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Genetic discrimination by means of DNA/DNA binding

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1. INTRODUCTION

The degree of genetic relationship between two organisms does not always reflect their genealogical history. Litter-mates in an outbred strain of mice may differ more genetically than two members of an inbred strain separated by many generations. Even speciation does not necessarily indicate that the two populations involved have achieved a greater degree of genetic remoteness than exists within each, since in theory a single gene difference may be enough to establish reproductive isolation.

Genetic relationship may be estimated in terms either of the proportion of genes held in common, or of the proportion of genetically relevant DNA held in common. The first measure can so far be applied only in the special case of co-isogenic lines, members of which are reckoned to differ from one another at a single gene locus only. The second measure became for the first time a practical possibility when Schildkraut, Marmur & Doty (1961) reported that denatured (i.e. single-stranded) bacterial DNA from different sources would form duplex strands of mixed origin, providing the two bacterial species concerned were not too distantly related. McCarthy & Bolton (1963) used the degree of binding of DNA fragments, and of messenger RNA, onto denatured DNA immobilized in agar, as a measure of the genetic relationships between several strains of bacteria belonging to a single family. The proportions of DNA or RNA bound between different strains agreed well with their relative taxonomic positions.

More recently, Hoyer, McCarthy & Bolton (1964) have extended their studies to animals, and have shown that DNA/DNA binding can also be used to compare different mammalian species and even different vertebrate classes. Whether similar methods can be used to estimate the degree of genetic relationship between more closely related species, or between inbred strains or selected lines within a species, will depend in part on the degree of discrimination which can be achieved.

The present study was concerned in the main with a single pair of species, laboratory mice (*Mus musculus* L.) and rats (*Rattus norvegicus* L.). Now classified in different genera, both species were at one time included in the single large

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genus *Mus*. Using the degree of DNA/DNA binding between these two species as a yardstick, we have explored some possible ways by which the resolving power of the method may be increased.

The term 'hybridization' has often been used to denote the formation of duplex strands of mixed origin ('hybrid strands'), whether the 'parent' DNA came from the same or different species. We have preferred the less evocative term 'binding' in this context, since 'hybridization' is used widely by geneticists in a different (though perhaps related) sense.

2. MATERIAL AND METHODS

Preparation of DNA-agar

High molecular weight DNA was prepared from the livers of mice and rats, by a phenol-extraction procedure (Walker & McLaren, 1965). The mice were of diverse origin; the rats were from a single laboratory colony; both were starved for 24 hours before being killed. In preparing the DNA-agar, we followed the methods of McCarthy & Bolton (1963). The DNA (molecular weight $\sim 10^7$) was denatured by heating at 100°C. for 10 min. at a concentration of 1 mg./ml. in 0·01 × SSC (SSC is 0·15 M NaCl, 0·015 M Na citrate). After denaturation it was immediately mixed with an equal quantity of 8% agar (Oxoid 'Ionagar' No. 2), and cooled rapidly. It was then sieved twice, thoroughly washed with 2 × SSC at 60°C. to remove inadequately trapped DNA (10–20%), and stored at 4°C.

Preparation of radioactive DNA fragments

Using the same procedure, DNA was prepared from rat and mouse cells grown in cultures to which ^{32}P orthophosphate (Radiochemical Centre) had been added a few days before harvesting. The mouse cells belonged to the L strain of tissue culture cells, derived originally from mice of the C3H inbred strain (Hsu & Kellogg, 1960); the rat cells belonged to a strain (SRL/1) isolated by Miss Daniels at the Strangeways Laboratory, Cambridge. The radioactive DNA, dissolved in 2 × SSC, was degraded to a molecular weight of about 5×10^5 by ultrasonic treatment, precipitated with ethanol, and resuspended in 0·01 × SSC after centrifugation. It was then denatured by heating at 100°C. for 10 min., mixed with 4 × SSC to give a final molarity of 2 × SSC, and stored at −10°C.

Binding

Our method of binding is modified from that described by McCarthy & Bolton (1963). Standard amounts of DNA-agar (usually 0·3–0·5 mg. DNA in 1 g. wet weight of agar) were mixed with standard amounts of ^{32}P-labelled DNA fragments (1–5% of the amount of DNA trapped in the agar, in 0·3–0·5 ml. 2 × SSC), and incubated overnight at 60°C. in water-jacketed glass columns. In the morning those DNA fragments not bound were washed out with ten successive 10 ml. fractions of 2 × SSC at 60°C.; a further five fractions of 0·01 × SSC at 75°C. washed out the remaining DNA fragments, since at this temperature and molarity the

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DNA duplexes formed during incubation are separated into single strands once again. The radioactivity in each of the fifteen fractions was counted, using a liquid scintillation counter. The sum of the radioactivity in fractions 11–15 divided by the sum of the radioactivity in fractions 1–15 gives an estimate of the percentage B of radioactive DNA fragments bound.

If the molecular weight of the DNA fragments ('short DNA') is too high, they will be trapped in the agar at 60°C., whether or not they are bound to the long DNA. They will therefore give a spuriously high B value. Our present sonication procedure (Walker & McLaren, 1965) ensures that the DNA fragments tested against agar alone (i.e. containing no long DNA) give a B value of less than 2%.

Recovery

Most of the unbound DNA fragments appear in fraction 1, most of the bound ones in fractions 11 and 12. The DNA fragments in these fractions may be recovered and incubated again with DNA-agar. After removal of an aliquot for estimation of the radioactivity, fraction 1 is dialysed overnight to remove the salt, and all three fractions are freeze-dried to a powder which is then resuspended in the required volume of 2 × SSC.

A fuller description of the methods used is given in Walker & McLaren (1965).

