22 May 1964, Volume 144, Number 3621 SCIENCE

CURRENT PROBLEMS IN RESEARCH

A Molecular Approach in the Systematics of Higher Organisms

DNA interactions provide a basis for detecting common polynucleotide sequences among diverse organisms.

B. H. Hoyer, B. J. McCarthy, E. T. Bolton

Systematics is the scientific study of the kinds and diversity of organisms and of any and all relationships among them.—G. G. SIMPSON, *Principles of Animal Taxonomy*

The demonstration that deoxyribonucleic acid (DNA) is the essential material of heredity (*1*), the finding that DNA is a duplex structure (*2*) capable of decomposition and reconstitution (*3*), and the discovery that "hybridization" between single-stranded DNA components from different origins can occur (*4*) provide a physical-chemical means for assessing genetic relatedness among species (*5*, *6*). In 1945 Simpson commented: "Although (probably) homologous genes can occasionally be recognized in distantly related animals by their phenotypic expressions, the homologization of genetic structure throughout any considerable generic or higher unit appears at present to be an impossible goal" (*7*). This statement is now open to serious challenge, and the earlier optimistic idea (*8*) that species relationships might be established through the detection of common genetic material is at last amenable to experimental test.

Dr. Hoyer is a staff member of the U.S. Public Health Service's Rocky Mountain Laboratory, Hamilton, Montana, and currently also a research associate with the Carnegie Institution of Washington, Washington, D.C. Dr. McCarthy and Dr. Bolton are staff members of the Carnegie Institution.

The "homologization of genetic structure" by physical-chemical means which can overcome the barrier of reproductive isolation inherent in the biology of different living forms has already been accomplished among bacteriophages (*9*) and among bacteria (*6*). Homologies between the DNA's of bacteriophages and host bacteria have also been demonstrated (*10*), and the probable usefulness of a similar physical-chemical approach for the systematics of higher taxonomic categories has been indicated (*11*). It is our purpose in this article to discuss the principle, and to present some illustrative results, of an approach which depends upon the idea that the total genetic potential of an organism is represented in DNA and that polynucleotide sequences held in common between species are indicative of similar genes.

The principle of the method here employed is symbolized by the series of pictographs shown in Fig. 1.

1) Duplex DNA of high molecular weight ($> 10^7$) is heated in order to separate the individual polynucleotide strands; the hot solution is cooled quickly so that the strands will remain separated.

2) The separated single strands are then immobilized (*12*) in agar.

3) Separately prepared radioactive DNA is degraded by mechanical shear force to fragments of molecular weight $\sim 0.5 \times 10^6$ (*6*), and these in turn are heated and quickly cooled to provide separated single-stranded fragments of DNA which, because of their relatively small size, may diffuse freely through the gel structure.

4) Upon incubation of these radioactive single-stranded fragments of DNA with the high-molecular-weight strands of DNA immobilized in agar, recombination will occur, provided that the homologies between the added DNA fragments and the fixed DNA are great enough to permit duplex reformation. The conditions of incubation might as a matter of choice be lax, which would allow relatively indiscriminate combination among interacting macromolecules, or, by contrast, be demanding, which would permit only those DNA molecules having the greatest sequence complementarity to be involved in a stable union. Under lax conditions the DNA's of very diverse creatures might be expected to interact, while under more stringent conditions only those DNA's having the most intimate molecular homologies would be expected to combine. In this article attention is focused principally upon duplex structures which are established by, and which survive, incubation at 60°C, in 0.3*M* NaCl, "overnight," a relatively restrictive condition which minimizes adventitious associations.

DNA-agar gels are prepared by mixing a hot solution of single-stranded DNA with a hot solution of agar and chilling the mixture (Fig. 2). The resulting slab of DNA-agar gel is forced through a screen and thus reduced to granules. These DNA-agar gel particles are suspended in buffer, thoroughly washed, and then incubated with sheared fragments of radioactive single-stranded DNA. After incubation the suspension is transferred to a thermostatically controlled vessel and washed with several portions of the incubation buffer solution; then the hybridized components are eluted by alteration of the temperature or ionic strength (or both) of the eluting solution (*12*). The several fractions so collected are assayed for radioactivity.

Experimental Considerations

In the assessment of genetic relatedness the experimental approach is necessarily one of quantitative comparison of a heterologous reaction with the homologous reaction. The quality of these comparisons will therefore depend upon the extent to which factors influencing the reaction, other than those resulting directly from differences in base sequence, can be controlled. In the paragraphs that follow, the effect of some of these parameters on the fraction of labeled DNA bound to a DNA-agar column is considered. Conditions are described under which reproducible data are obtained.

The renaturation of DNA requires an elevated temperature. In all the experiments described, incubation was carried out at 60°C. From the work of Marmur and Doty (*13*) it appears that this temperature is close to optimal for renaturation of DNA of the base composition found in higher animals (~42 percent guanylic and cytidylic acids, or 42 percent GC). In the system described, the rate of the reaction is limited by the diffusion in and out of the gel particles as well as by the concentrations of DNA in the gel and of the DNA fragments in the solution. Under the conditions normally employed the diffusion seems to be the rate-limiting factor, for the rates of reaction, with the same concentrations of DNA, are very similar whether the DNA originates from a virus (*14*), a bacterium (*6*), or a higher animal. The different order of complexity in these various genomes affects the concentration of individual segments but does not change the effective rate of reaction.

The rate of reaction of labeled fragments of mouse DNA with mouse DNA in agar is shown in Fig. 3. Two experiments are illustrated, in which different DNA-agar preparations were used. In one experiment 1 μg of labeled DNA was used in each incubation, and in the other series 10 μg was used. The results are essentially identical and fall on a single curve. The half-time for the reaction is about 2 hours and is independent of the amount of labeled DNA between 1 and 10 μg. The reaction is

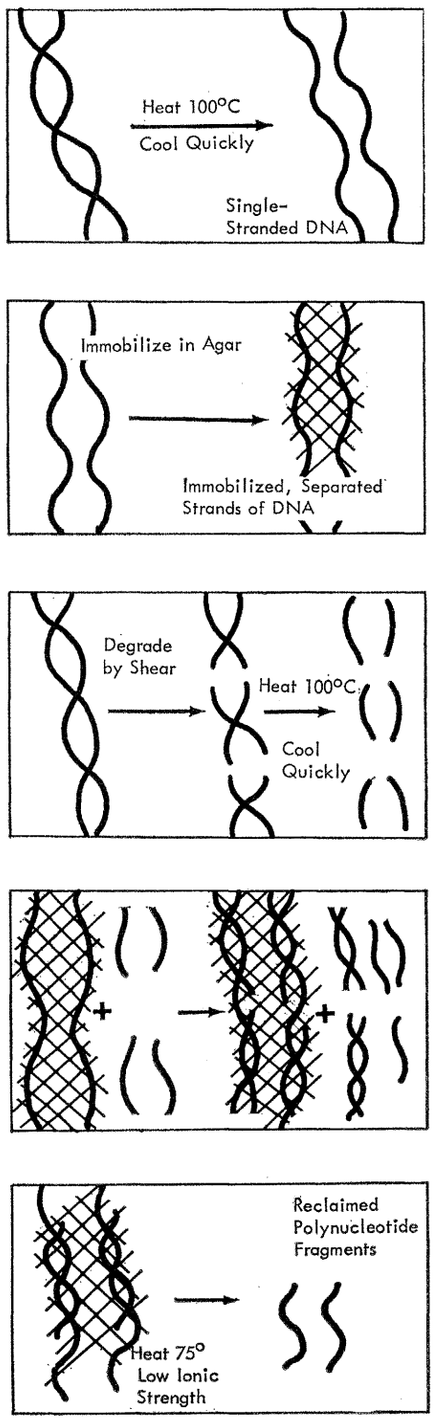

Fig. 1. Diagrammatic representation of the principle of the DNA-agar procedure.

