficient for washing the contents of 12 incubation tubes with $2 \times SSC$, can be suspended in a standard Grant water bath (model U3H). The first 60 vials are replaced by a further 60 vials for the $0.01 \times SSC$ washes.

RESULTS AND DISCUSSION

Thermal chromatography at a constant salt concentration. If native DNA of low molecular weight is adsorbed on to hydroxyapatite, the phosphate concentration adjusted to 0.06M and the temperature gradually increased, some of the fragments will eventually be denatured and eluted from the column. With a further increase in temperature more fragments are eluted until no DNA remains adsorbed. Miyazawa & Thomas (1965) showed that in bacterial and bacteriophage DNA the earlier-eluted fragments are rich in adenine and thymine (A+T) and the later in guanine and cytosine (G+C). Our experiments show that mammalian DNA is similarly heterogeneous and that fractions with a mean base composition of 33–52% G+C (on a molar basis) can be obtained (Fig. 1). Since it is

therefore relatively easy to separate from mammalian DNA fractions with differing base compositions, we can test the variation of other properties of these fragments. It has already been shown (Walker & McLaren, 1965a; McLaren & Walker, 1965) that there is a fraction of the mammalian genome that fails to form specific duplexes in the DNA-agar technique even when DNA preparations from the same species are incubated together. The results of an experiment designed to test whether these non-

Fig. 1. Base composition of thermal-chromatogram fractions from mouse DNA. ●, Cumulative sum of the radioactivity eluted at the temperatures stated from a hydroxyapatite column. Five 1 ml. washes with preheated 0.08 M-phosphate were made at each temperature. ■, Base compositions determined on Dowex (formate form) for the fractions stated. No other fractions were measured. The slope of the line is 1.6 moles of G+C/100 moles of nucleotides/°C. ○, Base composition of the unfractionated DNA placed at the temperature at which half the DNA had melted. A total of 1.4 mg. of DNA was used for this experiment; one-tenth of each thermal fraction was used for base-ratio determinations and the remainder for the agar incubations shown in Fig. 2.

reactive elements are evenly distributed throughout the fractions differing in base composition are shown in Fig. 2. The same fractions as those measured in Fig. 1 were incubated in agar with high-molecular-weight DNA from the same (mouse) and another (rat) species of rodent. As the G+C content of the fractions increases above 46%, there is a rapid falling-off of their binding capacity whether they are incubated in agar with DNA from the same or a different species, but below 46% G+C the fractions behave very differently with the two DNA preparations. As between mouse and rat, therefore, most of the differences that can be detected by this method appear to occur in the A+T-rich fractions.

This more detailed experiment thus confirms earlier results (McLaren & Walker, 1966) in which more heterogeneous chromatogram fractions had been used. It also shows that we can fractionate DNA fragments not only according to their overall nucleotide composition but also according to the distribution among the fragments of nucleotide sequences that are common or special to different organisms.

High-temperature salt fractionation of denatured DNA. It has already been shown (Walker & McLaren, 1965b) that denatured mouse DNA can be separated into two fractions, and that the minor fraction (approx. 10%), although it is eluted at the same phosphate concentration as native DNA (i.e. 0.3 M-phosphate), cannot be considered native. This is known to be so because spectroscopic melting curves determined at 260 mμ show that the temperature of half-melting (T_m) is 5.5° less than that of native DNA of the same molecular-weight distribution, and because this fraction, unlike native DNA, is very efficient at forming stable duplex molecules in DNA-agar experiments.

Joint experiments with Dr R. Britten in Washington have established that this minor component renatures at the same rate as the satellite

Table 1. *Base composition of mouse DNA after various fractionation and other procedures*

All results are based on the radioactivity due to ^{32}P appearing in the chromatogram peaks. Lines 2 and 3 refer to a typical agar incubation experiment with homologous DNA fractions. Lines 4, 5 and 6 are from experiments similar to those illustrated in Fig. 1. The DNA used was denatured, except in line 1.

