Thermal Stabilities and Species Specificities of Reannealed Animal Deoxyribonucleic Acids*

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ABSTRACT: Single-stranded mouse deoxyribonucleic acid (DNA) fragments incubated at 55, 60, or 65° in double-strength standard saline–citrate solution partially lose their capacity to react subsequently with mouse DNA single strands embedded in agar. The extent of this loss is time and concentration dependent. Incubated fragments fully regain their original capacity to bind to DNA–agar when heated to 100° and rapidly cooled. If heated between the incubation temperatures and 100°, an increased capacity of the previously incubated fragments to bind to DNA–agar is observed and a melting profile of the product formed in free solution may be constructed. The midpoints of the melting profiles (T_m) increase as the incubation temperature is raised but, in all cases, are lower than the T_m of native DNA. Fragments recovered after reaction with DNA–agar and subsequently incubated in free solution form products which have a T_m 3–4° lower than the DNA fragments from which they originated. This implies a selection of a special class of animal DNA fragments which react with DNA in agar.

The proportion of mouse DNA fragments binding to mouse DNA–agar at 40° (81%) is considerably greater than the fraction reacting at 67° (21%). A large proportion (66%) of chicken DNA fragments, for example, also bind to mouse DNA–agar at 40° while at 67° only 0.9% reacts. When a mixture of ^{3}H-labeled mouse DNA fragments and ^{32}P-labeled rhesus monkey DNA fragments are incubated with mouse DNA–agar, the ratio of $^3H/^{32}P$ bound is 7 at 40° and 35 at 67°. When the same labeled pair is similarly examined with rhesus monkey DNA–agar the $^3H/^{32}P$ bound falls from about 6 at 40° to 1 at 67°. Therefore, the ability to discriminate among animal DNA's with the DNA–agar technique increases as a function of the incubation temperature.

Denaturation and renaturation of viral and bacterial deoxyribonucleic acids (DNA's) have been extensively examined (Marmur and Doty, 1961, 1962; Doty *et al.*, 1960; Marmur and Lane, 1960). Similarly, considerable attention has been given to the denaturation of animal DNA's (Marmur and Doty, 1962). However, studies of the renaturation of animal DNA have been hampered by the small degree of reannealing observed by classical means (Marmur and Doty, 1961). Bacterial and animal DNA–DNA interactions may now be detected using the DNA–agar technique (Bolton and McCarthy, 1962; Hoyer *et al.*, 1964; Walker and McLaren, 1965). Some of the properties of these interactions are the subject of this communication.

Methods presently available for the examination of polynucleotide interactions include equilibrium sedimentation (Hayashi and Spiegelman, 1961; Yankofsky and Spiegelman, 1962), the determination of enzymatic stability of reaction products (Geiduschek *et al.*, 1962), and retention on nitrocellulose filters (Nygaard and Hall, 1963). These methods all depend upon the free solution interaction of either DNA with DNA or DNA with ribonucleic acid (RNA). In free solution the proportion of polynucleotide sites available for reaction is determined by the rate of reformation of DNA duplexes between homologous DNA strands. Complex networks of incompletely reannealed DNA strands appear to be the product of the reaction of long strands of animal DNA's (Eigner, 1963). More recently Britten and Waring (1965) have overcome some of the difficulties of free solution interaction of animal DNA's by prolonged incubation of DNA fragments of molecular weight less than 10^6.

Individual DNA strands may be restrained from interaction with themselves if they are embedded in or on cellulose strands (Bautz and Hall, 1962), agar particles (Bolton and McCarthy, 1962), or nitrocellulose filters (Gillespie and Spiegelman, 1965). The DNA–agar method has been applied to the study of animal DNA–DNA interactions (Hoyer *et al.*, 1964; Walker and McLaren, 1965). Labeled DNA fragments from various animals have been examined for their ability to react with DNA embedded in agar, and the thermal stability of the reaction products has been examined. Nearly all previous observations were made at one incubation temperature (60°), which was chosen to correspond to the reaction optimum for DNA's of 42% G+C[1]

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[1] Abbreviations used: G, guanosine; C, cytidine; MEM, minimal essential medium; SSC, 0.15 M NaCl–0.015 M sodium citrate; HMP, 0.0025 M Na_2HPO_4–0.005 M NaH_2PO_4–0.001 M EDTA; TCA, trichloroacetic acid.

composition. Although the specificity of the animal DNA–DNA interaction is well substantiated, the physical properties of the reaction products are not well understood.

We have employed the agar technique as an indicator of the properties of the free solution reaction products of animal DNA's. Specificity of the binding of various animal DNA fragments to DNA in agar has been determined for different incubation temperatures.