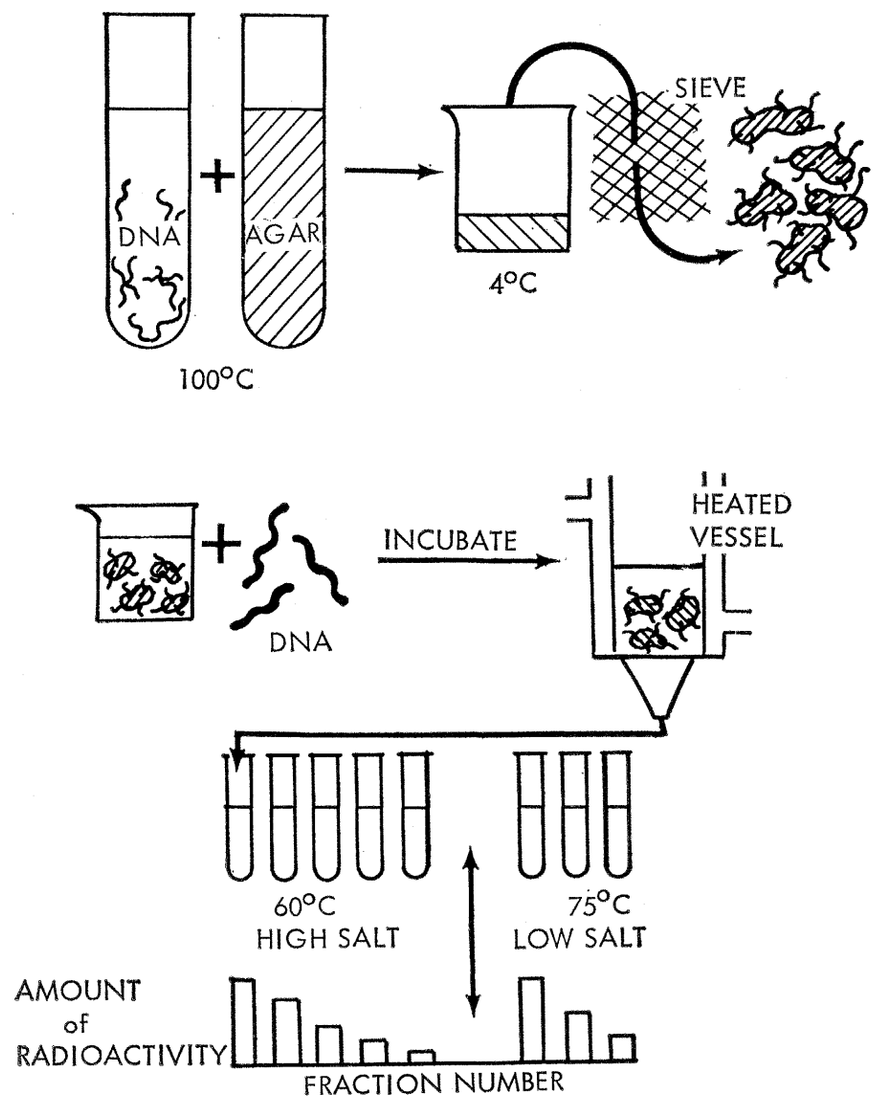

Fig. 2. Representation of the stages in the DNA-agar procedure.

complete after 8 hours. However, it has proved convenient to allow the reaction to proceed overnight, and 16 hours has been chosen as the standard incubation period.

The cessation of the reaction when only some 20 percent of the total labeled DNA fragments are combined with the trapped DNA is a result of another competing reaction. Free fragments of DNA are able to find a complementary strand in solution as an alternative to seeking a complementary site in the trapped DNA. The resulting duplex of DNA fragments is then unable to combine with the trapped DNA (*15*). The relative extent of these two reactions depends upon the amount of DNA fragments compared with the amount of DNA present in the agar gel and upon the fraction of the total liquid volume in the reaction mixture occupied by the gel. It should be noted in passing that in experiments where RNA is the radioactive component in the reaction mixture and a DNA-RNA hybrid is formed, this type of competing reaction does not occur. The RNA molecules are apparently representative of only one of the two strands in DNA (*15, 16*), and therefore no RNA-RNA interaction occurs. In this case 50 percent or more of the RNA may be bound to the DNA in agar (*11, 12*).

If the number of sites provided by the DNA in the agar is large as compared to the concentration of free DNA fragments, then a high percentage of binding is to be expected. Efficiencies of 80 percent can be easily achieved with virus DNA (*10*) or bacterial DNA (*6*). When animal DNA is used under identical conditions, about 25 percent of the labeled DNA fragments are bound. However, under other conditions (see Table 6) a larger proportion may be bound. Figure 4 shows the proportion of mouse DNA fragments bound to mouse DNA in agar as a function of the relative amounts of labeled and of high-molecular-weight DNA. The fraction bound continues to rise as the relative quantity of labeled DNA is reduced, but it levels off as the ratio of quantities falls below 0.01. Ratios of free to trapped DNA lower than 0.01 cannot in fact be achieved, since the embedded DNA slowly leaches out, raising the effective concentration of free DNA. During the course of an overnight incubation at 60°C, thermal degradation may liberate as much as 10 percent of the trapped DNA. This effect would also influence the rate of the reaction, which is found to be the same whether 1 or 10 μg of labeled DNA is used.

The relative extent of the competing reaction in solution will depend on the proportion of the time the DNA fragments spend inside the gel particles. Thus, in very moist incubation mixtures, fewer of the DNA fragments will react with the trapped DNA. The amount of binding decreases as the ratio of the total liquid volume to the volume of the agar gel increases (Fig. 5). However, where more than one-third of the labeled DNA can be expected to react with the trapped DNA, large variations will result from differing water contents of the DNA-agar preparations, and also from the nonuniform penetration of the small added volume of liquid into the particles of agar. It is usually preferable in comparative experiments to add a volume of liquid equal to the volume of the gel, since, as is clear from Fig. 5, minor variations in this added volume will have minimal effects on the fraction bound.