3. RESULTS

Mouse–mouse binding

Provided that the ratio of short to long DNA does not exceed about 1:10, the value of B is independent of the absolute quantities of either short or long DNA in the incubation mixture: that is, a constant proportion of the radioactive DNA

Table 1. *Incubation of mouse DNA and* E. coli *DNA*

Experiment	Short DNA		Long DNA		B(%)
	Origin	Amount (mg.)	Origin	Amount (mg.)	
11	mouse*	0·025	MOUSE	0·50	27·2
11	mouse	0·025	*E. COLI*	0·50	2·6
22	*E. coli**	0·05	MOUSE	0·20	2·3
22	*E. coli*	0·05	*E. COLI*	0·50	52·6
23	*E. coli*	0·05	*E. COLI*	0·50	51·0

* When the short mouse and the short *E. coli* DNA were incubated on plain agar (containing no long DNA), 0·5% and 0·4% respectively of the fragments were trapped.

fragments are bound (Walker & McLaren, 1965). But this proportion is far from 100%, even when short and long DNA both come from the same species.

Figure 1 illustrates a single experiment, comparing the trapping of fragments of labelled mouse DNA on plain agar, and on agar containing long mouse DNA.

Figure 2 shows the distribution of estimates of B for thirty mouse–mouse experi-

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ments. Only estimates based on a total of more than 5000 counts have been included, so the error due to counting should be small. The great majority of the estimates lie between 28% and 34%, with a population mean of 31·4%, and a standard deviation of 3·7%.

Comparison of within- and between-species binding

Mouse DNA was reciprocally tested with DNA from a distantly related organism, *Escherichia coli* (Table 1). As expected, the *B* values in both directions were very low, though significantly above the level of trapping in plain agar. In contrast,

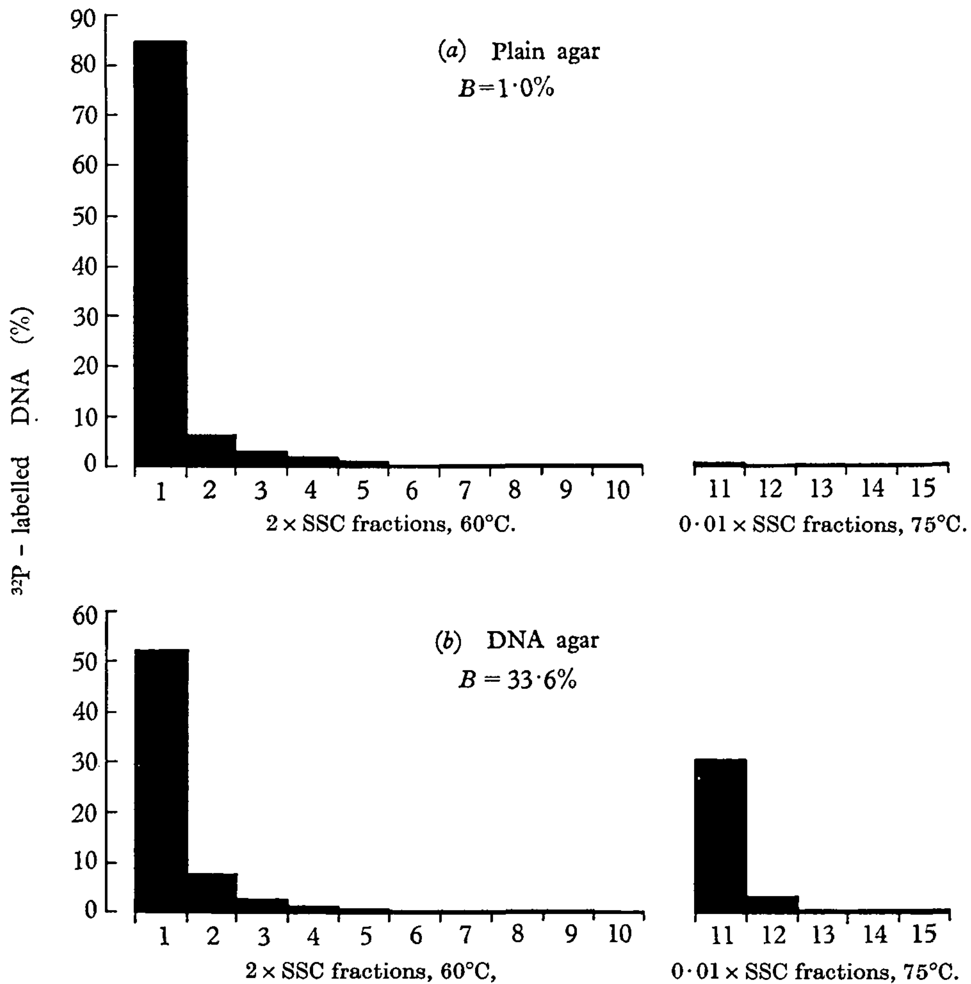

Fig. 1. The distribution of radioactivity in ten 2 × SSC washings at 60°C., followed by five 0·01 × SSC washings at 75°C., when ^{32}P-labelled mouse DNA fragments are incubated with either (*a*) agar alone, or (*b*) agar containing long mouse DNA. *B* is the counts in fractions 11–15 as a percentage of the total counts, i.e. the proportion of DNA fragments which are bound.

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the proportion of *E. coli* DNA fragments bound by long *E. coli* DNA was high, around 50%.