Materials and Methods

DNA Preparation

DNA for embedding purposes was prepared from cell nuclei by the method of Berns and Thomas (1964), as modified by McCarthy and Hoyer (1964). Radioactive DNA was prepared from embryonic or adult tissue culture exposed to [^{14}C]thymidine, [^{3}H]thymidine in complete Eagle's MEM with 5% fetal bovine serum, or [^{32}P]orthophosphate, the latter with phosphate-free Eagle's MEM and 2% fetal bovine serum. Labeled DNA in SSC was fragmented by mechanical shear of 10,000–15,000 psi to a molecular weight of about 0.4×10^6 (McCarthy and Bolton, 1964).

Preparation of DNA Agar and Agar Incubation

DNA was embedded in 4% Oxoid Ionagar No. 2 as described elsewhere (Bolton and McCarthy, 1962) and pressed through a stainless steel screen. DNA trapped in agar was assayed spectrophotometrically by dissolving 0.5 g of agar in hot 5 M sodium perchlorate. All agar preparations used in these studies contained 200–500 μg of DNA/g of agar.

Equal volumes of labeled fragments and agar were incubated for ~16 hr in 5-ml siliconized screw-cap vials. The "tea bag" procedure (McCarthy and Hoyer, 1964) was used to elute the radioactive fragments which, in turn, were counted on membrane filters after precipitation with 5% TCA in the presence of 50 μg of phenol-extracted RNA carrier. "Bound" fragments used for subsequent studies were recovered by precipitation with 2 volumes of cold ethanol in the presence of 0.1 M NaCl, resuspension in distilled water, and dialysis against two-times SSC.

Melting of Native and "Reannealed" Animal DNA Fragments

Native DNA fragments and the product of a 72-hr free solution reaction were examined by modifying a technique reported by Geiduschek *et al.* (1962). Samples of native DNA fragments or products of free solution[2] reactions were rapidly cooled in an ice–water bath, adjusted to the desired concentration in two-times SSC, and dispensed into screw-cap vials which were placed in a thermostatically controlled water bath. The bath temperature was increased in stepwise increments and held for 15 min after each increment. One vial was removed from the bath just preceding the next increment and rapidly cooled in ice water. A portion of its contents was added to DNA agar in a 5-ml siliconized screw-cap vial, incubated at the desired temperature overnight, and eluted as described above.

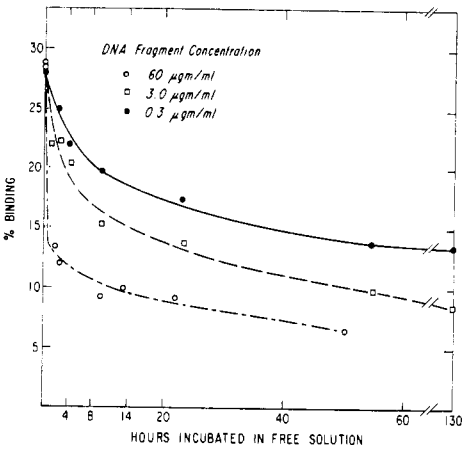

FIGURE 1: Free solution incubation of labeled mouse DNA fragments using mouse DNA–agar as indicator. Heat-denatured ^{3}H mouse DNA fragments in two-times SSC were allowed to react in the absence of DNA–agar for varying periods at concentrations of 60, 3, and 0.3 μg/ml. At the designated times, aliquots were removed and quenched, and a 0.25-ml sample was added to 0.25 g of mouse DNA–agar containing 320 μg of mouse embryo DNA/g of agar. DNA fragment concentration at the time of addition to agar was 0.3 μg/ml. All samples were incubated overnight at 60° and eluted from agar as outlined in "Materials and Methods."

Results

The Free Solution Reaction

Presence of Free Solution Reaction. While the reaction of labeled bacterial and viral DNA fragments with their homologous DNA–agars may involve a large proportion of the labeled fragments, a smaller proportion of animal DNA fragments react under similar conditions. The existence and extent of DNA–DNA interaction *external* to the agar as one cause of the lower binding of animal DNA was first investigated. Tritiated C-57 mouse embryo DNA fragments, at concentrations of 60, 3, and 0.3 μg/ml, were denatured in two-times SSC by heating to 100° for 15 min. After rapidly cooling in an ice–water bath, the denatured fragments were portioned into screw-cap vials which were incubated at 60°. At intervals, individual vials were removed and cooled in an ice–water bath. The concentration of DNA was adjusted to 0.3 μg/ml with two-times SSC and a 0.25-ml sample was added to 0.25 g of mouse embryo DNA–agar and incubated

[2] The term "free solution product" is used to indicate the partially duplexed DNA fragments re-formed in the absence of embedded DNA. "Reannealing" of animal DNA fragments under the conditions employed herein probably results in partial reformation of the interstrand structure of the native DNA.