It is often important to know not only how much DNA is trapped in the agar but also what fraction of it is available for duplex formation. In the case of bacterial DNA this fraction was shown to be greater than 80 percent by determination of the maximum amount of homologous DNA fragments which could be bound (*15*). A similar experiment with mouse DNA is shown in Fig. 6. In this case a maximum amount of DNA equivalent to about 70 percent of that embedded in the agar was fixed when very large amounts of DNA fragments were used. An assay of the availability of the

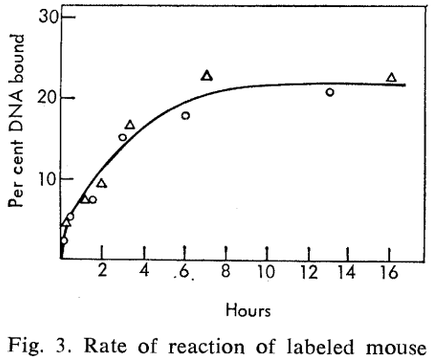

Fig. 3. Rate of reaction of labeled mouse DNA fragments with mouse DNA trapped in agar. The percentage of the labeled DNA bound in a duplex with the DNA in the agar is plotted as a function of the time of incubation at 60°C. Two experiments are plotted, in both of which 0.50 gram of agar gel was incubated with 0.50 milliliter of saline citrate, of double the standard concentration, containing the DNA fragments: (circles) 1 μg of C^{14}-labeled (2500 count/min μg) DNA fragments prepared from mouse L cells incubated with agar containing 192 μg of DNA per gram; (triangles) 10 μg of P^{32}-labeled (100 count/min μg) DNA fragments from mouse embryos, incubated with agar containing 150 μg of DNA per gram.

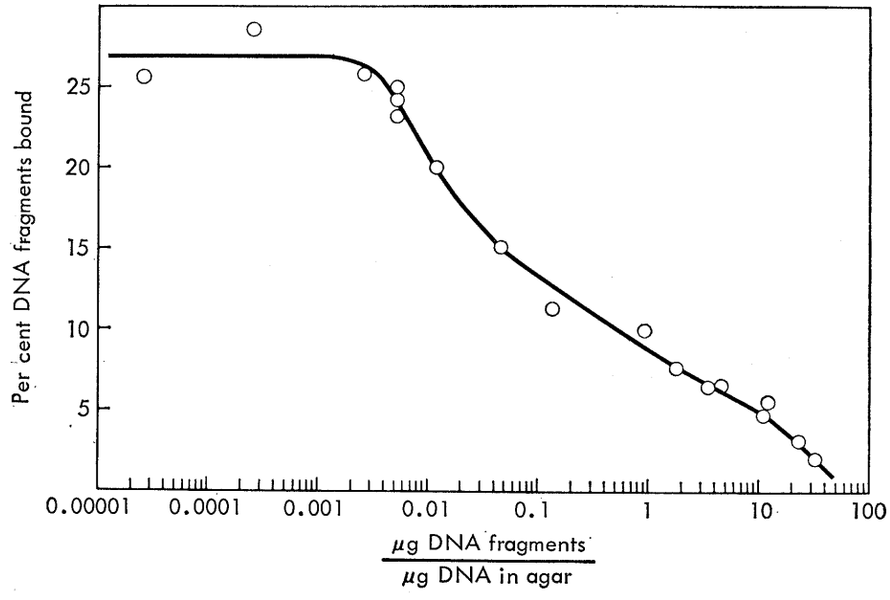

Fig. 4. Dependence of the fraction of mouse DNA fragments duplexed with mouse DNA in agar on the ratio of the amount of DNA fragments to the amount of entrapped DNA. The fraction of DNA fragments bound was determined when various quantities of P^{32}-labeled (200,000 count/min μg) DNA fragments from mouse L cells were incubated with 0.50 gram of agar containing 200 μg of mouse DNA in a total volume of 1.0 milliliter.

trapped DNA for duplex formation can also be made by means of the perchlorate procedure for the determination of DNA in agar (*11*). Thus, without the use of radioactivity, the DNA present in the agar gel may be measured after incubation with various amounts of homologous or heterologous DNA fragments, simply by dissolving it in sodium perchlorate after washing it free of unreacted DNA fragments.

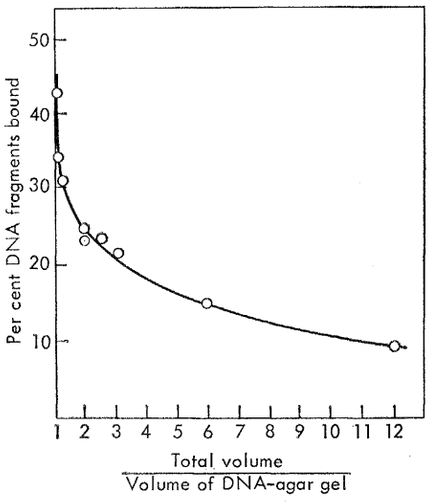

Fig. 5. Dependence of the fraction of mouse DNA fragments duplexed with mouse DNA in agar on the ratio of the volume of DNA-agar gel to the total volume in the incubation mix. One microgram of C^{14}-labeled (2500 count/min μg) DNA prepared from mouse L cells was incubated with 0.50 gram of agar containing 192 μg of mouse DNA at 60°C for 16 hours, with various volumes of double-strength saline citrate added. The percentage of the labeled DNA bound is plotted as a function of the fraction of the total volume occupied by the gel particles.

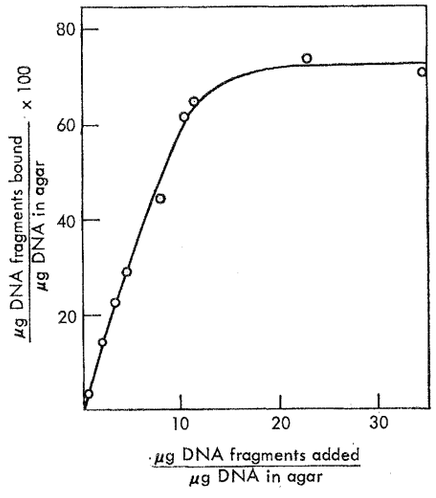

Fig. 6. Saturation of sites in an agar gel containing mouse DNA. The incubations contained 0.50 gram of agar with 192 μg of mouse DNA and various amounts of C^{14}-labeled mouse DNA fragments in a total volume of 1.25 milliliters.

Formation of Hybrid Duplexes by Animal DNA

In comparing polynucleotide homologies among different DNA's it is difficult to obtain routinely many DNA-agar preparations with predetermined DNA content and uniform binding characteristics. In spite of this limitation, in the case of bacterial DNA at least, it has proved possible to measure homologies among DNA's by comparing the binding of a single preparation of labeled DNA fragments with various preparations of DNA agar (*6*). This approach can provide useable data in comparisons among higher forms at higher taxonomic levels. However, the inherent variability in the quality of the animal DNA preparations impairs the precision of these measurements, so that, particularly where more closely related forms are to be compared, the results obtained are only semiquantitative.