Type of mouse DNA used	Molar base composition (%)					Ratios	
	C	A	G	T	C+G	C/G	A/T
(1) Native, high-molecular-weight	22.5	30.1	19.4	28.1	41.9	1.16	1.07
(2) Unbound in DNA-agar	21.6	28.5	19.4	30.5	41.0	1.10	0.90
(3) Bound in DNA-agar	21.1	28.4	18.8	31.7	39.9	1.10	0.90
(4) Hydroxyapatite thermal chromatogram at 82.5°	17.9	32.2	15.7	34.2	33.6	1.14	0.94
(5) Hydroxyapatite thermal chromatogram at 85.0°	19.8	31.7	17.4	33.3	37.2	1.14	0.95
(6) Hydroxyapatite thermal chromatogram at 95.0°	24.0	26.2	23.4	26.3	47.5	1.03	1.0

Fig. 2. Effect of incubating the hydroxyapatite fractions illustrated in Fig. 1 with DNA-agar. Nine-tenths of each thermal-chromatogram fraction was dialysed against $0.01 \times \text{SSC}$, denatured by heating at 100° for 10 min. and made up to $2 \times \text{SSC}$ before overnight incubation with DNA-agar. ●, Homologous mouse DNA; ○, heterologous rat DNA. Percentage binding is the fraction of the radioactive chromatogram fractions remaining stable at 60° after incubation. —, Base composition of the fractions, transferred from Fig. 1.

band that he isolated from denatured mouse DNA by caesium chloride density-gradient procedures. Our earlier results (Walker & McLaren, 1965b), in which the fraction of the minor component did not decrease as the concentration of DNA was lowered, appear to have been due to renaturation occurring at the elution front of the 0.12 M -phosphate washes. If denatured DNA is run on to the column at 70° in 0.12 M -phosphate instead of 0.03 M -phosphate as in the original procedure, then the increase in size of the fraction eluted at 0.3 M -phosphate shows normal second-order kinetics.

If we first remove the rapidly renaturing fraction and then incubate the remainder in high salt concentration (0.3 M -sodium chloride) for 18 hr. at 60° , more of the DNA will renature as shown by the fractionations illustrated in Fig. 3. Without incubation little or none of the DNA is eluted at 0.3 M -phosphate, but after 18 hr. incubation a part of the DNA now appears at this position, and the magnitude of this renatured fraction depends on the concentration of the DNA during incubation (Fig. 3 inset).

As in the first section, we have tested the fractions that renature at fast and medium rates with the DNA-agar technique. Table 2 shows that the two renaturing fractions bind almost equally well with homologous DNA. Presumably the conditions for agar binding, which include overnight incubation at 60° in $2 \times \text{SSC}$, are not stringent enough to distinguish between them. On the other

Fig. 3. Salt chromatograms on hydroxyapatite at 70° of 0.12 M -phosphate fractions previously separated at 70° on hydroxyapatite. ↓ ↓, Indicated salt fractions; ●, a second fractionation of DNA (0.14 mg./ml.) that followed the first without an intermediate incubation; ○, a second fractionation that followed the first after incubation for 18 hr. at 60° in 0.3 M -NaCl- 0.01% sodium dodecylsulphate. After incubation, the DNA was diluted threefold with 0.03 M -phosphate before being adsorbed on to the hydroxyapatite. The inset includes two other parallel experiments; ▲, percentage of DNA eluted at 0.30 M -phosphate after incubation at the stated concentration.

hand, there is a significant difference in the between-species incubations; the fast-renaturing mouse fraction is more different from rat DNA than is the fraction with the medium rate of renaturation.

The thermal chromatography procedure, discussed in the first part of the Results and Discussion section, provides a means of comparing the range of stabilities of renatured DNA molecules. It is thus analogous to an optical melting curve but can be applied to isotopically labelled material. In Fig. 4 we have compared the stabilities of the two renaturing fractions with those of their parent native DNA.

The elution curves of the two renaturing fractions are almost identical. It is therefore probable that they include DNA fragments of the same range of base composition and of perfection of match between sequences, despite the great differences in concentration and incubation time that preceded their formation. Further, there is an almost identical temperature difference (5.5°) between the melting temperature (T_m) of the renatured fractions and that of native DNA as there was in the optical measurements quoted above. It should also be noted that the T_m of the native DNA as measured by thermal chromatography (89.5° in 0.12 M -Na $^+$) is

Table 2. *Binding capacity of mouse DNA fragments that renature at a medium rate after incubation, when tested in the DNA-agar procedure against mouse and rat DNA*

For comparison we include the binding capacities of the two fractions obtained without prior incubation, taken from McLaren & Walker (1966). Fractions 1 and 2 were first fractionated at 70° to remove the 10% of fast-renaturing DNA (as in Fig. 5a) and then incubated at 60° for 18 hr. in 0.3M-NaCl at a concentration of 0.5 mg./ml. before re-fractionation at 70° on hydroxyapatite. Then 0.5 ml. of the fragments fractionated a second time at the indicated phosphate concentration was added at a concentration of 0.01 mg./ml. to each agar incubation mixture. In fractions 3 and 4, the DNA was denatured at 0.12 mg./ml. and then immediately run on to the column without further incubation. The DNA-agar contained high-molecular-weight DNA from either mouse or rat, as indicated.