The results of incubating mouse DNA and rat DNA (Table 2) show that (1) the proportion of short DNA fragments bound is lower in between-species than in

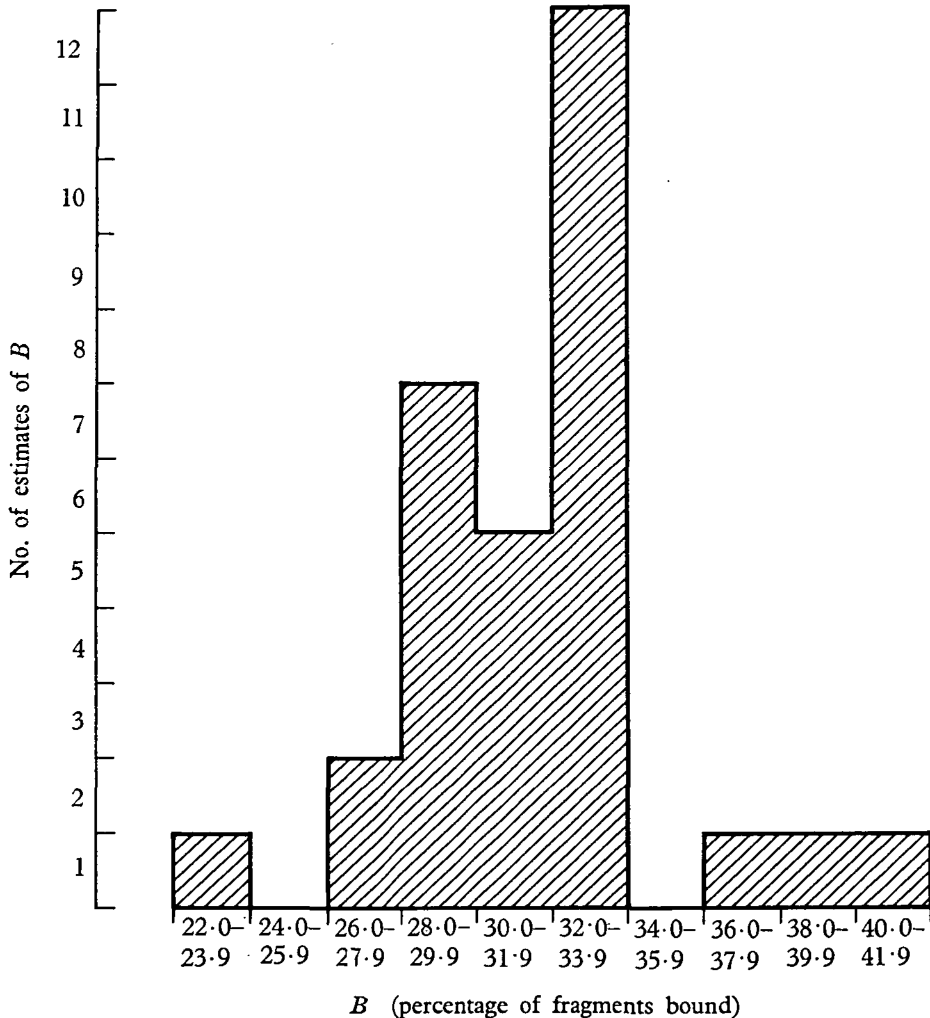

Fig. 2. The distribution of estimates of B from thirty incubations of ^{32}P-labelled mouse DNA fragments with agar containing long mouse DNA.

within-species tests, and (2) long rat DNA is less effective than long mouse DNA in binding short DNA fragments from either source.

In Table 3, the sources of variation in the reciprocal incubations from Table 2 are examined. After angular transformation of the percentages, an analysis of variance was carried out. The difference between within- and between-species incubations and the source of long DNA were both, as expected, highly significant; on the other hand the source of short DNA was not significant.

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Note that relative binding ability cannot be adequately assessed if short DNA from one source only is available. If short mouse DNA alone had been used in this experiment the ratio of between-species to within-species binding would have been estimated at 49%; while if short rat DNA alone had been used, the corresponding estimate would have been 93%. The discrepancy arises from the greater binding capacity of long mouse DNA. This can be allowed for by carrying out reciprocal incubations, using short DNA from both species, and deriving combined estimates of within-species and between-species binding. When this is done for the three reciprocal experiments in Table 2, the ratio of between-species to within-species binding is 68%, 64% and 73% in experiments 42, 50 and 60 respectively. Assuming that binding efficiency reflects DNA homology, we might be tempted to conclude that nearly 70% of the DNA of mice and rats is common to both species. The validity of such a conclusion is discussed later.

In what follows we are not concerned with estimating the degree of relationship between rats and mice, but with selecting a procedure which will maximize the apparent difference between the two species, that is which will increase the power of resolution or discrimination of the technique.

Table 2. *Incubation of mouse DNA and rat DNA*

	Amount of short DNA (mg.)		Amount of long DNA (mg.)		*B*%			
Experiment	mouse	rat*	MOUSE	RAT*	mouse/MOUSE	rat/MOUSE	mouse/RAT	rat/RAT*
11	0·025	—	0·50	0·25	27·2 (1)†	—	17·3 (1)	—
23	0·01	—	0·50	0·23	34·3 (2)	—	28·3 (2)	—
42	0·01	0·01	0·30	0·20	36·7 (3)	27·3 (3)	17·9 (3)	29·2 (3)
50	0·01	0·01	0·33	0·41	36·3 (2)	24·7 (2)	20·1 (1)	33·5 (1)
60	0·01	0·01	0·40	0·30	39·5 (1)	28·7 (1)	24·5 (1)	32·9 (1)

* The source of long and short DNA, here and in other tables, is indicated in capital and lower case type respectively: thus mouse/RAT indicates that short fragments of mouse DNA have been incubated with long rat DNA.

† The number of replicates averaged for each estimate of *B* is given in parentheses.

