Plant Physiol. (1967) 42, 959–967

Relatedness Among Plants as Measured by the DNA-Agar Technique

Arnold J. Bendich[1] and Ellis T. Bolton
Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D. C. 20015

Received March 22, 1967.

Summary. An improved method for extraction of plant DNA is described. Quantitative species comparisons based on DNA-DNA hybridization are reported for several members of the family *Leguminosae* and for barley, wheat and rye. A maximum of about 10 % homology in DNA polynucleotide sequences is found between monocotyledons and dicotyledons tested, whereas 20 to 90 % homology is observed within a family. Species compared using a DNA fraction enriched for redundant polynucleotide sequences generally appear to be more closely related than when whole DNA is used. DNA-DNA hybridization may be useful in systematic and evolutionary study of plants, and also as a possible screening procedure for interfertility of species.

Homologies among the nucleic acids of viruses, of bacteria, and of animals have been established and a quantitative means for the assessment of genetic relationship among species has been described (5, 7). The development of an improved method for the isolation of high molecular weight plant DNA has now permitted application of the DNA-agar procedure to studies of genetic relatedness among higher plants. This report describes the DNA isolation procedure and presents some results of comparisons of polynucleotide sequence similarities in plant nucleic acids.

The design of these experiments was to react radioactive DNA fragments of one species with homologous DNA in agar in the presence of increasing amounts of non-radioactive DNA fragments from the same or from different species. Whenever the mixture contains radioactive and non-radioactive fragments which are similar in nucleotide sequence, the non-radioactive fragments will compete with the radioactive ones for sites on the DNA immobilized in agar (5). Hence, the amount of radioactivity bound to the DNA-agar would be decreased and the decrement serves as a measure of the relatedness of the DNA from the 2 sources.

Materials and Methods

Preparation of Plant DNA. The method described below for the isolation of DNA from seedlings of *Pisum sativum*, var. Alaska has been successfully applied to other legumes and to representative ferns, gymnosperms and angiosperms. In general, it is advantageous to use young and rapidly-developing tissues such as seedlings, although fully-expanded leaves of several dicotyledons and monocotyledons were also useful sources of DNA.

Ten g[2] fresh weight of pea seedlings from which the cotyledons have been stripped were minced with scissors. Five to 10 ml of a solution containing 1 % sodium dodecyl sulfate, 0.1 M disodium EDTA, and 3 × SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate) were added to the minced seedlings in a mortar, and vigorous grinding at room temperature was carried out for 1 minute. The resulting thick paste was transferred to a glass stoppered bottle containing an equal volume of chloroform containing 1 % octanol. The mixture was shaken rapidly by hand for 30 seconds and then centrifuged briefly to separate the phases. The upper aqueous layer which contains the DNA was poured into a pre-heated bottle and incubated for 5 minutes at 72° in a water bath. The time required for the procedure to this point was not allowed to exceed 4 minutes. This extract was quickly cooled in an ice bath and adjusted to 1 M sodium perchlorate, and shaken with an equal volume of chloroform-octanol and recentrifuged. The aqueous layer was removed to a beaker. Two volumes of 95 % ethanol were layered over the extract, the layers slowly mixed, and the DNA fibers were removed with a glass rod. Should the DNA concentration be low, the DNA precipitate will not form fibers efficiently. In this event the

[1] Present address: Department of Microbiology, University of Washington, Seattle, Washington 98105.

[2] Hundreds of grams of plant tissue (including tissues which are difficult to grind because of a fibrous nature) may readily be processed if the tissue is frozen at dry ice temperature and brought to a fine powder by pulverizing in a chilled blender or mortar. Except for scaling up the volumes of solutions, the remainder of the procedure is the same.

alcohol-precipitated nucleic acid can be collected by centrifugation and dissolved in a small amount of 0.1 × SSC from which the DNA usually can be reclaimed by winding on a glass rod again after bringing the solution to 1 × SSC and adding 2 volumes of ethanol. The fibrous DNA was transferred to a vessel containing 0.1 × SSC and the DNA dissolved by gentle shaking. Tightly spooled DNA requires more time for dissolving than does loosely spooled or clumped DNA. The procedure to this point usually requires about one-half hour.

DNA was further purified by treatment with ribonuclease (20–50 μg/ml, one-half hour at 37°) which was previously heated to 100° for 5 minutes to inactivate possible DNAase contaminants, and by shaking with chloroform-octanol, and again precipitating with ethanol. Deproteinization by shaking with water-saturated phenol is useful when the DNA is contaminated with plant pigments, and when the presence of RNAase is undesirable.

The yield of DNA varied considerably depending upon the species, and particularly upon the stage of development of the tissue. Typically, 2 to 3 mg of highly purified DNA were extracted from 10 g fresh, young pea leaves, roots, or tendrils, while only 0.1 mg of DNA was isolated from 50 g of mature forms of a common fern (*Woodwardia* Sp.).