This feature is indicated by the following experiments. Mouse L-cell DNA was labeled with either C^{14}-thymidine or P^{32}-orthophosphate. Mixtures of the two radioactive DNA preparations were incubated either with agar containing mouse, hamster, or guinea pig DNA or with agar lacking DNA. The results of Table 1 show that each labeled mouse DNA preparation binds less to the hamster and guinea pig DNA than to the mouse DNA. A measure of the extent of homology between the mouse and these other rodents may be obtained from the relative extents of the reactions. Clearly, the hamster is more closely related to the mouse than the guinea pig. Estimates of the homology afforded by the two differently labeled DNA preparations are 65 and 52 percent, respectively, for the hamster and 22 and 25 percent for the guinea pig.

To improve the quantitation it is desirable to devise a procedure which can compensate for variations in the DNA preparations. This has been accomplished by mixing DNA fragments, labeled with different radioisotopes, and incubating the mixture with various DNA-agars to test their relative binding reactions. An illustrative experiment is described next.

A mixture of P^{32}-labeled mouse DNA fragments and C^{14}-labeled human DNA fragments from HeLa cells was incubated overnight at 60°C with agar containing either mouse DNA or human DNA. Figure 7 shows the results of these experiments. The agar containing human DNA bound 19.5 percent of the human DNA fragments, as compared to 4.6 percent of the mouse fragments. Conversely, 4.4 percent of the human DNA fragments and 18.3 percent of the mouse DNA fragments were bound to agar containing mouse DNA. The relative extents of the heterologous and homologous reactions in the two experiments are in good agreement. A similar finding is described in Table 2. Thus these two species appear to have in common

Table 1. Reaction of a mixture of C^{14}-labeled and P^{32}-labeled mouse DNA fragments with various DNA-agars. Mixtures of 1.0 μg of C^{14}-labeled (2500 count/min μg) and 0.1 μg of P^{32}-labeled (10,000 count/min μg) mouse DNA fragments in 0.5 ml of double-strength saline citrate were incubated with and eluted from (see Fig. 7 for details) 0.50 gram of any one of various DNA-agars or of agar lacking DNA. Labeled fragments were prepared from mouse L-cell cultures exposed to $10^{-6}M$ C^{14}-thymidine in Eagle's medium with 2 percent calf serum or to P^{32}-orthophosphate (25 μc/ml) in phosphate-free Eagle's medium with 2 percent calf serum.

DNA in agar	DNA (μg/g of agar)	Labeled DNA bound (%)	
		C^{14}	P^{32}
Mouse	280	27	23
Hamster	370	14	15
Guinea pig	220	6	6
None	0	1.0	0.5

Table 2. Reaction of C^{14}-labeled human DNA fragments and P^{32}-labeled mouse DNA fragments with various DNA-agars. DNA-agars (0.50 g) or agars lacking DNA (0.50 g) were incubated with a mixture of C^{14}-thymidine-labeled (220 count/min μg) HeLa cell DNA fragments (8 μg) and P^{32}-labeled (100 count/min μg) BALB/C mouse embryo DNA fragments (15 μg) in 0.6 ml of double-strength saline citrate. The procedure is described in detail in Fig. 7.

DNA in agar	DNA (μg/g of agar)	Labeled DNA bound (%)	
		C^{14}	P^{32}
Human	650	18	5
Mouse	1020	6	22
Rhesus monkey	450	14	8
Rat	350	3	14
Hamster	370	3	12
Guinea pig	280	3	3
Rabbit	390	3	3
Bovine	720	5	4
Salmon	600	1.5	1.5
Escherichia coli	400	0.4	0.4
None	0	.4	.4

some 20 to 25 percent of their polynucleotide sequences. It is worth noting that such agreement between reciprocal reactions can occur only where the total complement of DNA in the two species is very similar.

Stability of Hybrid DNA Duplexes

It would be of great interest to determine how similar two sequences of nucleotides must be to form duplex structures under the conditions here described. The specificity exhibited in the reactions among nucleic acid preparations in which the overall nucleotide composition is similar has certainly been amply demonstrated (*6, 12*), but it is not clear how many minor differences in the nucleotide sequence can be tolerated. If the duplex structure formed in heterologous reactions contained relatively large mismatched regions which are looped out of the main helical structure, it would seem possible that this structure would be detectably less stable than the structure formed by homologous DNA fragments. This possibility was tested by comparing the stability, at increasing temperature, of the duplex formed by rhesus monkey DNA fragments with mouse DNA in agar with the stability of the duplex formed by homologous mouse DNA fragments, as described next.

A mixture of C^{14}-labeled mouse DNA fragments and P^{32}-labeled rhesus monkey DNA fragments was incubated with 0.5 gram of agar containing 192 μg of mouse DNA. The agar gel was then washed with ten 15-milliliter fractions of saline citrate of 0.1 standard concentration (*17*), at 46°C. Subsequently three 10-milliliter fractions were collected, at each of several higher temperatures (Fig. 8, top). The contents of C^{14}- and P^{32}-labeled DNA in each fraction were then determined. It has been shown for bacterial DNA-DNA and DNA-RNA duplex structures that the fractionation temperature depends on the base composition of the duplex structure (*18*). Thus, DNA fragments appear at a temperature that is characteristic for the percentage of guanylic acid and cytidylic acid in the molecules. In the case of the preparations of animal DNA in agar, however, it appears that the DNA is not so firmly entrapped, so that there is, superimposed on the distribution of DNA fragments according to their composition, a trail resulting from loss of entrapped DNA and radioactive DNA associated with it. Nevertheless, while only some 5 percent of the monkey DNA appears in the fractions eluted at temperatures higher than 51°C, compared with more than 20 percent of the mouse DNA, the heterologous structures appear to be equally stable. Thus, at temperatures of 56°C and higher the ratio of P^{32} to C^{14} is essentially constant. Apparently, about one-fifth of the monkey DNA sequences can form heterologous duplex structures whose stability is comparable to that of homologous duplexes.

In Fig. 8 (middle) a similar experiment is illustrated, in which the P^{32}-labeled DNA originated from a bacterium of the Providence strain whose overall base composition is close to that of mammals. In this case, essentially no P^{32} appears in the fractions selected at higher temperatures and the pattern given by the C^{14}-labeled mouse DNA is quite similar to that in Fig. 8 (top). In the experiment of Fig. 8 (bottom) both the C^{14}- and the P^{32}-labeled DNA fragments were of mouse origin; hence the percentages of the two isotopes in the various fractions were identical.