Table 3. *Analysis of variance in reciprocal mouse–rat incubations (experiments 42, 50 and 60, Table 2)*

Source	Degrees of freedom	Mean square	*P*
Between- v. within-species	1	259·5	< 0·001
Long mouse v. long rat	1	99·0	< 0·001
Short mouse v. short rat	1	0·09	n.s.
Between experiments	2	8·68	n.s.
Group-experiment interactions	6	2·41	n.s.
Within groups, within experiments	10	1·24	

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The extent to which between-species binding departs from within-species binding (standardized at 100%) for any given procedure may be termed the discrimination factor for that procedure. Thus, in the simple reciprocal binding technique described above, between-species was 70% as effective as within-species binding. This corresponds to a discrimination factor of 30%. In order to increase the discrimination factor it is necessary to depress between-species relative to within-species binding. We have tried two methods of doing this: (1) by isolating and using for further incubations that fraction of the DNA of each species which is characteristic of that species alone, and is not held in common by both species; and (2) by blocking the binding reaction with unlabelled DNA fragments from the other species.

DNA fractionation

When DNA fragments are incubated with long DNA immobilized in agar, the bound fragments (from the 75°C. washings) and the unbound fragments (from the 60°C. washings) may be separately recovered, and each subsequently incubated again with a fresh sample of long DNA in agar. The unbound fraction will be contaminated with a small percentage of bound fragments, since 10–15% of the long DNA leaches out of the agar during the 60°C. washings, presumably carrying with it any DNA fragments bound to it.

Table 4. *Percentage* B *of short mouse DNA fragments bound in second cycle, after recovery of bound and unbound fractions from first cycle*

		Bound fractions		Unbound fractions	
		Long DNA in 2nd cycle		Long DNA in 2nd cycle	
Experiment	Long DNA in 1st cycle	MOUSE	RAT	MOUSE	RAT
13	MOUSE	49·2	—	13·3	—
13	MOUSE	34·1	—	14·4	—
13	MOUSE	—	—	9·6	—
27	MOUSE	48·1	—	6·9	—
27	MOUSE	47·8	—	10·0	—
17	MOUSE	48·5	34·7	7·9	6·5
29	MOUSE	37·2	—	5·4	—
29	MOUSE	49·8	—	9·7	—
17	RAT	33·2	35·6	15·3	5·6
29	RAT	49·8	—	20·4	—
29	RAT	36·8	—	15·9	—

The bound and unbound fractions of short mouse DNA incubated with long mouse DNA behave very differently from one another when tested a second time with long mouse DNA (Table 4). An average of between 40% and 50% of the bound fraction is bound again on the second cycle, compared with only about 10% of the unbound fraction (some 3–4% of this will be due to contamination with bound fragments, as explained above). Apparently the initial preparation of DNA

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fragments is heterogeneous, containing a considerable amount of DNA which is capable of little or no duplex formation under the conditions of incubation.

When short mouse DNA is incubated with long rat DNA, the unbound fraction should also contain that part of the total mouse DNA which is mouse-specific, and which therefore cannot bind to rat DNA. This mouse-specific DNA should be bound if the fraction is then incubated with long mouse DNA, but not if it is again incubated with long rat DNA. Table 4 suggests that the unbound fraction from a primary incubation of short mouse and long rat DNA does indeed bind more on the second incubation with long mouse than with long rat DNA, and also more than does the unbound fraction of short mouse initially incubated with long mouse DNA.

Table 5 shows the results of a reciprocal mouse–rat two-cycle experiment. Whether the initial incubation has been a within-species or a between-species one,

Table 5. *Two-cycle discrimination: the percentage of short mouse and rat DNA fragments bound in second cycle, after recovery of bound and unbound fractions from first cycle (experiment* 42)

1st cycle			Long DNA in 2nd cycle		Combined estimate of B		
Short DNA	Long DNA	Fraction taken	MOUSE	RAT	Within species	Between species	Discrimination factor
mouse	MOUSE	Bound	35·5	28·8	43	35	19%
rat	RAT	Bound	41·2	49·5			
mouse	RAT	Bound	43·8	34·2	42	35	17%
rat	MOUSE	Bound	35·8	39·8			
mouse	MOUSE	Unbound	12·1	7·6	11	9	18%
rat	RAT	Unbound	10·5	9·7			
mouse	RAT	Unbound	26·6	5·1	20	8	60%
rat	MOUSE	Unbound	10·4	13·9			

the *bound* fractions discriminate poorly between the two species on the second cycle (43% versus 35%; 42% versus 35%). The unbound fractions from initial within-species combinations give uniformly low B values on the second cycle (11% versus 9%). But the unbound fractions from initial *between-species* combinations behave as predicted: the second-cycle B values average 8% in the between-species, but 20% in the within-species situation. This represents a discrimination factor of 60% as compared with about 30% for a single reciprocal incubation.

The same procedure may be extended to a third cycle. Since we may initially take either short mouse or short rat DNA, combining it at each of three incubations with either long mouse or long rat DNA, and since from each of the first two incubations we may select either the bound or the unbound fractions for further testing, there are sixty-four variants possible. The B values from one such experiment are listed in Table 6, in which each line represents four variants, together yielding one discrimination factor. For example, line 6 signifies that if short mouse

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Table 6. *Three-cycle discrimination: the percentage of short mouse and rat DNA fragments bound in third cycle, after recovery of bound and unbound fractions from first and second cycles* (*experiment* 42)