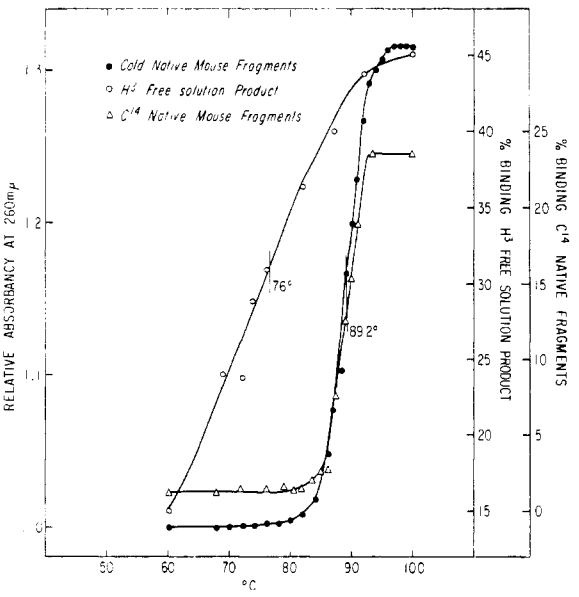

FIGURE 2: Thermal denaturation of the 60° free solution product and native DNA fragments. Denatured ^3H mouse DNA fragments in two-times SSC were incubated at 60° for 3 days at a concentration of 6 μg/ml. These labeled fragments and native DNA fragments labeled with [^{14}C]thymidine were exposed to successively higher temperatures for 15-min periods and quenched in ice. Samples (0.25 ml) of the fragments were then added separately to 0.25 g of mouse embryo DNA-agar. The ^3H-labeled fragments were added to DNA-agar containing 270 μg/g of agar; the ^{14}C-labeled fragments were added to DNA-agar containing 120 μg/g of agar. Elution of fragments from the DNA-agar was done in the usual manner. Unlabeled mouse DNA fragments at a concentration of 150 μg/ml in two-times SSC were thermally denatured and monitored in a Gilford spectrophotometer.

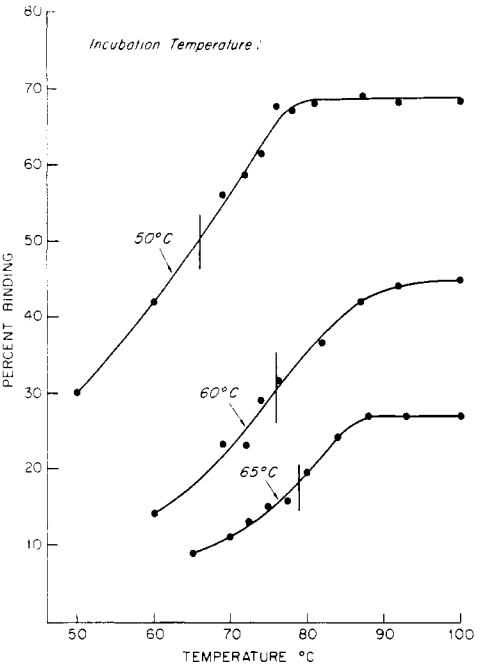

FIGURE 3: Thermal denaturation of 50, 60, and 65° free-solution reaction products. Denatured ^3H mouse DNA fragments in two-times SSC were incubated at 50, 60, and 65° for 3 days in the absence of DNA-agar at a concentration of 6 μg/ml. These fragments were individually exposed to successively higher temperatures for 15 min at which time samples were removed and quenched in ice. Samples (0.25 ml) of labeled fragments (0.075 μg) were added to 0.25 g of mouse embryo DNA-agar containing 270 μg of DNA/g of agar. Overnight incubation and two-times SSC elutions were carried out at 50, 60, and 65°, respectively. Final elutions with SSC/100 were done at 70°.

overnight at 60°. The proportion of labeled fragments bound was then determined. The results of this experiment are illustrated in Figure 1. The initial proportion of fragments bound to DNA-agar (28–29%) was reduced by nearly 80% in the case of fragments incubated at a concentration of 60 μg/ml (lower curve, Figure 1) and a 40% reduction in binding was observed at 0.3 μg/ml (upper curve, Figure 1). It should be noted that most DNA-agar incubations are performed with labeled DNA concentrations of 0.05–1.0 μg/ml. These data support the view that DNA–DNA interaction external to the agar occurs to an appreciable extent even at a low concentration of DNA fragments and that this external reaction may be in part responsible for the low levels of binding (25–40%) observed with animal DNA fragments.