The temperature at which the duplex

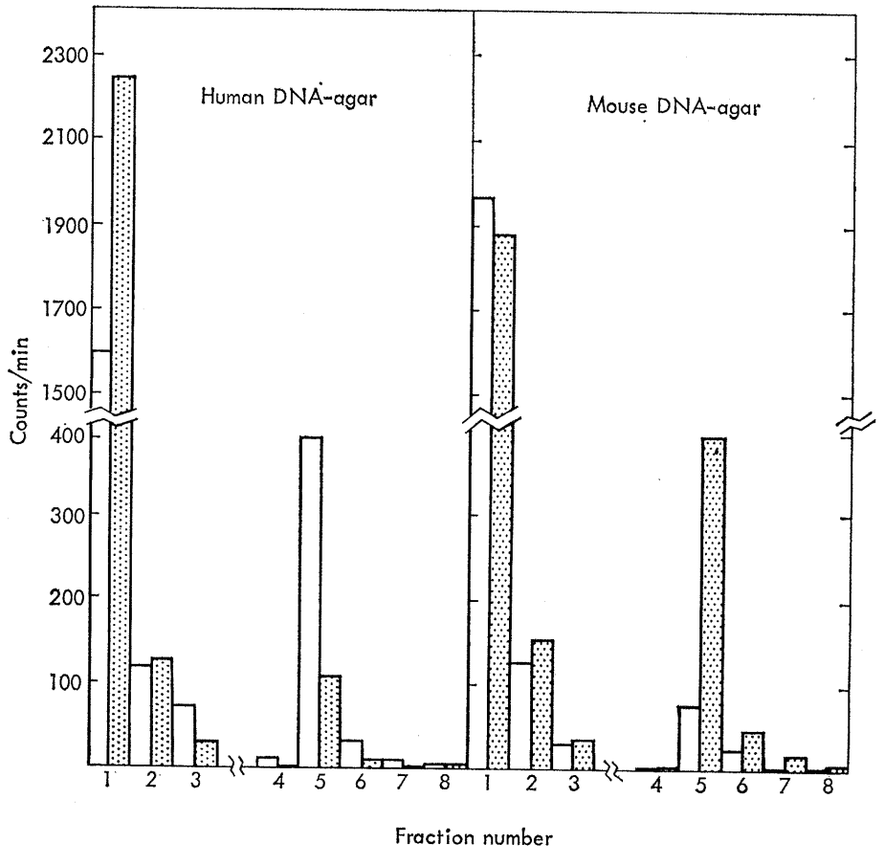

Fig. 7. P^{32}-labeled mouse (strain BALB/C) DNA fragments and C^{14}-labeled HeLa cell (human) DNA fragments mixed in double-strength saline citrate were heated for 5 minutes at 100°C and rapidly cooled; 0.5 milliliter of this mixture, which contained 25 μg of mouse DNA (with radioactivity of 100 count/min μg) and 10 μg of human DNA (with radioactivity of 220 count/min μg), was incubated for 16 hours at 60°C with 0.50 gram of agar containing mouse DNA (466 μg/g) or agar containing human DNA (656 μg/g). The incubated preparations were put into heated, water-jacketed columns and washed at 60°C with double-strength saline citrate. Three 15-milliliter fractions were collected, the agar was washed with six or seven 15-milliliter volumes of double-strength saline citrate, and a final fraction was collected. Four successive 7.5-milliliter fractions of standard saline citrate diluted 100-fold were then collected, at 72° to 73°C. Fractions were collected every 10 to 15 minutes at 60°C and every 5 minutes at 72° to 73°C. The DNA fragments were precipitated with 5 percent trichloracetic acid in the presence of 100 μg of yeast RNA, collected on membrane filters, dried, and counted in a liquid scintillation counter (*11*). The labeled mouse DNA (shaded areas) was prepared from mouse embryos from mice daily injected intraperitoneally with P^{32}-orthophosphate (1 mc) for from 1 to 3 days. The labeled HeLa cell DNA (open areas) was prepared from cells exposed for several generations to about $10^{-6}M$ C^{14}-thymidine in Eagle's medium with 5 percent calf serum added.

structures of this experiment dissociate can be compared with the temperature of dissociation expected for DNA duplexes of this base composition. Under identical conditions duplexes of *Escherichia coli* DNA (50 percent GC) dissociate at a mean temperature of 74°C (*18*). In addition, it is known that temperatures of dissociation for molecules differing by 1 percent of guanylic and cytidylic acid content are separated by about 1°C. Thus, duplexes of animal DNA (about 42 percent GC) should decompose at a mean temperature of about 65°C. If allowance is made for the nonspecific elution due to loss of DNA, it may be seen that both the homologous and the heterologous duplexes are dissociated at about this temperature. Certainly there are some duplexes of each kind that are stable at somewhat higher temperatures. Although this test is unsatisfactory in detail, the general trend of the evidence points to the conclusion that both homologous and heterologous duplexes have the properties expected of well-formed helical structures.

Table 3. Reaction of P^{32}-labeled rhesus monkey DNA fragments and C^{14}-labeled mouse DNA fragments with various DNA-agars. DNA-agars (0.50 g) were mixed with 0.5 ml of double-strength saline citrate which contained either 0.1 μg of P^{32}-labeled (27,000 count/min μg) rhesus monkey DNA fragments alone or a mixture of this preparation with 1.5 μg of C^{14}-thymidine-labeled (2500 count/min μg) mouse L-cell DNA fragments. Incubation and washing were performed as in Fig. 7. The rhesus monkey DNA was prepared from primary kidney cells labeled for several generations in phosphate-free Eagle's medium to which 5 percent calf serum with P^{32}-orthophosphate (25 μc/ml) had been added.

DNA in agar	DNA (μg/g of agar)	Labeled DNA bound (%)	
		C^{14}	P^{32}
Rhesus monkey	380		30
Mouse	280		8
Mouse	280	26	6
Armadillo	410	7	6
Chicken	180		3

Table 4. Reaction of H^3-labeled green monkey DNA fragments and P^{32}-labeled human DNA fragments with human and green monkey DNA-agars. The DNA-agars (0.50 g) were incubated with a mixture of H^3-labeled (22,000 count/min μg) green monkey DNA fragments (1 μg) and P^{32}-labeled (10^5 count/min μg) HeLa cell DNA fragments (0.2) μg). The monkey DNA was prepared from primary green monkey kidney cells to which 5 percent calf serum with H^3-thymidine (5 μc/ml) had been added. The labeled HeLa cell DNA was from cells grown in phosphate-free Eagle's medium to which 2 percent calf serum and P^{32}-orthophosphate (25 μc/ml) had been added.

DNA in agar	DNA (μg/g of agar)	Labeled DNA bound (%)	
		H^3	P^{32}
Human	750	25	28
Green monkey	640	23	20

Polynucleotide Homologies among Vertebrate DNA's

In order to explore the extent of the similarities among a wide variety of vertebrates, a number of comparisons have been attempted. The results, summarized in Tables 2 and 3, were obtained by allowing labeled mouse, monkey, or human DNA fragments to interact with a series of representative vertebrate DNA's in agar. In most incubations a mixture of two different labeled DNA fragments was used, in order to provide maximum confidence in the data.