	1st cycle		2nd cycle		3rd cycle: Short mouse onto long		3rd cycle: Short rat onto long		Combined estimate of B	
	Long DNA	Fraction taken	Long DNA	Fraction taken	MOUSE (within)	RAT (between)	RAT (within)	MOUSE (between)	Within-species	Between-species
(1)	Within	Bound	Within	Bound	61·3	49·8	51·8	37·5	57	44
(2)	Within	Bound	Between	Bound	66·8	31·6	42·2	42·2	55	37
(3)	Between	Bound	Within	Bound	41·3	46·3	52·5*	68·0*	47	57
(4)	Between	Bound	Between	Bound	51·4	43·4	45·0	50·1	48	47
(5)	Within	Bound	Within	Unbound	41·2	13·4	24·5	24·2	33	19
(6)	Within	Bound	Between	Unbound	31·7	13·4	26·3	21·6	29	17
(7)	Between	Bound	Within	Unbound	42·2	22·6	23·0	27·9	33	25
(8)	Between	Bound	Between	Unbound	40·6	14·5	37·4	28·5	39	21
(9)	Within	Unbound	Within	Bound	39·4	32·5	34·0	31·9	37	32
(10)	Within	Unbound	Between	Bound	44·0	41·0	24·9	24·0	34	33
(11)	Between	Unbound	Within	Bound	49·8	28·6	46·5	28·0	48	28
(12)	Between	Unbound	Between	Bound	46·4	37·0	32·6	33·5	39	35
(13)	Within	Unbound	Within	Unbound	7·8	5·6	6·9	7·4	7	7
(14)	Within	Unbound	Between	Unbound	8·6	4·6	8·8	7·3	9	6
(15)	Between	Unbound	Within	Unbound	10·5	4·8	8·5	5·1	9	5
(16)	Between	Unbound	Between	Unbound	19·0	4·8	15·0	4·5	17	5

* These values are from a later experiment, as the corresponding variants in experiment 42 were lost.

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is incubated initially with long mouse DNA, and the bound fraction tested against long rat DNA, the unbound fraction from this second incubation will give B values of 31·7% and 13·4% on long mouse and long rat DNA respectively; and *mutatis mutandis*, short rat DNA will give third cycle B values of 21·6% and 26·3% on long mouse and long rat DNA respectively. As before, both the within-species and the between-species B values are lower on long rat than on long mouse DNA. When the results for long mouse and long rat DNA are combined, the within-species and between-species B values for this variant are 29% and 17% respectively, giving a discrimination factor of 41%.

The first four lines of Table 6 show the effect of selecting the bound fractions from each of the first two cycles. The third-cycle B values are high, but discrimination is poor. Line 4 is of particular interest, since here the bound fractions at each stage were from between-species incubations. This procedure represents a successive isolation of that fraction of the DNA which is common to both rats and mice. As might be expected, by the third cycle this fraction is capable of virtually no discrimination between mouse and rat DNA.

The last four lines of Table 6 are concerned with the fractions of DNA which were bound on neither the first nor the second cycle. The third-cycle B values are even lower than those of the second cycle (Table 5), presumably because the contamination with bound fragments is by now very small. The DNA fraction which had been rejected in two successive between-species incubations (line 16) gave a third-cycle discrimination factor of 71%.

Fractions which were bound on the first cycle, unbound on the second, or vice versa, are listed in lines 5–12 of Table 6. Note that the fractions which are rejected on the second cycle, having already been bound once, give fairly high B values. This is very different from the fractions rejected on the first cycle, which as we have seen show a very low degree of binding thereafter.

From the discrimination point of view, the most important fraction is that which remains unbound in an initial between-species incubation and which therefore contains the 'species-specific', maximally discriminant DNA. On a second incubation, this may either be exposed again to long DNA of the other species, in order to remove by binding any residual DNA held in common by the two species, and the unbound fraction again selected (line 16); or it may be incubated with long DNA of its own species and the bound fraction selected (line 11). This should eliminate to some extent the 'random noise' effect of the 'unbindable' DNA. These two procedures were applied to three separate samples of mouse and three of rat DNA, in a further three-cycle experiment. Table 7 shows that a consistent level of discrimination between long mouse and long rat DNA was achieved on the third cycle by both procedures, though the level was higher for the variant involving two successive between-species rejections.

Blocking experiments

If long DNA in agar is incubated for 24 hours at 60°C. with short unlabelled DNA fragments, washed with 2 × SSC to remove any unbound fragments, and incubated

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Table 7. *Comparison of two different three-cycle discrimination procedures (experiment 92). Three replicates with short mouse and three with short rat DNA were done by each procedure, keeping the replicates separate from one another through all three cycles*

Procedure (see Table 6)	3rd-cycle B values: Short mouse		Short rat		Average B values		Discrimination factor
	MOUSE	RAT	RAT	MOUSE	Within-species	Between-species	
(11)	53·9	31·0	39·3	26·3			
	36·4	24·3	39·4	22·2	43·2	25·9	40%
	50·4	26·7	39·6	24·7			
(16)	14·7	5·4	10·3	4·2			
	19·8	7·2	8·1	3·0	13·5	4·6	66%
	19·0	4·1	9·0	3·8			

Procedure 11. Cycle 1: Between-species incubation, take unbound fraction.
Cycle 2: Within-species incubation, take bound fraction.
Procedure 16. Cycles 1 and 2: Between-species incubation, take unbound fraction.