Fraction of mouse DNA fragments	Fragments bound (%)	
	Mouse DNA	Rat DNA
After prior incubation		
(1) 0.12M-Phosphate	17.2	15.0
(2) 0.30M-Phosphate	45.6	16.4
Without prior incubation		
(3) 0.12M-Phosphate	26.1	20.0
(4) 0.30M-Phosphate	45.1	8.7

considerably higher than that from the comparable optical measurement (79.6° in 0.1M-Na⁺). This may be accounted for by the free Ca²⁺ ions in the column material, but it also may reflect the ability of hydroxyapatite to stabilize partially denatured molecules. The melting temperature may then be a function not of mean composition but of the presence of small regions rich in guanine and cytosine.

These results show that by selecting the time of incubation and the concentration of denatured DNA it is possible to purify, in part at least, DNA fragments that have a wide spectrum of renaturation rates, and that though these fractions may be similar in their chemical properties they differ interspecifically, i.e. in those properties peculiar to DNA fractions from mouse and rat.

Salt chromatograms at low temperature. Bernardi (1965) used increasing phosphate concentration to elute DNA from hydroxyapatite at room temperature. Most of his denatured DNA was eluted at 0.15–0.5M-phosphate and he reported that rechromatography of these peaks resulted in a similar distribution to that in the original chromatogram. This is opposite to the situation when high-temperature chromatography is used, since Walker & McLaren (1965b) showed that at 70° the fractions appeared at the same position when run a second

Fig. 4. Thermal chromatograms of native and renatured fractions of mouse DNA that have been ultrasonically treated. In each set of measurements the DNA fragments were at a concentration of 0.5 mg./ml. of 0.03M-phosphate. ●, DNA fragments left in the native state; ▲, heat denatured only; △, heat denatured and then incubated in 0.3M-NaCl-0.01% sodium dodecyl sulphate for 18 hr. The third alternative was diluted threefold with 0.03M-phosphate and dialysed against 0.03M-phosphate in the cold before being adsorbed on to the column at room temperature. The remainder were diluted threefold and adsorbed directly. The temperature was raised to 70° and the column was washed with 0.12M- and 0.16M-phosphate, as in our standard stepwise elutions. The column was then washed with 0.08M-phosphate and the temperature was increased in 2.5° increments. The graphs show the cumulative total of the fragments eluted in this last phase of the procedure.

time. A reasonable explanation of Bernardi's (1965) results is that at room temperature secondary structure is stabilized by hydrogen-bonding between so few bases that many possible conformations are possible. Hydroxyapatite separates the DNA on the basis of the particular conformations present at the time of elution. After elution the DNA may rearrange itself to give the same spectrum of single and partially duplex molecules as in the original unfractionated preparation. At higher temperatures the number of possible stable duplex conformations is more restricted, since it depends on the presence of longer complementary sequences, and these will not be uniformly distributed among the DNA fragments.

Fig. 5. Stepwise salt chromatograms of denatured DNA at two temperatures. $\downarrow\downarrow$, Indicated salt fractions. (a) Initial 70° chromatogram; (b) the 0.12M-phosphate fraction from (a) rechromatographed at 40°; (c) the 0.12M-phosphate (●) and the 0.30M-phosphate (○) fractions from (b) rechromatographed at 40°. Neither the initial nor the fractionated DNA was incubated at high temperatures before fractionation, and the DNA samples were at the following concentrations (mg./ml.) before threefold dilution and application to the column: (a) 3×10^{-1} ; (b) 6×10^{-3} ; (c) 2×10^{-4} (0.12M) and 1×10^{-4} (0.3M).