Preparation of ^{32}P-labeled DNA. Seeds were allowed to germinate in a moist inert supporting medium such as pearlite or vermiculite, after previously having been soaked in 1 % clorox (NaOCl) for 20 minutes. When the seedlings were approximately 2 cm high, 10 or 20 were removed to a beaker after thoroughly washing their roots free of supporting medium. The roots were covered with distilled water (usually 3–5 ml) and carrier free $^{32}PO_4$ (1 mc) was added. The water was replenished as required. After 3 to 7 days when the plants had doubled or tripled their mass, they were washed free of radioactive solution. The remaining seed coats and cotyledons were removed and the whole plant was minced with scissors and the ^{32}P-labeled DNA was extracted as described above. Various preparations, depending on the age of the plants, the species, and the duration of labeling have yielded DNAs containing from 1000 to 50,000 cpm/μg.

Purified radioactive pea DNA was examined to determine whether the ^{32}P was in the DNA and not in other contaminating cell components. Deoxyribonuclease digestion rendered most of the radioactivity trichloroacetic acid soluble, whereas ribonuclease treatment left the radioactivity precipitable on cellulose nitrate filters (table I). Chromatography on G-100 Sephadex of heat denatured ^{32}P pea DNA showed the radioactivity to be associated with 260 mμ-absorbing material and in the high molecular weight fraction.

DNA-Agar Procedure. The DNA-agar method of Bolton and McCarthy (1) was used to measure homologies among various plant DNAs following the procedures described by Hoyer, McCarthy, and Bolton (5) in comparing DNAs of vertebrates. Calculation of percent homology in the DNA of 2 sources is as follows: W = % binding of ^{32}P labeled DNA to DNA-agar; X = % binding of ^{32}P labeled DNA to DNA-agar in the presence of n μg heterologous unlabeled DNA; Y = % binding of ^{32}P labeled DNA to DNA-agar in the presence of n μg homologous unlabeled DNA; % homology $= \frac{W-X}{W-Y} \times 100$.

Preparation of Aggregate and Supernatant Fractions of DNA. ^{32}P-DNA was sheared at 10,000 to 15,000 p.s.i. in the French pressure cell (11) and the resulting fragments (about 800,000 daltons) were heated for 5 minutes at 100° in 2 × SSC, and quickly placed in a 60° water bath for overnight incubation at a concentration of 30 to 250 μg/ml. The suspension was then transferred to a 2 ml or 5 ml centrifuge tube and 2 × SSC added at room temperature to fill the tube. The tube was centrifuged in the Spinco Model L centrifuge at 30,000 or 35,000 rpm for 20 minutes. The aggregate pellet could be seen at the bottom of the tube. The supernatant liquid was poured off or if the aggregate pellet was small, it was drawn off. The pellet was washed into a vessel with 2 × SSC, and both the aggregate and supernatant fractions were heated in a boiling water bath for 5 minutes and quickly cooled to inhibit renaturation. The amount of total DNA trapped in the aggregate varied from 10 to 65 %, depending on the initial concentration. Up to 90 % of the

Table I. *Results of Nuclease Action on ^{32}P-DNA*

The reaction mixtures were incubated 30 minutes at 37°. Carrier RNA was added and precipitated. The numbers represent percent of control radioactivity trapped on cellulose nitrate filters.

	Percent counts remaining after treatment		
	Control*	Deoxyribonuclease**	Ribonuclease***
Preparation A	100	34	99
Preparation B	100	27	98

* Control: ^{32}P-Pea DNA.
** Deoxyribonuclease: ^{32}P-Pea DNA + DNAase (30 μg/ml) in 10 mM $MgCl_2$.
*** Ribonuclease: ^{32}P-Pea DNA + RNAase (30 μg/ml).

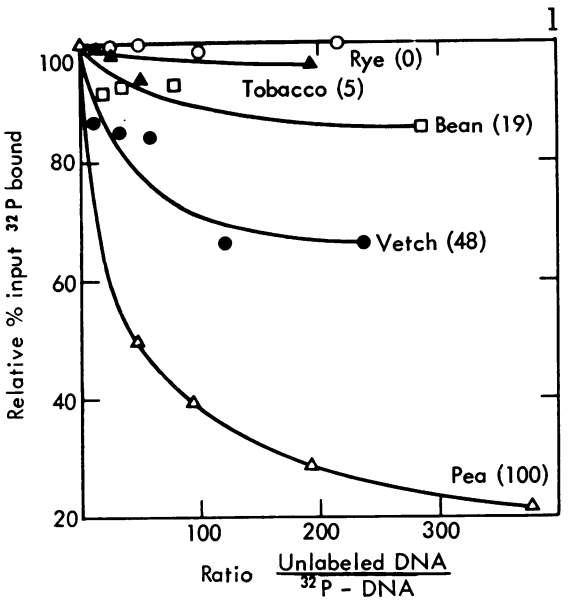

FIG. 1. Competition by unlabeled DNA fragments in the reaction between ^{32}P-labeled pea DNA fragments and agar containing high-molecular weight single-stranded pea DNA. One-half or one microgram ^{32}P-labeled pea DNA fragments (640–3800 cpm/μg) was incubated with 0.3 to 0.5 g agar containing 86 to 184 μg pea DNA in the presence of varying quantities of unlabeled DNA fragments from the species indicated. The total volume was 0.6 to 1.0 ml. Percentage (relative to homologous test in absence of unlabeled DNA) of labeled DNA fragments bound is plotted against the ratio of unlabeled DNA/^{32}P-labeled DNA.