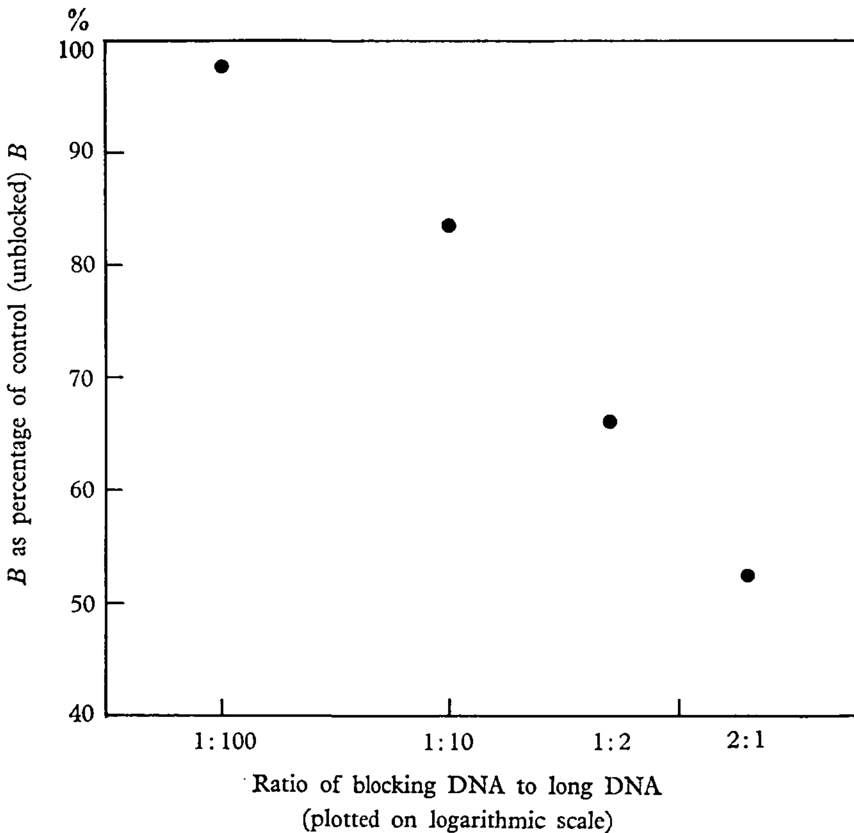

Fig. 3. The effect of incubating long mouse DNA with varying relative concentrations of short unlabelled mouse DNA ('blocking DNA'), prior to testing with short labelled mouse DNA. B is the proportion of labelled DNA bound by the long DNA.

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for a further period with short labelled DNA fragments, the proportion of labelled DNA bound should be lower than usual because some of the sites on the long DNA should be blocked, and therefore unavailable for further binding.

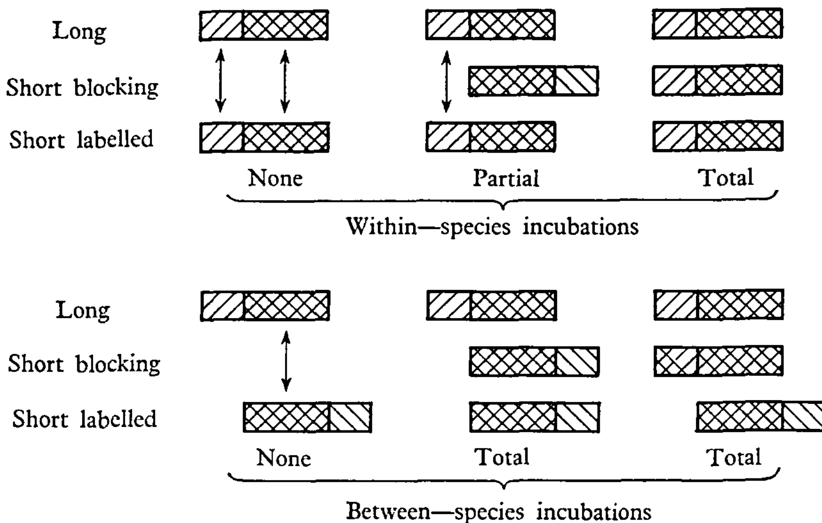

Fig. 4. Different possible blocking situations, using DNA from two species. The cross-hatched portions represent that fraction of the DNA which is common to both species; arrows show where binding of labelled DNA fragments can occur.

Figure 3 shows the results of an experiment where the long DNA, the short blocking DNA, and the short labelled DNA, were all derived from the same species (mouse). Clearly the higher the concentration of blocking DNA, the more was binding depressed. But even when the amount of blocking DNA used was twice that of the long DNA, the *B* value fell only to just over half its control level. Concentration studies (Hoyer *et al.*, 1964; Walker & McLaren, 1965) suggest that the blocking DNA would need to be about twenty-five times as concentrated as the long DNA for blocking to be maximal.

Where DNA from different species is used, we have the possibility of 'partial blocking'. If the long DNA and the short labelled fragments are more similar to each other than either is to the blocking DNA, some of the sites will remain unblocked whatever the concentration of blocking DNA. But if the blocking DNA resembles either of the other two kinds of DNA as or more closely than they do one another, all the potentially combining sites can in principle be blocked. Such a situation we call 'total blocking', even where the relative concentration of blocking DNA is such that blocking is in fact far from total. The various possible blocking situations are illustrated diagrammatically in Fig. 4.

The results of blocking experiments using both mouse and rat DNA are listed in Table 8. The following points may be noted: (1) The long mouse DNA used in experiment 22 was less effective in binding than that used in the other two experiments; (2) as before, long rat DNA was less effective in binding than long

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mouse DNA, and it is therefore only possible to compare the results from the two experiments (54 and 61) where reciprocal mouse/rat incubations were done; (3) in the four blocking situations where comparison was possible, experiment 54, which had the higher blocking ratio, consistently yielded lower B values than experiment 61; (4) total blocking effectively reduces the B value, whether in the within-species or in the between-species situation; (5) partial blocking, which is possible only in a within-species combination (Fig. 4), also reduces the B value, but less than does total blocking.