Thermal Stability of Native and "Reannealed" Mouse DNA Fragments. Thermal denaturation of native and "reannealed" animal DNA's can be compared by measuring the resultant binding of labeled fragments to DNA-agar following exposure to successively higher temperatures.

NATIVE FRAGMENTS. Native mouse DNA fragments labeled with [^{14}C]thymidine were suspended in two-times SSC, dispensed into separate screw-cap vials, and denatured in a thermostatically regulated water bath, as described in Methods. Samples were added to mouse DNA-agar and incubated at 60° overnight. The ability of the fragments to bind to DNA-agar thus served as the index for melting of the native fragments and made it possible to plot their thermal denaturation profile. The midpoint of the thermal denaturation curve (T_m) determined by the DNA-agar procedure was 89°, the same value observed spectrophotometrically with similar fragments in two-times SSC (Figure 2). A temperature spread of approximately 13° (6.5° on either side of the T_m) was found with both procedures.

"REANNEALED" FRAGMENTS. Denatured ^3H mouse DNA fragments were allowed to incubate at 60° in two-times SSC for 3 days at a concentration of 6 μg/ml. The DNA concentration was then adjusted to 0.3 μg/ml

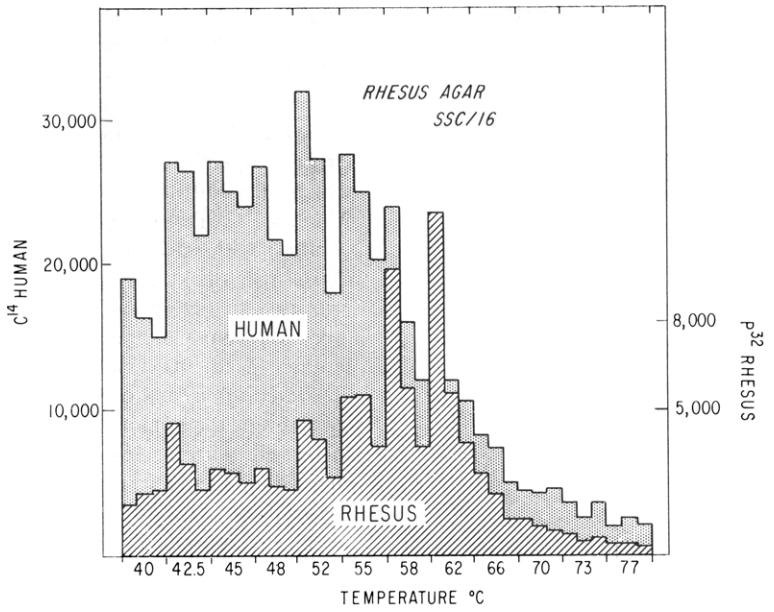

FIGURE 4: Temperature elution profiles of rhesus monkey and human DNA fragments from rhesus monkey DNA–agar. HeLa cell (human) [^{14}C]thymidine-labeled (100 μg, 4100 cpm/μg) and rhesus monkey ^{32}P-labeled DNA fragments (10 μg, 8000 cpm/μg) were denatured, quenched, and mixed in 0.5 ml of times SSC. The mixture was added to 0.50 g of rhesus monkey [^{3}H]thymidine-labeled DNA embedded in agar (160 μg/g, 700 cm/μg) and incubated overnight. The unbound fragments were washed free at 60° and the temperature lowered to 40°, whereupon a series of washes with twofold decreases in salt concentration to SSC/16 were made. The temperature was then increased stepwise and three washes with 15 ml of SSC/6 were made at 10 min/wash. The eluted fragments were collected on membrane filters and counted for 30 min in a liquid scintillation counter set to discriminate among the isotopes. Loss of ^{3}H from the agar monitored the leaching of DNA from the agar; 55.5% of the embedded DNA leached out during the entire elution procedure: 11.5% loss occurred during the times SSC wash and decrease to SSC/16; 5.5% loss to 55°; 15.5% loss from 55 to 66°; 23% from 66 to 77°.

and the sample placed in a water bath and "melted out" as described in the preceding paragraphs. As can be seen from Figure 2, the midpoint of the thermal denaturation curve so obtained is 13° below that of the native DNA fragments which indicates imperfect reannealing. Britten and Waring (1965), using optical parameters, have noted a free solution product with similar thermal properties.