This hypothesis can be investigated by studying the reproducibility of chromatogram fractions at an intermediate temperature. After a preliminary fractionation at 70° (Fig. 5a), which eliminates the 10% of renatured mouse DNA (at 0.3M-phosphate) that has secondary structure stable at this temperature, we fractionated the single-stranded molecules at 40° (Fig. 5b). This shows that 11% of the remaining fragments form secondary structure stable above 0.16M-phosphate, and another 30% have the presumably less perfect structures that nevertheless allow them to remain adsorbed on to hydroxyapatite at 0.12–0.16M-phosphate.

The peaks eluted at 0.12M- and 0.30M-phosphate were then rerun a third time (Fig. 5c). It was found that, whereas the low-salt fraction is eluted with almost identically the same pattern as on the second fractionation, the high-salt fraction gave a very different pattern with a pronounced maximum at the high-salt position.

Table 3. *Effect of dilution on the proportion of DNA eluted at different salt concentrations during fractionation at 40° on hydroxyapatite*

The 0.12M-phosphate fraction from a previous 70° fractionation was diluted fourfold with distilled water, giving a concentration of 0.0034 mg./ml. Samples were further diluted as shown, and all four samples denatured by heating at 100° for 10 min. before being adsorbed on to hydroxyapatite.

Further dilution ...	DNA eluted (%)			
	$\times 1$	$\times 10$	$\times 100$	$\times 1000$
Phosphate concn. at which DNA was eluted				
0.16M	35.9	38.1	37.6	38.9
0.30M	3.1	2.7	5.0	4.4

We then repeated the first 40° fractionation on a different batch of DNA to determine whether the pattern of peaks was dependent on the concentration at which they were adsorbed on to the hydroxyapatite and therefore whether the secondary structure revealed was likely to be inter- or intra-molecular. In this experiment we diluted the 0.12M-phosphate fraction over a 1000-fold range and heat-denatured each sample before adsorbing it on to the same amount of hydroxyapatite. There is no evidence that the amount of secondary structure increases with concentration (Table 3).

We also repeatedly fractionated the 0.12M-phosphate fractions at 40° from this same batch of DNA and found that after three fractionations the radioactivity appearing at 0.16M-phosphate and 0.12M phosphate fell to 9.6% and 1.6% respectively.

We conclude that, even at the comparatively low temperature of 40°, there is some restriction on the possible duplex conformations that can form within these DNA fragments. It should therefore be possible to use the method to separate DNA fragments with secondary structure stable within a narrow temperature range, and it is likely that the higher-temperature components will be richer in guanine and cytosine.

Hydroxyapatite columns provide a simple method of separating DNA into fractions that differ in base composition, in rate of renaturation or in the secondary structure of the denatured molecule. The examples described in this paper show how earlier observations (Walker & McLaren, 1965a; McLaren & Walker, 1966) can be extended. Thus we have found that the large fraction of DNA that does not bind in agar and the sequences common to rat and mouse are distributed non-randomly among DNA fragments of differing base composition.

The renaturation behaviour of certain fractions of mammalian DNA is explained by the findings of

Britten (1965) and Waring & Britten (1966), who have measured the rates of renaturation of various fractions isolated from the DNA of higher organisms. The results indicate that there are classes of DNA fragments that have sequences sufficiently similar to allow them to renature, and that the proportion of the genome in each class, having a different rate of renaturation, is widely variable. Thus in the mouse there are perhaps a total of 10^6 copies of sequences of about 250 nucleotides, which account for the rapidly renaturing fraction that Britten (1965), Walker & McLaren (1965b) and Flamm, Bond, Burr & Bond (1966) have found. More slowly renaturing fractions, such as those described in the present paper, contain classes that are less frequently repeated in each genome, and the fractions that do not renature in these or the DNA-agar experiments have probably only a few copies in each chromosome set.

We have recently provided striking evidence for the simplicity of the fast-renaturing fraction from mouse DNA, since we have been able to isolate and characterize its complementary chains (Flamm, McCallum & Walker, 1967).

Most of the results described in the present paper have been obtained by thermal chromatography and by salt fractionation at high temperatures on hydroxyapatite. The third method in which denatured DNA is separated on its ability to form intrastrand secondary structure appears, at present, less useful. It does show that denatured DNA contains molecules, with varying abilities to coil

back on themselves and form secondary structure, stable above 40° . It is likely that this property reflects their base composition, and therefore the procedure is a less elegant analogue of the thermal chromatography method, which, however, can only be applied to native or renatured DNA.

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