DNA could be trapped in an aggregate pellet when long DNA strands were used in place of sheared DNA fragments.

The radioactivity in both the aggregate and supernatant fractions was released by DNAase when treated, and passed through cellulose nitrate filters. Non-radioactive DNA aggregate and supernatant fractions were prepared in a similar way, and the concentration at incubation was 500 μg/ml.

Results

Figure 1 shows the results of interactions of several plant DNAs with that of the pea. Considering the homologous pea-pea interaction as a standard for comparison, and using the criteria of Hoyer, McCarthy, and Bolton (5) for DNA similarity, it can be seen that the nucleotide sequences in the DNA of the hairy vetch (*Vicia villosa*) are similar to about one-half of those of pea, while only about one-fifth of the pea sequences are found in beans (*Phaseolus vulgaris*). A species of tobacco (*Nicotiana glauca*) DNA interacts to a much smaller extent and almost no interaction can be detected between rye (*Secale cereale*, var. Rosen) and pea DNA.

Comparisons of the similarities in the DNAs of rye, barley (*Hordeum bulbosum*, var. Betzes) and wheat (*Triticum vulgare*, var. Seneca) have also been made. It can be seen in figure 2 that the DNA of the wheat reacts more extensively with rye DNA than does barley DNA. Approximately 60 % of the nucleotide sequences in the rye DNA appear similar to those in barley, while about 75 % of the rye sequences are similar to those of wheat.

Table II summarizes the results of other similar competition experiments.

Discussion

DNA Extraction. Extraction of DNA in high yields is desirable in choosing the species against which others may be compared. In our procedure peas lend themselves well to DNA extraction. Close relatives of the pea, such as pole beans (*Phaseolus vulgaris*), black-eyed cow peas (*Vigna sinensus*), clover (*Trifolium repens*), and peanuts (*Arachis hypogea*) prove to be relatively poor sources, while hairy vetch, and all the cereal grains tried, were quite good sources. Young tissue was better than older tissue for DNA extraction. The most important factor in our procedure is the speed with which the soluble extract, with its DNA, can be

Table II. *Competition Comparisons of the DNAs of the Members of the* Leguminosa *Family and the Cereal Grains*

^{32}P-DNA and DNA in agar	Heterologous competitor	Percent homology
A) Pea	Pole beans (*Phaseolus vulgaris*)	19
	Hairy vetch (*Vicia villosa*)	48
	Tobacco (*Nicotiana glauca*)	5–10
	Rye (*Secale cereale*, var. Rosen)	0–5
B) Hairy Vetch	Pea (*Pisum sativum*, var. Alaska)	54
C) Rye	Barley (*Hordeum bulbosum*)	59, 56
	Seneca wheat (*Triticum vulgare*, var. Seneca)	75
	Chinese Spring Wheat (*Triticum vulgare*, var. Chinese Spring)	74
D) Barley	Rye	70, 78
	Seneca Wheat	73
	Pea	10
E) Seneca Wheat	Pea	10
	Barley	80
	Rye	95, 90

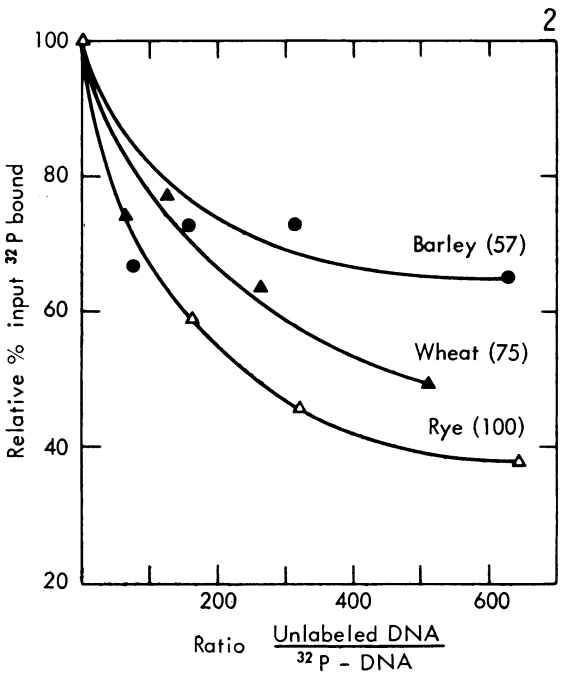

FIG. 2. Competition by unlabeled DNA fragments in the reaction between ^{32}P-labeled rye DNA fragments and agar containing high-molecular weight single-stranded rye DNA. One quarter and 0.41 μg of ^{32}P-labeled rye DNA fragments (2600 and 8400 cpm/μg) was incubated with 0.5 g agar containing 18 μg rye DNA in the presence of varying quantities of unlabeled DNA fragments from the species indicated. The total volume is 1.0 ml. Coordinates as for figure 1.