The effect of temperature on the formation of the free solution DNA–DNA product was examined by allowing denatured ^{3}H mouse DNA fragments to react at a concentration of 6 μg/ml in two-times SSC at 50, 60, or 65° for 3 days. After adjusting the DNA concentration to 0.3 μg/ml, the samples were thermally denatured as described above, quenched in an ice–water bath, and added to mouse DNA–agar. The DNA–agars were then incubated and washed at the same temperatures at which the free solution products had been formed. Figure 3 shows that the thermal denaturation curves obtained in each case have midpoints reflecting the free solution and agar incubation temperatures. Midpoints of the thermal denaturation curves from the 50, 60, and 65° incubations are 66, 76, and 79°, respectively. In addition, the 20–25° temperature spread of the melting profile also indicates a greater heterogeneity of duplex structure than is found in native DNA fragments which melt over a narrow 13° range. It is also evident from this figure that binding of fully denatured DNA fragments to DNA–agar is inversely related to the temperature of incubation (*e.g.*, 70% binding of mouse fragments at 50°). The enhanced binding at the lower temperatures of animal DNA to DNA embedded in agar will be discussed more fully below.

Nature of DNA Fragments Binding to DNA–Agar

Hoyer *et al.* (1964) and Walker and McLaren (1965) demonstrated enhanced binding of animal DNA fragments which previously had bound to DNA–agar. These latter authors examined the thermal properties of the bound DNA fragments by directly "melting" them out of DNA–agar. They washed the agar with SSC/10 and collected fragments released at each temperature increment. Melting homologous and heterologous animal DNA fragments directly out of agar (Figure 4) was difficult to reproduce in our hands, particularly at temperatures exceeding 70° when much of the embedded DNA leached out of the agar. A true equilibrium was not attained, as evidenced by failure of the three successive washes to effectively remove labeled fragments at the selected temperatures.

Consequently, a different technique was employed

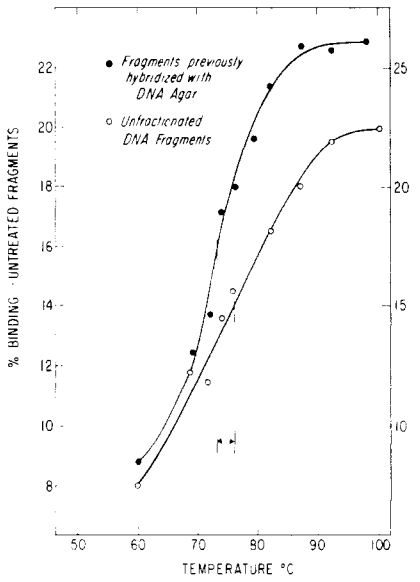

FIGURE 5: Thermal denaturation of "bound" and unfractionated DNA fragments. ^{32}P mouse embryo DNA fragments (105 μg) in 2.00 ml of two-times SSC was added to two 1.00-g samples of mouse DNA–agar and incubated overnight at 60°. The 70° SSC/100 eluate was pooled from the two incubations (total of 15 μg) and the labeled fragments were precipitated with 2 volumes of cold ethanol in the presence of 0.1 M NaCl. The precipitate was resuspended in 1.0 ml of distilled water, dialyzed against two-times SSC, thermally denatured, and incubated for 3 days at 60°. Unfractionated ^{32}P mouse DNA fragments were incubated at 60° in free solution for 3 days at a concentration of 6 μg/ml. Both types of fragments were thermally denatured using mouse DNA agar, containing 270 μg of DNA/g of agar as indicator, as described in Figure 2.

which eliminated some of these difficulties. Bound, labeled mouse fragments were recovered by eluting the DNA–agar with SSC/100 at 70°, dialyzed against two-times SSC, and incubated in free solution at 60° for 3 days. It is evident from Figure 5 that the free solution product generated from labeled DNA fragments which had previously bound to DNA–agar has a T_m 3° lower than the product formed from the original fragments. This suggests a difference between these two classes of DNA fragments and implies a selection of a particular type of DNA during the course of the fragment DNA–agar reaction.

If the bound fragments have properties demonstrably different from those of the whole preparation, a gradual loss of ability of the unbound portion to combine with DNA should be observed on successive recovery and reincubation. Walker and McLaren (1965) showed that the unbound portion, indeed, loses its ability to recombine with DNA–agar. However, animal DNA embedded in agar has a tendency to leach out during incubation and the recovered, unbound, labeled DNA fragments could be contaminated with leached DNA which would act as a competitor and reduce subsequent binding of these fragments. Several cycles of recovery of unbound DNA and reincubation with DNA–agar might indicate successively reduced reaction with embedded DNA because of the presence of successively larger proportions of leached DNA. An experiment which rules out this possibility was done by incubating 2 μg of ^{14}C-labeled human DNA fragments (12,500 cpm/μg) with each of two 1.00-g samples of human DNA–agar (224 μg of DNA/g). One of these samples was used to determine the initial binding of the ^{14}C-labeled fragments and the other provided the "unbound" fragments. One-half of the unbound fragments recovered after each of the three successive incubations was frozen; the remainder was thermally denatured and incubated with a new sample of DNA–agar. Each succeeding incubation mixture, then, contained about one-half the concentration of labeled fragments present in the previous incubation mixture. At the end of the experiment 0.25 μg of ^{32}P-labeled human DNA fragments was added to each stored ^{14}C-labeled sample, melted, quick cooled, added to separate samples of DNA–agar, and examined for binding as described in Methods. Therefore ^{32}P-labeled, unselected fragments served as a monitor for possible competition by leached DNA present in the ^{14}C-labeled unbound fraction. Table I indicates that the unbound DNA gradually loses its binding capacity upon reincubation while the ^{32}P-labeled fragments exhibit nearly constant binding. These data suggest that leaching of DNA out of the agar plays a minor role in reducing binding by competition. Instead, it seems likely that a class of DNA fragments finds binding sites and participates in the DNA–agar reaction.