Homologies between human and mouse DNA and a series of DNA's embedded in agar are recorded in Table 2. It is clear that significant interaction occurred when the mixture was incubated with all the embedded DNA's except the bacterial DNA. The most extensive cross-reaction involving the human DNA was obtained between human DNA and rhesus monkey DNA. Among the rodents, rat DNA exhibited the greatest affinity for the mouse DNA, and hamster DNA exhibited somewhat less. Interestingly, there is no more apparent homology between guinea pig or rabbit DNA and mouse DNA than between the DNA's of primates or even-toed ungulates and mouse DNA. Table 3 illustrates the amount of cross-reaction which occurred when P^{32}-labeled rhesus monkey DNA was incubated with various unlabeled DNA's. It is evident from these results that there is greater homology between the various mammalian DNA's than between mammalian and chicken DNA or, as shown in Table 2, salmon DNA.

The results presented in Table 2, which indicate a high degree of similarity in the polynucleotide sequences of human and rhesus monkey DNA, have been confirmed by comparisons of human and green monekey DNA, as shown in Table 4. Clearly, the quality of the agar gel preparation containing human DNA is superior to that of the preparation containing monkey DNA, so that in the former the absolute amount of each of the labeled DNA's bound is higher. Nevertheless, a consistent measurement of the overlap in polynucleotide sequences may be obtained from the relative amounts of human and monkey DNA bound in the two separate experiments. These calculations suggest that common sequences make up some 85 to 90 percent of the total.

Table 5. Reaction of C^{14}-labeled mouse DNA fragments, previously bound to agar containing bovine DNA, with various DNA-agars. Mouse L-cell DNA fragments (20 μg) (see Table 2) were allowed to react for 16 hours with bovine DNA (1000 μg) in 1.00 g of agar at 60°C. The bound DNA fragments eluted at 75°C in saline citrate of 0.01 standard concentration contained 4.2 percent of the total radioactivity. The previously bound DNA fragments were boiled for 5 minutes in double-strength saline citrate and rapidly cooled; 1.5 ml of this preparation (0.1 μg DNA) was added to each 1.00 g of the DNA-agars.

DNA in agar	DNA (μg/g of agar)	Labeled DNA bound (%)
Bovine	1000	29
Guinea pig	220	29
Mouse	280	42
Salmon	600	11

Table 6. Reaction of P^{32}-labeled and H^3-labeled green monkey DNA fragments with DNA-agars. The P^{32}-labeled fragments had been previously selected through reaction with agar containing mouse DNA. Five-microgram fragments of P^{32}-labeled (100,000 count/min μg) green monkey DNA in 1 ml of saline citrate of double the standard concentration (*17*) were incubated for 16 hours at 60°C with 400 μg of mouse embryo (strain C57B1) DNA in 1.00 gram of agar. Bound DNA fragments (containing 8.9 percent of the radioactivity) were eluted at 75°C with saline citrate of 0.01 standard concentration after the agar had been thoroughly washed with double-strength saline citrate at 60°C. The bound P^{32}-labeled DNA fragments were mixed with fragments of H^3-thymidine-labeled (22,000 count/min μg) green monkey DNA, and 0.6 ml of the mixture (0.015 μg of P^{32}-labeled DNA and 0.1 μg of H^3-labeled DNA) in double-strength saline citrate was incubated overnight at 60°C with 0.50 gram of the DNA-agars.

DNA-agar	DNA (μg/g of agar)	Labeled DNA bound (%)		Enrichment factor*
		P^{32}	H^3	
Armadillo	410	18	4	3
Calf	720	25	6	3
Chicken	180	7	1	5
Hamster	370	30	7	3
Human	750	61	40	1
Mouse	400	38	7	4
Rabbit	390	23	4	4
Salmon	600	18	2	6
Green monkey	640	52	33	1

* Enrichment factor=[P^{32}-bound/H^3-bound (heterologous)]/[P^{32}-bound/H^3-bound (homologous)].

Common Nucleotide Sequences Conserved by Vertebrates

Perhaps the most notable feature of the data presented in Tables 2 and 3 is the relatively constant degree of homology between the different orders of mammals. Except for pairs of closely related animals, the extent of homology indicated by the heterologous reactions is approximately one-fifth. This observation raises the question of whether there exists among the various animals a particular class of nucleotide sequences which have been retained during the diversification of the vertebrate forms.

The reaction between labeled DNA fragments and a heterologous DNA in agar is a purification procedure for recovering those portions of the labeled DNA which have the closest homology to the embedded DNA. Since it is possible to recover these DNA fragments, they may be reincubated with the same or other DNA's in agar. If there are common segments among the various DNA's, a larger proportion of the reclaimed fragments will react in each of the secondary incubations. For example, when labeled mouse DNA fragments were selected by reaction with bovine DNA, recovered, and reincubated with several DNA's, an increased reaction was observed not only with bovine DNA but also with guinea pig DNA (Table 5). In addition, the bovine and guinea pig DNA's reacted to the same extent. The greater reaction with the parent mouse DNA probably reflects imperfect selection of the labeled fragments during the initial incubation. The considerably increased reaction with the salmon DNA indicates that the homology between mouse and salmon DNA must reside in the sequences selected by reaction with the bovine DNA.

In a more extensive investigation, P^{32}-labeled green monkey DNA was first made to react with mouse DNA embedded in agar. The selected fragments were then mixed with unselected fragments of H^3-labeled green monkey DNA. The mixture was allowed to incubate with various DNA-agar preparations. Inclusion of the unselected fragments of H^3-labeled green monkey DNA in the incubation mixture provided a means of compensating for the variability in binding capacity among the different DNA-agar preparations. The results, presented in Table 6, show that in every case the selected P^{32}-labeled fragments reacted to a greater extent than did the total H^3-labeled DNA. Thus, the initially selected fragments were greatly enriched in nucleotide sequences common to the various DNA's. The factor of enrichment varies from 3 or 4 among mammalian DNA's to 5 or 6 for the DNA's of birds or fishes.

Base sequence complementarity among nucleic acid molecules is also studied by means of competition reactions. In this case similarity is measured by the extent of interference (with a homologous reaction) resulting from the presence in the reaction mixture of an excess of unlabeled heterologous nucleic acid (*6*). This approach has two great practical advantages: study of a series of genetic relationships requires only a single labeled nucleic acid preparation and a single preparation of DNA-agar. The advantage of eliminating some of the labeled DNA's is obvious, but perhaps equally