Table 8. *Blocking experiments. The blocking ratios (short blocking DNA : long DNA) for experiments* 22, 61 *and* 54 *were* 1:2, 1:1 *and* 2:1 *respectively. (Here and in Table* 9, *the source of short blocking DNA is printed in bold type)*

Type of experiment	Type of block	Short labelled DNA	Short blocking DNA	Long DNA	B (%) in experiment 22	61	54	Combined estimate of B (from 61 and 54 only)
Within-species	None	mouse	—	MOUSE	24·3	34·6	34·4	33%
		rat	—	RAT	—	30·9	31·0	
		Reciprocals combined				*32·7*	*32·7*	
	Partial	mouse	**rat**	MOUSE	17·5	31·7	22·3	26%
		rat	**mouse**	RAT	—	24·9	23·4	
		Reciprocals combined				*28·3*	*22·9*	
	Total	mouse	**mouse**	MOUSE	8·4	23·7	20·5	20%
		rat	**rat**	RAT	—	19·8	16·5	
		Reciprocals combined				*21·7*	*18·5*	
Between-species	None	mouse	—	RAT	—	21·2	14·8	21%
		rat	—	MOUSE	—	23·1	25·1	
		Reciprocals combined				*22·1*	*19·9*	
	Total	mouse	**mouse**	RAT	10·9	—	10·1	15%
		rat	**rat**	MOUSE	—	18·0	18·1	
		Reciprocals combined				—	*14·1*	
	Total	mouse	**rat**	RAT	9·0	15·9	8·8	15%
		rat	**mouse**	MOUSE	—	14·3	20·6	
		Reciprocals combined				*15·1*	*14·7*	

If blocking is to be used to discriminate between DNA from two different sources, the relevant comparison must be between a partially blocked and a totally blocked combination. Since there are one partial and three total blocking situations, three comparisons are possible. These correspond to comparing the DNAs to be discriminated as long DNA, short blocking DNA, or short labelled DNA. In each case the other two test components are held constant. In Table 9 the combined data from the two reciprocal experiments of Table 8 (54 and 61) have been rearranged to illustrate these three possibilities.

It appears that DNAs from different organisms can be more sensitively distinguished in a blocking experiment if they are used as the source of long DNA

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or short labelled DNA than if they are used as blocking DNA. This is largely because in the latter situation the comparison is between two within-species incubations, while in the two former situations, the effect of comparing a within-species and a between-species incubation reinforces the discrimination due to blocking.

The discrimination factors achieved by these means (42%) are certainly in excess of those obtained from simple reciprocal incubation, but they are lower than those yielded by two- and three-cycle fractionations. Possibly, however, a higher relative concentration of blocking DNA would make possible a higher degree of resolution.

Table 9. *The degree of discrimination achieved by blocking experiments, using the three different possible comparisons. The reciprocal data from experiments* 54 *and* 61 *(Table* 8*) have been combined*

Discrimination based on	Type of block	Short labelled DNA	Short blocking DNA	Long DNA	Combined estimate of B (from Table 8)	Discrimination factor
Long DNA	Partial	mouse	**rat**	MOUSE	26%	42%
		rat	**mouse**	RAT		
	Total	mouse	**rat**	RAT	15%	
		rat	**mouse**	MOUSE		
Short blocking DNA	Partial	mouse	**rat**	MOUSE	26%	23%
		rat	**mouse**	RAT		
	Total	mouse	**mouse**	MOUSE	20%	
		rat	**rat**	RAT		
Short labelled DNA	Partial	mouse	**rat**	MOUSE	26%	42%
		rat	**mouse**	RAT		
	Total	rat	**rat**	MOUSE	15%	
		mouse	**mouse**	RAT		

4. DISCUSSION

How much validity can be attached to statements of the type: 'Nearly 70% of the DNA of mice and rats is common to both species'? The basic tenet, that the degree of binding between samples of DNA from different organisms reflects similarities in base sequence, which in turn reflect genetic relationships, has been firmly established in micro-organisms; there seems little reason to doubt that similar phenomena in higher organisms are similarly based. But we have no information on how divergent in base sequence two samples of DNA need to be before binding is no longer possible. The labelled DNA fragments used in our experiments have an average molecular weight, before denaturation, of about 500,000. This represents less than 1000 nucleotides per single-stranded fragment, of the order of three medium-sized genes. What proportion of these thousand bases need to pair with complementary bases on the long DNA strands for the

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fragment to count as bound? We know that the answer to this question will vary with the conditions of the experiment (e.g. the temperature and salt concentration during incubation); the conditions which we have used are those recommended by Bolton & McCarthy (1963), and claimed by Hoyer *et al.* (1964) to be rather rigorous, in the sense that they will permit only those duplexes which have a high degree of complementarity to form stable hybrids.

We may therefore interpret the statement quoted above to mean: 'Under the given experimental conditions, the degree of homology between rat and mouse DNA is sufficient to permit 70% as much duplex formation with DNA of the other species, as with DNA of the same species.' At the level of simple incubation experiments, within-species differences may be ignored: our labelled fragments of DNA from L-strain tissue culture cells bind equally well on DNA from the livers of random-bred mice as on DNA from L-strain cells (Walker & McLaren, 1965). A further assumption is made, namely that the stability of between-species duplexes is no different from that of within-species duplexes. *A priori*, this might seem unlikely; however, Hoyer *et al.* (1964) searched for a difference, but were unable to find one. There is also the possible complication that the 'unbindable' fraction of DNA which we have demonstrated may turn out not to be randomly distributed with respect to species-specific DNA. If this were so, any estimates of genetic similarity based on DNA/DNA binding would of course be gravely biased.

In spite of these evident difficulties, DNA/DNA binding has a more compelling claim than DNA/RNA binding to reflect genetic relationship. DNA/RNA binding is essentially a function of phenotypic similarity, along with all other aspects of the phenotype; but DNA/DNA binding, subject to the qualifications made above, is a function of genotypic similarity. The two measures give very similar results in bacteria (McCarthy & Bolton, 1963), but it would be surprising were they to coincide also in higher organisms.