brought to the heating step which apparently inactivates the deoxyribonucleases present. The time for grinding, first chloroform deproteinization, and transfer of the soluble extract to a 72° bath should be kept at a minimum (ca. 3–4 mins, if possible). This time factor depends on the species; pea requires less haste. Pressure cells and blenders apparently shear the DNA and makes extraction difficult. Grinding by hand with a mortar and pestle proved best.

Species Comparisons. The interactions of the DNAs of the cereal grains are especially intriguing because fertile hybrids of wheat and rye are a commonplace while hybrids of barley and rye are not known. Figure 2 shows wheat DNA to be more similar to rye DNA than is barley DNA. The possibility is thus offered that quantitative comparisons of DNA homologies may serve as indicators of genetic compatibility between plant species. It might be feasible, for example, to provide the plant breeder with a choice based on DNA relatedness among possibilities for successful crosses which would have a high probability of giving rise to desirable offspring.

The pea, vetch, and bean are members of the family *Leguminosae* and their DNAs interact to a greater degree than does the DNA from a dicotyledon of a different family (*N. glauca*) or that from a monocotyledon, the rye. It would appear, therefore, that such interactions might usefully be applied to chart systematic affinities and possible evolutionary relationships among higher plants. Within the family *Leguminosae* the relative genetic diversity as measured by these DNA interactions is at least as great as exists among orders of mammals as distantly related as mice and men (5). The basis for such diversity is not known. It may be that since peas and beans are cultivated forms and therefore strongly influenced by human intervention, they have been subjected to a kind of directed evolution. In view of this consideration it would be of interest to compare DNA homologies among a series of feral species of plants. Given an adequate archaeological and paleontological record of the probable evolutionary progress of such forms, it ought to be possible to assess how profoundly man may have influenced the average rate of evolution within a group of plants.

Aggregate DNA Fraction. Britten and Waring (2) have observed that denatured DNA from higher organisms forms 3-dimensional networks when incubated in solution under renaturation conditions. These networks, or aggregates, are hyperpolymers apparently held together by interstrand complementary nucleotide sequences. They can be collected as gelatinous precipitates by high speed centrifugation. The amount of DNA trapped in such an aggregate depends upon concentration, conditions of incubation, gravitational force during centrifugation, and molecular weight of the strands. As shown below, such aggregate DNA reacts with high efficiency in the DNA-agar procedure. Reaction in both the DNA-agar procedure and in the aggregation phenomenon between single DNA strands is one of renaturation, or partial renaturation, of the strands. It had been previously thought that the complexity of DNA from higher organisms was the reason why complementary polynucleotide sequences failed to find one another and renature at a detectable rate under renaturation conditions (6). Britten and Waring (2) have found the renaturation rate of some higher organism DNAs comparable to that of bacterial DNA. They concluded that this anomalously high renaturation rate was due to a redundancy of similar sequences present in the DNAs with some regions having hundreds of thousands of close copies. It was thought that these redundant sequences of about 2×10^5 daltons were the ones reacting in an overnight DNA-agar or aggregation incubation. The remaining less redundant or unique sequences would renature too slowly under these incubation conditions and would not participate in the reaction. There would be a gradation in redundancy of sequence among segments as opposed to a sharp distinction between aggregatable and non-aggregatable segments. This would lead to populations of se-

quences with the ones best able to form a duplex with DNA-agar appearing in the aggregate pellet and the ones less able to do so in the supernatant fraction. Therefore the aggregation procedure should prove a technique for separating regions of DNA based on redundancy of sequences.