TABLE I: Progressive Reduction in Binding of ^{14}C Human DNA Fragments Recovered from "Unbound" Portions Remaining after three Successive Reactions with Human DNA–Agar.

	^{14}C Hybridization	Cor[a] ^{14}C Binding	^{32}P[b] Hybridization
Original	19	19	19
First transfer	12	12	20
Second transfer	9	9	20
Third transfer	6	8	16

[a] Corrected for reduction in binding as monitored by the ^{32}P fragments. [b] ^{32}P human DNA fragments (0.25 μg) were added to the "unbound" recovered ^{14}C DNA fragments after each transfer. The ^{32}P fragments then served to monitor the possible influence of leached high molecular DNA on the binding of the ^{14}C fragments.

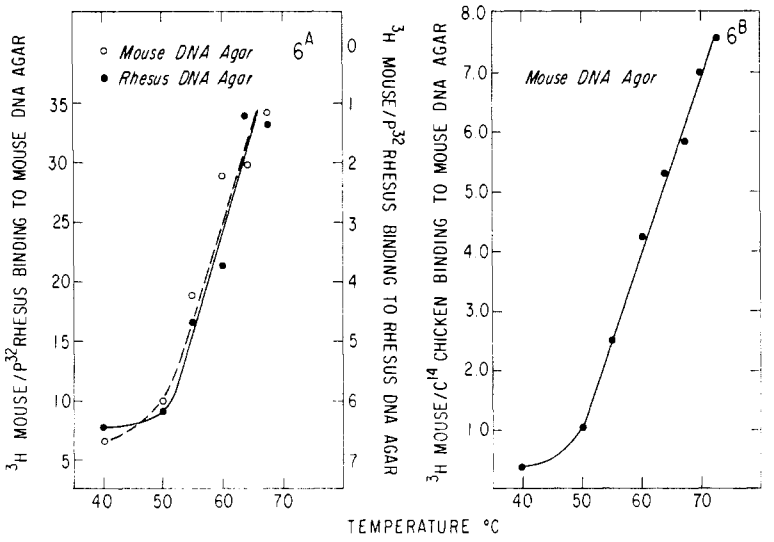

FIGURE 6: Specificity of DNA–agar hybridization as a function of temperature. (A) 0.25-ml samples of a mixture of denatured ^{3}H mouse (0.3 μg/ml), and ^{32}P rhesus DNA (0.4 μg/ml) fragments were incubated overnight at the indicated temperatures with either mouse embryo or rhesus DNA–agar containing 270 and 210 μg of DNA/g of agar, respectively. (B) 0.25-ml samples of a mixture of denatured ^{3}H mouse (0.3 μg) and ^{14}C chicken DNA (0.6 μg) was incubated with 0.25 g of mouse embryo DNA–agar at the indicated temperatures. Ratio of hybridized counts is expressed as a function of incubation temperature in each case.

Effect of Temperature on the DNA–Agar Reaction

Both the thermal stability of free solution products and the proportion of fragments bound to DNA–agar are influenced by the incubation temperature (Figure 3). To investigate this phenomenon as it pertains to the specificity of binding of different DNA fragments to DNA embedded in agar, 0.25-ml samples containing 0.3 μg of ^{3}H mouse embryo DNA and 0.4 μg of ^{32}P rhesus DNA in two-times SSC were added to 0.25 g of either mouse embryo or rhesus DNA–agar and incubated overnight at several different temperatures. The results are presented in Table II. With mouse DNA–agar, for example, binding of fragments at 40° is high in both the homologous and heterologous reactions (90.2 and 81.8% for mouse and rhesus fragments, respectively). Under more stringent incubation conditions (68°) hybridization of both types of fragments to either DNA–agar is lowered but the ratio of bound homologous/bound heterologous radioactivity

TABLE II: Hybridization of Animal DNA to DNA–Agar as a Function of Temperature.