The sensitivity of the method could be increased either by improving the technique so as to cut down experimental error, or by adapting it so as to increase the resolving power. The sources of experimental error are considered more fully elsewhere (Walker & McLaren, 1965). They include small contributions from counting error and from variations in the length of the DNA fragments; larger contributions from variations in the wetness of the incubation mixture, and from variable amounts of the long DNA leaching out of the agar; and a very large contribution from variations in binding ability among different long DNA preparations (especially those from different species). The last factor, the explanation of which is still unknown, can be allowed for by testing labelled DNA fragments from different organisms on samples of the same DNA-agar preparation; or, better, by the elegant technique of Hoyer *et al.* (1964), in which DNA fragments from different organisms are labelled with different isotopes, and tested simultaneously, on the same sample of DNA-agar.

The present paper has been concerned more directly with ways of increasing the resolving power of the technique. We have shown that discrimination between mouse and rat DNA can be improved by blocking the binding reaction with rela-

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tively large amounts of unlabelled short DNA. Still greater discrimination can be achieved by recovering the bound and unbound DNA fragments, and subjecting them to a second and even a third incubation. These experiments incidentally provide direct evidence that mice and rats do both possess some DNA which is absent from the other, as well as a larger fraction common to both species.

One unexpected finding was that the initial preparations of DNA fragments are not homogeneous. Some DNA fragments are bound, and on recovery continue to show a good proportion of binding; but the DNA (or at any rate the radioactivity) which fails to be bound on the first cycle appears to be virtually incapable of binding thereafter. We are still uncertain as to the cause of this heterogeneity (see Walker & McLaren, 1965).

The existence of a DNA fraction which is 'unbindable' under the experimental conditions used may be relevant to the findings of Cowie & McCarthy (1963). They incubated λ phage DNA fragments with long *E. coli* DNA, and found an initial binding factor of 26%. The reciprocal experiment was not carried out, nor were λ and *E. coli* DNA fragments incubated simultaneously on the long *E. coli* DNA, so there is no direct evidence as to the binding ability of the long DNA. On a second cycle of incubation, the bound fraction gave a *B* factor of 45%, and the initially unbound fraction a *B* factor of 8%. These values are strikingly reminiscent of our own second-cycle values when mouse DNA fragments are incubated with long mouse DNA (Table 5). The authors' conclusion that only about 33% of the λ DNA was homologous to *E. coli* DNA should therefore be viewed with reserve.

In so far as they overlap, our findings support those of Hoyer *et al.* (1964). Their binding factors (*B* values) are consistently lower than ours, no doubt because their incubation mixtures were wetter. They find, as we do, a higher proportion of binding on the second cycle after recovery of the bound DNA fragments; but they did not recover the unbound fragments. They report that fragments of mouse DNA give a *B* value of 14% with long rat DNA, compared to 22% with long mouse DNA. This difference, though of roughly the same order of magnitude as we found, is not in itself evidence of any genetic differences between rats and mice: it could be due merely to differences in binding ability between the long rat and long mouse DNA, since they, like us, found that long DNA from different species differed widely in binding ability. They did not do the reciprocal experiment, using fragments of labelled rat DNA.

For a given relative concentration of blocking DNA, Hoyer *et al.* (1964) report a more extensive decrease in binding than we have found. This may be because they did not wash off the unbound blocking DNA before adding the labelled material, but incubated all three kinds of DNA together. In such a situation, the blocking DNA may prevent the labelled fragments from binding onto the long DNA not only by occupying some of the sites on the long DNA, but also by itself combining with the labelled fragments.

In their discussion, Hoyer *et al.* state that they have been unable to distinguish between DNA from the green monkey and from the rhesus monkey. Presumably this refers to experiments using a simple reciprocal incubation technique. How

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small a genetic difference between two groups could be detected with the aid of the discrimination procedures described in the present paper remains to be determined.

SUMMARY

Under suitable experimental conditions, short denatured DNA fragments may be bound by high molecular weight denatured DNA immobilized in agar ('long DNA'). The proportion of fragments bound depends in part upon the degree of taxonomic relationship between the organisms from which the two DNA components are taken. Before the method can be used as a measure of genetic relationship between closely related species or between intra-specific groups, its resolving power requires to be increased. The present study explores some modifications of the method from this point of view, using the degree of binding between DNA from laboratory mice and rats as a model.

Long rat DNA was less effective than long mouse DNA in binding short DNA fragments from either source. It was therefore necessary to test reciprocally, using both short mouse and short rat DNA on long DNA from each species. Combining reciprocals gives a measure of the proportion of fragments bound in within-species and in between-species combinations. With rats and mice, between-species binding was at about 70% of the within-species level. The discrepancy could be increased, and hence the discriminating power of the method improved, if the binding reaction was partially or totally 'blocked' with an excess of unlabelled DNA fragments in an appropriate species combination.

Even when short and long DNA came from the same source, only about a third of the fragments were bound. The bound and unbound fractions were recovered, and each reincubated with a fresh sample of long DNA. The two fractions then behaved very differently, suggesting that the original DNA preparation is heterogeneous. The initially unbound DNA showed a very low binding ability in subsequent incubations and may be considered virtually 'unbindable'.

When the first incubation is a between-species one, the unbound fraction should include not only this 'unbindable' DNA, but also any DNA characteristic of that species alone, and not held in common by both species. If it could be isolated, this DNA fraction might be expected to discriminate maximally between the two species. By using three successive cycles of incubation, a fraction was obtained which showed considerably improved discriminating power, in that it showed only about 30% as much binding in between-species as in within-species combinations.

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