Labeled DNA. The extent to which ^{32}P-labeled plant DNA will bind to DNA-agar depends upon the length of time the seedlings grow in the presence of the isotope. This was shown in the following way. Young pea seedlings were grown in $^{32}PO_4$ and water. At different time intervals several plants were removed from the ^{32}P-medium, the DNA extracted, and aggregate and supernatant fractions of the ^{32}P-DNA were prepared as described above. The specific radioactivities (count per min per μg DNA) were determined for the whole DNA as well as the supernatant and aggregate fractions separately. The results of this experiment, shown in table III, indicate that the ratio of specific activity of the supernatant to that of the aggregate falls as the duration of labeling increases. In another experiment the DNAs from pea plants grown in the presence of label for 1.8 days and 5 days were isolated and separated into aggregate and supernatant fractions. The ability of these fractions as well as that of whole DNA to bind to pea DNA-agar is shown in table IV; the aggregate fraction binds to DNA-agar much better than does the supernatant fraction. The whole DNA is intermediate in binding ability and binds 7.5 % in the case of the 1.8-day labeled material, and 22 % with the 5-day labeled DNA. Figure 3 shows the results of competition experiments involving these fractions. It can be seen that the supernatant fraction has very few nucleotide sequences similar to those in the aggregate fraction. The observed drop in the supernatant competitor DNA curve may be due to imperfect separation of the 2 fractions, and thus would represent an aggregate DNA contaminant in the supernatant fraction. The whole competitor DNA curve quickly approaches the aggregate competitor DNA curve as would be expected since it contains the same DNA fraction, albeit in lower concentration than does the aggregate fraction. In other experiments it was shown, in agreement with Britten and Waring (2), that over 90 % of high molecular weight DNA can be trapped in the aggregate fraction; this probably is due to longer denatured regions trapped with contiguous short duplex structures. The same phenomenon was exhibited by labeled wheat, barley, and rye DNAs. The specific activity ratios of supernatant to aggregate fractions ranged from 1.87 with 7-day labeled rye to 3.3 with 4.7-day labeled barley. Aggregate and supernatant DNA

Table III. *Effect of Length of $^{32}PO_4$ Labeling Period on Specific Activities of Pea DNA Fractions*

DNA isolated from pea seedlings after various times of growth in the presence of of $^{32}PO_4$. DNA preparations were heated to 100° for 5 minutes at the concentrations given, and incubated overnight at 60° in 2 × SSC. Aggregate fractions were collected by centrifugation for 20 minutes at 30,000 rpm in the Spinco SW-39 rotor. Separated aggregate and supernatant fractions were diluted, heated to 100° for 5 minutes, and quickly cooled.

Length of labeling period	Conc. at aggregation incubation	% DNA in aggregate	Specific activity whole DNA	Specific activity supernatant fraction	Specific activity aggregate fraction	Specific activity sup. / Specific activity agg.
hr	μg/ml	OD	cpm/μg pea DNA	units	units	
4.1	125	40	424	541	29.8	18.8
18.5	226	49	865	1498	211	7.1
43	213	53	431	621	188	3.3
125	112	47	422	459	255	1.76
Unlabeled pea DNA fragments	568	71	...	...	...	...

Table IV. *Unfractionated, Aggregate, and Supernatant DNA Binding to DNA-agar*

The experimental conditions were: 1 μg ^{32}P-pea DNA: Whole DNA, 1.8-day labeling period, 5000 cpm/μg; Whole DNA, 5-day labeling period, 2700 cpm/μg; Aggregate fraction, 1.8-day period, 300 cpm/μg; Aggregate fraction, 5-day period, 1600 cpm/μg; Supernatant fraction, 1.8-day period, 6000 cpm/μg; Supernatant fraction, 5-day period, 4400 cpm/μg. 0.5 g DNA-agar containing 74 μg pea DNA in each case. + 0.5 ml ^{32}P-DNA solution in each case. Aggregation incubation at 100 μg/ml for 5-day labeled DNA, and at 136 μg/ml for the 1.8-day labeled DNA.

	% Of input ^{32}P-pea DNA binding to pea DNA-agar	
	5 Day label	1.8 Day label
Unfractionated DNA	22	7.0
Aggregate fraction	46	48
Supernatant fraction	8.4	2.5

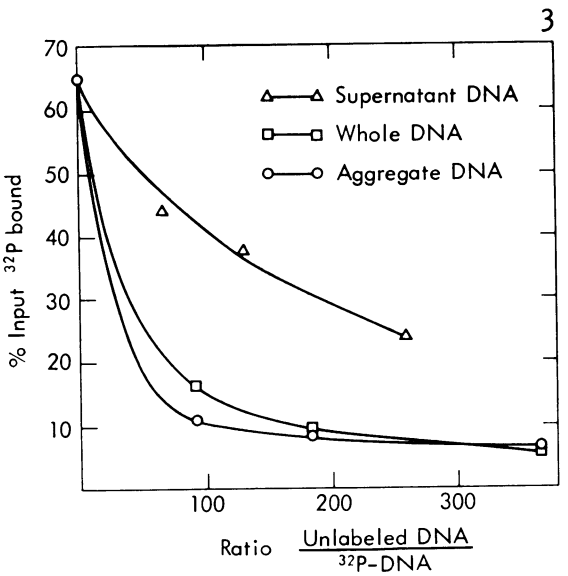

FIG. 3. Competition by unlabeled pea DNA fractions in the reaction between ^{32}P-labeled aggregate fraction pea DNA fragments and agar containing pea DNA. One-half gram of agar containing 74 μg pea DNA was incubated with 0.3 μg ^{32}P-labeled aggregate fraction pea DNA (800 cpm/μg) fragments in the presence of varying quantities of unlabeled DNA fragments from whole, aggregate and supernatant fractions. The total volume was 1.0 ml. The percentage of labeled DNA fragments bound is plotted against the ratio of unlabeled DNA/^{32}P-labeled DNA.

fractions from 5-day labeled wheat bound 34.5 and 7.4 % respectively to wheat DNA-agar.