Temp (°C)	Mouse DNA–Agar % Hybridization		Rhesus DNA–agar % Hybridization	
	^{3}H Mouse	^{32}P Rhesus	^{3}H Mouse	^{32}P Rhesus
40	90.2	81.8	79.0	81.0
50	67.8	47.2	49.1	55.1
55	58.1	26.5	19.6	37.6
60	42.2	12.5	7.6	32.0
64	37.6	8.0	—	—
68	5.6	0.9	2.4	18.4

TABLE III: Hybridization of Animal DNA to DNA–Agar as a Function of Temperature.

Temp (°C)	Fragment	% Binding		Counts Bound of ^{3}H/^{14}C, Mouse/chick
		Mouse DNA–Agar	Blank Agar	
40	Mouse	81.1	1.85	0.39
	Chick	66.1	1.16	
50	Mouse	56.2	0.97	1.01
	Chick	16.1	1.04	
55	Mouse	37.7	1.05	2.52
	Chick	4.1	0.79	
60	Mouse	28.9	0.91	4.26
	Chick	1.8	...	
64	Mouse	22.2	0.90	5.30
	Chick	1.0	0.13	
67	Mouse	20.6	...	5.82
	Chick	0.9	...	
70	Mouse	8.6	...	7.01
	Chick	0.3	...	
72	Mouse	5.1	...	7.66
	Chick	0.2	...	

is higher. Therefore, the discrimination between two animal DNA's increases as the incubation temperature is elevated. Also, the change in the ratio of homologous fragments bound/heterologous fragments bound is about the same between 40 and 68° with either mouse or rhesus DNA–agar (Figure 6A). In an analogous experiment performed with a mixture of ^{3}H mouse and ^{14}C chicken DNA fragments and mouse DNA agar, the ratio of ^{3}H bound/^{14}C bound is 0.4 at 40° and 5.8 at 67°, a 14.5-fold change (Table III, Figure 6B). A 4.3-fold change is observed at the same two temperatures with the more closely related mouse and rhesus monkey DNA's. Binding of DNA fragments to agar containing no DNA is only slightly increased at the lower incubation temperatures (Table III).

The differential binding of fragments to DNA–agar at various temperatures can also be utilized to determine relationships of DNA from different animal or plant sources. Using mouse DNA embedded in agar as a reference, the ratio of the 40/60° binding can be examined (Table IV). It is apparent that the homologous reaction (mouse on mouse) gives the lowest value, the most distantly related (barley), the highest, and the ratio of binding at these two temperatures for the other organisms examined lies at an intermediate position, depending on their relationship to the mouse. Hamster DNA, as would be expected, is more closely related to mouse than rhesus DNA; this has been corroborated in competition reactions (Hoyer *et al.*, 1964) employing DNA–agar. From data presently available, it would appear that a given agar preparation gives fairly constant 40/60° ratios with homologous DNA fragments prepared at different times and labeled with different isotopes (Table IV).

TABLE IV: Ratio of 40/60° Binding on Mouse DNA–Agar.

DNA Fragments	40° Binding/60° Binding
^{32}P Mouse	2.82
^{14}C Mouse	2.85
^{32}P Syrian Hamster	7.15
^{32}P Rhesus	12.09
^{14}C Chicken	64.0
^{32}P Barley	96.0

Discussion

The present study was initiated to examine some of the properties of animal DNA interactions. Animal DNA fragments of molecular weight 400,000 react with one another in free solution even at low concentrations (0.3 μg/ml). The extent of such DNA–DNA interaction at this concentration is sufficient to reduce subsequent binding to DNA–agar by 40%. The 40% reduction in binding observed at the DNA fragment concentration of 0.3 μg/ml explains, in part, the low capacity of animal DNA to react with DNA–agar as compared with bacterial and viral DNA's. Greater degrees of DNA–DNA interaction occur in free solution when the fragment concentration is raised to 60 μg/ml. Walker and McLaren (1965) also present data suggesting DNA–DNA interaction at a DNA concentration of 1 μg/ml. The failure to demonstrate the formation of a free solution product in SSC spectrophotometrically was reported by Eigner (1963), who worked with high molecular weight material. He noted nonspecific DNA aggregation at DNA concentrations greater than 50 μg/ml. Britten and Waring (1965) employing optical and isotopic techniques have been able to demonstrate species-specific free solution DNA–DNA products with animal DNA fragments of mol wt 4×10^5.