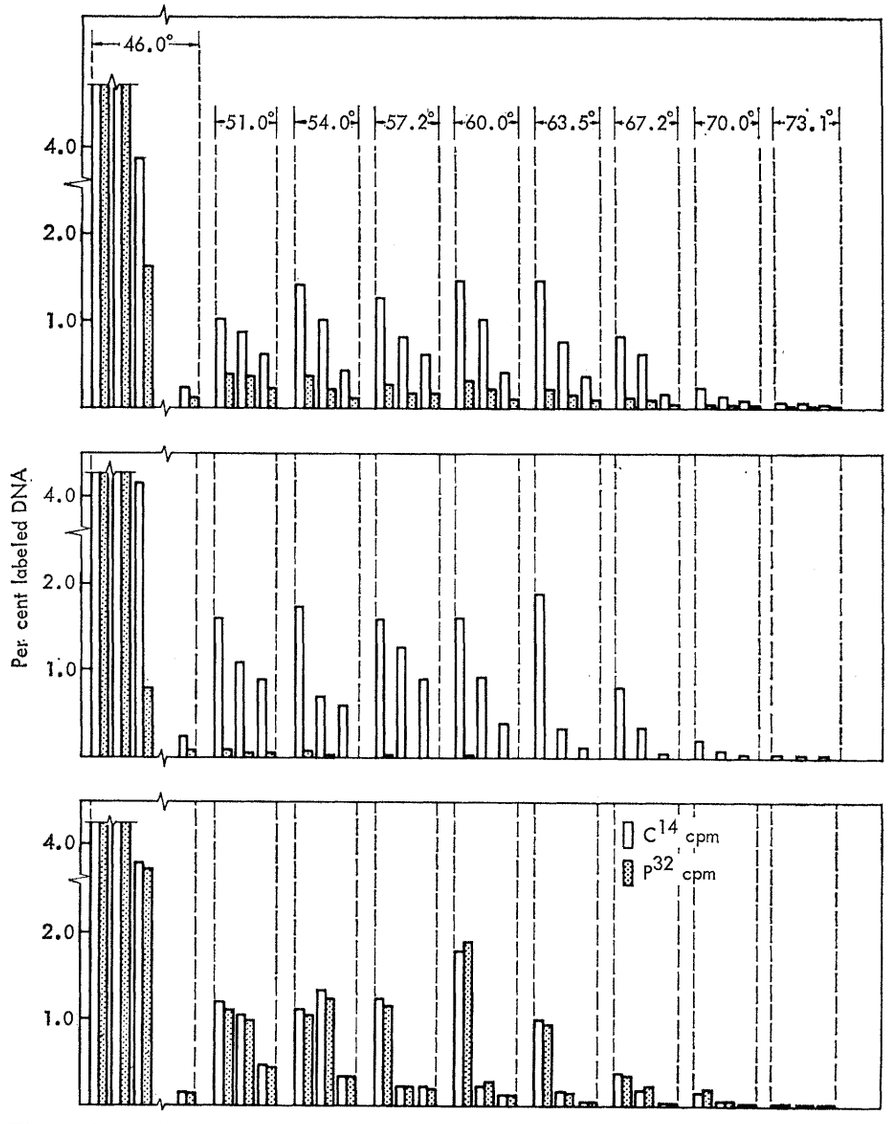

Fig. 8. Comparison of the stability of duplex DNA structures formed by homologous and by heterologous DNA fragments. A mixture of C^{14}- and P^{32}-labeled DNA fragments was incubated with 0.50 gram of agar containing 192 μg of mouse DNA, at 60°C for 16 hours. After the unbound DNA was removed by exhaustive washing, at 46°C, with standard saline citrate diluted tenfold, three 10-milliliter washes were then given at each of several higher temperatures. In each of the three experiments represented, 4 μg of C^{14}-labeled (2500 count/min μg) DNA fragments from mouse L cells was used. Added to this was (top) 1 μg of P^{32}-labeled (3000 count/min μg) DNA fragments from a primary culture of rhesus monkey kidney, or (middle) 0.5 μg of P^{32}-labeled (19,000 count/min μg) DNA fragments from bacteria of the Providence strain (guanylic acid, cytidylic acid, 41 percent) or (bottom) 0.07 μg of P^{32}-labeled (72,000 count/min μg) DNA fragments from mouse L cells.

important is the possibility of making comparative studies with aliquots of a single DNA-agar preparation. Much of the variability in the quality of the data obtained results from the number of different DNA-agar preparations used, each containing different amounts of trapped DNA, itself of variable molecular weight, and each subject to more or less loss of DNA during incubation. Although these effects can be maintained within acceptable limits by careful control of the preparation of DNA and DNA gels, it is nevertheless true that the most highly reproducible results are obtained with a single batch of DNA-agar.

The competition reaction has been used in the further study of the cross-reaction noted earlier between the DNA's of widely different vertebrates. The relationship of several of these DNA's to mouse DNA has been measured from the extent of competition exhibited in the homologous mouse-DNA reaction. A series of 0.50-gram aliquots of a single preparation of agar containing mouse DNA were incubated with 1 μg of fragments of C^{14}-labeled mouse DNA in the presence of various amounts of unlabeled DNA fragments prepared from various sources. The percentage of the labeled DNA fragments forming a duplex under these conditions is given in Fig. 9. Even large amounts of bacterial DNA from *Bacillus subtilis*, whose base composition resembles that of mammalian DNA, fail to inhibit the homologous reaction. There is thus no obvious nonspecific effect even where the competing DNA is several thousand times more abundant than the labeled component. On the other hand, the presence of large quantities of unlabeled mouse DNA reduces the amount of the labeled component bound, due to the saturation of sites in the trapped DNA. Where unlabeled hamster DNA is present this effect is shown for most of the sites in the mouse DNA, although even where excess quantities of hamster DNA are present, a sizable proportion of the sites remain available only to mouse DNA. With salmon DNA competition occurs for only a few of the sites in the mouse DNA, and the binding is slightly reduced.

In the case of the other three samples of unlabeled DNA used, from man, bovine, and guinea pig, the homologous reaction is inhibited to the extent of about one-third by the presence of moderate levels of any of the three, and the inhibitory effect is no greater if the amount is increased to 3 or 4 milligrams. The competitive effect of these three DNA samples is essentially identical, a finding which confirms the conclusion drawn from the data of Table 2 that these DNA's are equally related to mouse DNA. It is possible from this competition reaction to determine not only these quantitative similarities but also how much these individual similarities overlap one another. Thus, if the similarity in the hereditary material of the mouse and in each of the three other animals in fact reflects a common segment among all four animals, then the competitive effects being studied will not be additive. The extent of additivity in the competition reaction will be a measure of the extent of overlap in the genetic homologies being studied. These experiments, in which a mixture of two or more unlabeled DNA's was used as competitor, are described in Table 7. In the two experiments outlined, the inhibition occurring with a mixture of DNA's from the bovine and the guinea pig was tested at two different levels, and the reaction was also carried out in the presence of high levels of guinea pig, bovine, and human DNA. In none of these cases was there any convincingly greater effect where more than one DNA species was present. In experiment 2f of Table 7, DNA from a fourth animal, the hamster, was included in the mixture. This DNA, known to be more closely related to that of the mouse than the three other DNA's, did show greater inhibitory effect even in the presence of high concentrations of DNA from the three other species. This would seem to eliminate the possibility that a possible additive effect might be obscured in such a mixture of DNA's at high concentration.