It therefore appears that the aggregate fraction in these DNAs represents the major part of the DNA which will bind to DNA-agar, and shows very little homology to the supernatant fraction. In addition, the supernatant fraction contains DNA synthesized at least 18 times as rapidly, and probably much more, as the bulk of the DNA. This rapidly labeled small fraction of DNA depresses the percent of the total input of ^{32}P-DNA which binds to DNA-agar. Since the binding of ^{32}P-DNA to DNA-agar can vary greatly as the duration of labeling varies, a difference in percent binding of labeled DNAs between 2 species does not necessarily accurately reflect degree of relatedness in these DNAs. Competition curves such as those in figures 1 and 2 are based not on different preparations of labeled DNAs but rather on the comparison of unlabeled DNAs and should better represent DNA relatedness.

The rapidly labeled small fraction of DNA may be extranuclear. The presence of DNA in both plastids and mitochondria is now well substantiated (9, 10). Hotta, et al. (4) have reported a rapidly turning over fraction in wheat DNA which they suspect is plastid DNA. The following experiment was carried out to determine whether or not the rapidly labeled component was chloroplast DNA. Young pea plants were labeled with $^{32}PO_4$ for 6 days. The roots were separated from the shoots (only the seed was discarded) and DNA was extracted from each separately. The specific activity in the root DNA was 18,100 while that of the shoots was 2260 counts per minute per μg. One μg of sheared labeled root DNA was added to 205 μg unlabeled root DNA fragments and aggregation was carried out at 296 μg/ml. A similar preparation was made with labeled and unlabeled shoot DNA fragments under the same conditions.

Aggregate and supernatant fractions were separated and specific activities determined. The ratio of specific activities of supernatant to aggregate is 3.16 with roots, and 1.78 with shoots (table V). This indicates that the rapidly labeled component is more prevalent in roots than in shoots, and hence is unlikely to be chloroplast DNA. Furthermore, under identical conditions (0.4 μg labeled DNA with 44.4 μg DNA-agar) labeled root DNA fragments bound 26.4 % and labeled shoot DNA fragments bound 42.3 % to DNA-agar. Figure 4 shows that the DNAs from roots and shoots are indistinguishable as judged by competition curves. Unlabeled root and shoot DNA fragments compete with the labeled fragments equally well.

Table V. *Effect of Tissue Source on Specific Activities of Pea DNA Fractions*

One μg ^{32}P-shoot DNA fragments (2260 cpm/μg) added to 205 μg unlabeled shoot DNA fragments. One μg ^{32}P-root DNA fragments (18,100 cpm/μg) added to 205 μg unlabeled root DNA fragments. Both heated to 100° for 5 minutes at 296 μg/ml and incubated overnight at 60° in 2 × SSC. Aggregate fraction collected by centrifugation for 25 minutes at 35,000 rpm in the Spinco SW-39 rotor. Separated aggregate and supernatant fractions diluted, heated to 100° for 5 minutes, and quickly cooled.

	% DNA in fraction	Radioactivity in fraction	Specific activity	Specific activity sup. / Specific activity agg.
	OD		cpm/μg	
A) Roots Sup.	41.9	69	133	3.16
A) Roots Agg.	58.1	31	42.1	
B) Shoots Sup.	40.4	55	17.9	1.78
B) Shoots Agg.	59.6	45	10.1	