Walker and McLaren (1965) suggest that intrastrand, rather than interstrand, reactions of melted DNA fragments in free solution limit their ability to react with DNA embedded in agar. This interpretation is mainly based on the results of experiments where binding remains constant over ratios of DNA fragments/DNA in agar of 10^{-4} to 10^{-2}, as also noted by Hoyer *et al.* (1964). In the experiments of Walker and McLaren (1965) 300–500 μg of embedded DNA was used. Consequently, to maintain the 10^{-2} ratio, 3–5 μg of labeled DNA fragments should have been originally present in the volume external to the agar. If the embedded DNA leaches out of the agar [Walker and McLaren (1965) indicate that a maximum of 15% leaching does occur in their experiments], as much as 45–75 μg of "long mouse DNA" could be present as free strands external to the agar. Therefore, it is not surprising that a plateau is reached when the labeled fragments added contribute little to the total DNA external to the agar. Our data suggest that the extent of free solution interaction of DNA fragments is, indeed, concentration dependent, and that interstrand, not intrastrand, interactions reduce the binding of animal DNA fragments to DNA embedded in agar.

The thermal stability of the products of animal DNA interactions whether formed in free solution or with DNA trapped in agar is influenced by the temperature at which the product is formed. DNA fragments allowed to incubate in two-times SSC at 65° in the absence of DNA–agar have a higher T_m (79°) than those exposed to 50° (T_m = 66°). The binding of homologous and heterologous DNA fragments to DNA–agar is also temperature dependent. A large proportion of both homologous and heterologous labeled DNA fragments react with DNA–agar at 40°; at 70°, however, while a smaller proportion is bound, the homologous reaction is favored over the heterologous. It would appear that at higher temperatures of incubation relatively precise helical structures are formed approaching native DNA in thermal stability. At lower incubation temperatures,

less stringent conditions prevail and a product, probably containing many mismatched sequences, is formed. The ability to discriminate among animal DNA's also increases as the incubation temperature is raised. With mouse DNA embedded in agar, the ratio, mouse DNA fragments bound/chicken DNA fragments bound, increases from 1.0 at 40° to 14.5 at 67°. Under similar experimental conditions, the ratio of mouse DNA fragments bound/rhesus monkey DNA fragments bound also increases but the change is only 4.3. This is not surprising in view of the closer taxonomic relationship of the rhesus monkey and the mouse. Higher incubation temperatures should facilitate the discrimination between two very closely related DNA's (*e.g.*, human–chimpanzee or Syrian hamster–Chinese hamster).

Our data and those of Walker and McLaren (1965) both indicate that binding of labeled fragments to DNA–agar involves a selection of a particular class of DNA. Hoyer *et al.* (1964) noted enhanced subsequent binding of labeled animal DNA fragments which had previously been selected by hybridization with DNA–agar. Fragments which fail to react with DNA embedded in agar bind poorly on subsequent incubations, a phenomenon also noted by Walker and McLaren (1965). In addition, we also demonstrated that labeled fragments which previously had reacted with DNA–agar have a thermal denaturation profile with a T_m 3–4° lower than the DNA fragments from which they originated (Figure 5). Thus, it appears that a special class of fragments is selected from the labeled DNA pool and reacts with DNA embedded in agar. This process may involve a species of DNA fragments which is present in greater abundance and implies that redundant polynucleotide sequences are present in animal DNA; this latter conclusion substantiates the work of Britten and Waring (1965).

The reaction of labeled, single-stranded DNA fragments with DNA embedded in agar depends on the pairing of complementary strands. Viral and bacterial DNA's readily renature in free solution, forming products possessing physical and biologic properties similar to that of the native DNA. It was initially thought that the probable heterogeneity of animal DNA would preclude any renaturation of this material. The results obtained with the DNA–agar system (Hoyer *et al.*, 1964), however, demonstrated that animal DNA's can and do interact specifically with one another. Labeled animal DNA fragments which react with DNA embedded in agar are slightly different (Figure 5) from the initial labeled DNA preparation. In other words, a special portion of DNA readily reacts with DNA–agar leaving behind a fragment population with a lower binding capacity. Britten and Waring (1965) have concluded that many polynucleotide sequences occur repeatedly in the DNA of higher organisms as observed by the use of DNA fragments of about 4×10^5 daltons. They observed a fraction of mouse DNA, for example, constituting between 10 and 20% of the total cellular DNA, which rapidly renatures at 60° and forms a sharp band in CsCl at a density characteristic of native DNA. A very large number of copies of this satellite DNA appears to be present per cell. It would appear, therefore, that while the DNA content (and, hence, potential number of genes) of animal cells is approximately 10^3-fold greater than bacterial cells, redundancy of certain nucleotide sequences in the DNA of higher organisms permits the association of strands with approximately complementary sequences. DNA fragments reacting with DNA embedded in agar could represent those portions of the DNA which contain a great number of similar sequences; on the other hand, there may be relatively fewer common polynucleotide sequences in that fraction of the DNA which fails to react with DNA–agar.

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