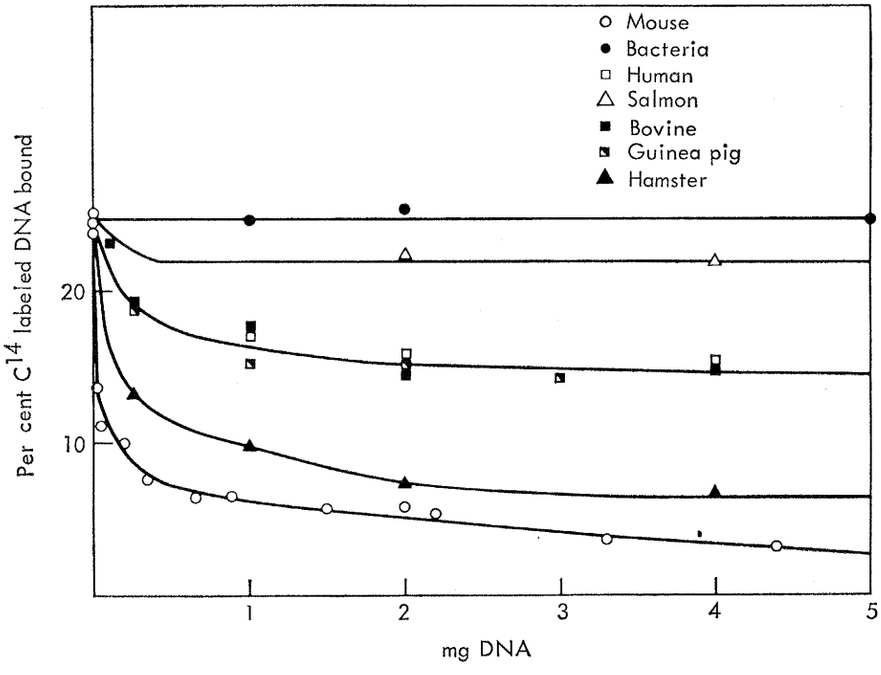

Fig. 9. Competition by unlabeled DNA fragments in the reaction between labeled mouse DNA fragments and agar containing mouse DNA. One microgram of C^{14}-labeled (2500 count/min μg) DNA fragments from mouse L cells was incubated with 0.50 gram of agar containing 192 μg of mouse DNA in the presence of varying quantities of unlabeled DNA fragments from the following sources: mouse, *Bacillus subtilis*, salmon, human, bovine, guinea pig, and hamster. The total volume was 1.25 milliliter. The percentage of labeled DNA fragments bound is plotted against the amount of unlabeled DNA present in the reaction mixture.

Conclusions

It is clear from the results presented that there exist homologies among polynucleotide sequences in the DNA's of such diverse forms as fish and man. These sequences represent genes which have been conserved with relatively little change throughout the long history of vertebrate evolution. Although we have no means yet of relating such genes to particular phenotypic expressions, it is conceivable that they are the determinants of the fundamental conservative characteristics of the vertebrate

Table 7. Competition by unlabeled DNA fragments in the reaction of labeled mouse DNA fragments with mouse DNA in agar. C^{14}-labeled mouse DNA fragments (1 μg) were incubated with 0.50 g of agar containing 192 μg of mouse DNA in each case. The total volume was 1.25 ml.

Experiment No.	Unlabeled DNA competitor (mg)				Labeled DNA fragments bound (%)
	Bovine	Guinea pig	Human	Hamster	
1a					24.2*
1b	0.25				19.2
1c	1.0				17.6
1d		0.25			18.9
1e		1.0			15.1
1f	0.25	0.25			15.9
1g	1.0	1.0			15.1
2a	2.0				14.5
2b		2.0			15.8
2c			2.0		15.7
2d	2.0	2.0	2.0		15.1
2e				1.0	9.9
2f	2.0	2.0	2.0	1.0	9.8

* Average of three determinations.

form—as, for example, bilateral symmetry, establishment of a notochord, and the presence of hemoglobin. The detection of such common polynucleotide sequences indicates at once that the method in its present form is especially suited to the quantitative determination of relationships among higher taxonomic categories. It is now possible to measure the extent of genetic relatedness *within* higher taxonomic categories, and it should also prove possible to arrive at an estimate of the relative genetic diversities in equivalent taxa of different groups. In this connection it has already been suggested that the genetic diversity among families of bacteria is relatively greater than the genetic diversity among all the major vertebrate classes (*19*).

In addition to DNA-DNA interactions it is possible to study RNA-DNA interactions. Thus, insofar as RNA molecules serve as the transcript of genetic information and, through their translation, give rise to the phenotype, a means is at hand for quantitatively comparing phenotypic as well as genotypic similarities among organisms. Such a comparison has already been reported for bacteria (*6*), where there is good reason to believe that essentially all of the genes are expressed through the production of RNA (*15*).

In the series of experiments discussed here a single criterion has been used for the detection of complementary polynucleotide sequences. Thus, those combinations which cannot survive a high salt environment at 60°C have been discarded, and those which have this minimum stability are grouped together. While such a criterion may be suitable for many types of comparisons, it should be realized that this is an arbitrary choice and that the data obtained cannot be treated as absolute measures of common nucleotide sequence.

It is not clear how similar such complementary sequences must be in detail, or how many noncomplementary base pairs can be tolerated. These considerations do not, however, detract from the significance of the relative numbers obtained in comparisons of two preparations of DNA fragments with one or more embedded DNA's. Furthermore, the large cross-reactions between human DNA and old-world-monkey DNA, and the lack of ability to distinguish at all between the rhesus and the green monkey, suggest that numerous refinements will be required if the method is to be applied to closely related forms.

The method described has several limitations in addition to its lack of discrimination among closely related organisms. It is not applicable to fossil materials or to preserved specimens because of the need for high-molecular-weight DNA as one of the reacting components. Neither is it yet suitable for studies among higher plants, since there exists no general method for preparing high-molecular-weight plant DNA. At present, tracer methods are not part of the usual armament of the systematist, and, although in principle radioactive nucleic acids are not necessary, in practice they provide the most convenient means of detecting polynucleotide homologies. There exists also the ever-present danger of uncritical acceptance of numerical data produced by sophisticated machines. Certainly, numbers on a scintillation spectrometer tape are not completely adequate descriptions of the differences between man and monkey! Simpson, wisely, has commented: "[others have suggested] that the ultimate (i.e. the touchstone?) for classification would be the complete DNA code. Certainly I can think of nothing more desirable as an addition to our criteria for classification, but I strongly doubt whether even that most desirable of criteria would be sufficient in itself. At high taxonomic levels, particularly, I suspect that interpretation of DNA resemblances and differences would be as difficult as interpretation of anatomical resemblances and differences and that the two would have to be combined, with each other and with all other classes of data, for the soundest results" (*20*). Nevertheless, at high taxonomic levels, particularly, the method discussed appears appropriate. The caliber of these preliminary results warrants the optimistic view that future developments will provide a fruitful addition to our criteria for the classification of living forms.

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