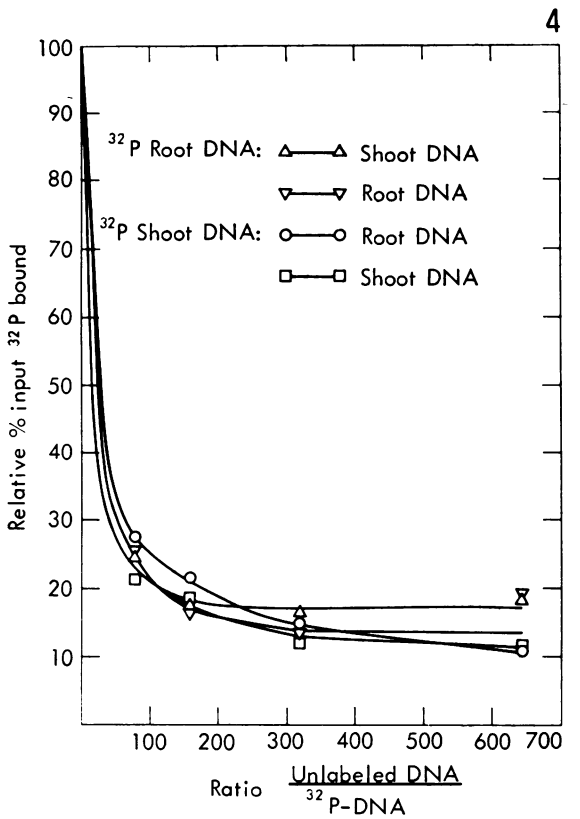

FIG. 4. Competition between unlabeled root and shoot pea DNA fragments in the reaction between ^{32}P-labeled root and shoot pea DNA fragments and agar containing pea DNA. Four-tenths μg ^{32}P-labeled pea DNA fragments from roots (12,500 cpm/μg) or shoots (2500 cpm/μg) was incubated with 0.3 g agar containing 44 μg pea DNA in the presence of varying quantities of unlabeled pea DNA fragments from roots and shoots. The total volume was 0.6 ml. The percentage of labeled DNA fragments bound is plotted against the ratio of unlabeled DNA/^{32}P-labeled DNA.

The identity of the small DNA component which incorporates ^{32}P quickly is not now known. But its apparent deleterious effect on this technique of preventing a disproportionately large number of counts from participating in hybridization can be minimized by long labeling periods, perhaps 7 days, to approach a "steady state" situation[3].

Aggregate DNA Species Comparisons. Some of the same competition experiments as in table II were carried out using aggregate ^{32}P-DNA instead of whole ^{32}P-DNA. Since the data from figure 3 indicate very little difference in competitive ability between unlabeled whole and unlabeled aggregate DNAs at high levels of competitor, whole unlabeled DNAs were used exclusively as competitor in the following experiments. The data in table VI represent homology values derived from competition curves using aggregate ^{32}P-DNA. These values are directly comparable to those in table II. In general the species appear more closely related using aggregate ^{32}P-DNAs than whole ^{32}P-DNAs. In other words, the more redundant sequences (aggregate fraction) are held in common between 2 species to a greater extent than are the less redundant ones (whole DNA). The preparation of an aggregate fraction selects the more redundant sequences which have been better conserved among species than overall DNA sequences.

Because of the small amounts of some of the labeled DNAs prepared, it was not practical to carry out the aggregation incubations at the same concentrations. As a consequence the amount of DNA trapped in each aggregate varied from 18 % with rye to 52 % with pea (table VI). This means that the rye aggregate preparation should contain DNA of more highly redundant and more closely similar polynucleotide sequences than that of pea. The most precise and the most numerous replicas would aggregate first. If these redundant sequences have been conserved relative to the rest of the DNA, species should appear closer in the DNA-agar technique using ^{32}P-aggregate DNA fractions than ^{32}P-whole DNAs, and discrepancies between the aggregate DNA and whole DNA values should be greatest when the fraction of whole DNA in the aggregate is lowest. In the cereal grains comparison of tables II and VI shows the discrepancies in percent homology between aggregate and whole DNA determinations are greatest with ^{32}P-rye (18 % in aggregate fraction), less with ^{32}P-barley (34 % in aggregate fraction), and none with ^{32}P-wheat (40 % in aggregate fraction).

Reciprocal Comparisons. The data in table II show that among the cereal grains the reciprocal experiments do not agree. That is, with labeled rye DNA and rye DNA-agar, unlabeled wheat competes 75 % as well as unlabeled rye, but with labeled wheat DNA and wheat DNA-agar, unlabeled rye competes 90 to 95 % as well as unlabeled wheat. This effect is also seen with rye and barley: 59 and 56 % versus 70 and 78 %, with the last pair, barley and wheat, 73 versus 80 % may be significant or due to experimental scatter. The same experiments in table VI using ^{32}P-aggregate fractions of the various DNAs show almost perfect reciprocal agreement. A model is proposed to interpret these data. Figure 5a is a schematic representation of the genomes of wheat and rye. The length of each line represents the amount of dissimilar DNA polynucleotide sequences. Identical or nearly identical genome segments would not add to the length of the line. The DNA-agar technique is not sensitive enough to differentiate between closely similar

[3] The possibility that it may be bacterial DNA cannot be excluded. A small amount of DNA from contaminating bacteria containing a substantial fraction of the ^{32}P in DNA would serve to raise the noise level in these experiments, but should not invalidate the relatedness values obtained with competition experiments.

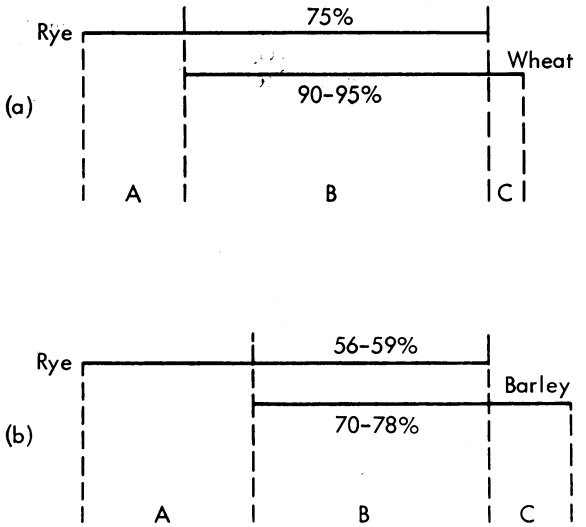

FIG. 5. Model of genomes of rye, wheat, and barley. The length of each line represents the amount of dissimilar DNA polynucleotide sequences. Identical repeated sequences would not add to the length of the line. Regions A and C represent sequences unique to the species. Region B represents those sequences held in common by the pair of species. See text for details.

polynucleotide sequences. Region B represents those nucleotide sequences held in common by the 2 species. On this model it can be seen that unlabeled wheat DNA would compete for sites on rye DNA-agar only 75 % as well as would unlabeled rye DNA. Unlabeled rye would compete 90 to 95 % as well as unlabeled wheat for sites in wheat DNA-agar. The supernatant fraction of rye DNA would occur as a part of region A, that of wheat DNA as a part of region C. Figure 5b is an analogous representation of the rye and barley situation. Again the supernatant fractions of rye and barley DNA would be found within regions A and C respectively. Preparation of aggregate DNA fractions would enrich for the redundant polynucleotide sequences (found within region B) and discriminate against the more unique ones (found within regions A and C). Consequently the reciprocal comparisons using ^{32}P-aggregate DNA fractions should agree better than those using ^{32}P-whole DNAs. The data in table VI show extremely close agreement in reciprocal comparisons of the cereal grains. Recently Rees et al. (8) have compared the amount of DNA per nucleus in 3 angiosperm genera using interference microscopy and Feulgen photometry. They have found striking differences in the DNA content per nucleus within the same genus: as much as 7-fold difference within the *Vicia* genus, without an increase in chromosome number. Differences in DNA content were also found within the genera *Lathyrus* (3.5-fold) and *Lolium* (30 %); the chromosome number being the same within each genus for the species reported. This finding would be consistent with the differences obtained in the reciprocal comparisons in the cereal grains.

The above model and discussion of the relationships of aggregate and supernatant DNA fractions may have practical importance in application of DNA-DNA hybridization to problems of plant taxonomy. By selecting particular conditions for formation of an aggregate fraction of DNA, species' differences could be either magnified or diminished. Conditions permitting only a very small portion of labeled DNA to be trapped in an aggregate preparation (e.g. higher temp of incubation) might bring distantly related species within the workable range for comparison, when use of whole labeled DNAs would preclude this. Also use of labeled supernatant DNA fractions might amplify subtle species' differences unnoticed by use of whole labeled DNA. With the advent of DNA-DNA hybridization techniques making use of DNA embedded on filters (3, 12) instead of in agar, the technical difficulties of the DNA-agar technique, such as the need for very high molecular weight DNA for agar, small numbers of incubations per day, and other specialized procedures, may be avoided and large numbers of comparisons should be possible.

Table VI. *Effect of Competition Curves in the DNAs of the Members of the* Leguminosa *Family and the Cereal Grains Using* ^{32}*P-aggregate DNAs*

^{32}P-aggregate DNA and DNA in agar	% Of total ^{32}P-DNA trapped in aggregate	Heterologous competitor	% Homology
A) Pea	52	Barley	0–7
		Hairy vetch	40
		Black-eyed cow pea (*Vigna sinensus*)	98–100
B) Rye	18	Barley	82
		Seneca wheat	86
C) Barley	34	Rye	84, 83
		Seneca wheat	80
D) Seneca wheat	46	Barley	78
		Rye	86

Literature Cited

1. BOLTON, E. T. AND B. J. MCCARTHY. 1962. A general method for the isolation of RNA complementary to DNA. Proc. Natl. Acad. Sci. U. S. 48: 1390–97.
2. BRITTEN, R. J. AND M. WARING. 1965. "Renaturation" of the DNA of higher organisms. In: Carnegie Inst. Wash. Year Book, 1964-1965. Garamond/Pridemark Press, Baltimore, Maryland. p 316.
3. DENHART, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23: 641–46.
4. HOTTA, Y., A. BASSEL, AND H. STERN. 1965. Nuclear DNA and cytoplasmic DNA from tissues of higher plants. J. Cell Biol. 27: 451–57.
5. HOYER, B. H., B. J. MCCARTHY, AND E. T. BOLTON. 1964. A molecular approach in the systematics of higher organisms. Science 144: 959–67.
6. MARMUR, J. AND P. DOTY. 1961. Thermal renaturation of deoxyribonucleic acids. J. Mol. Biol. 3: 585–94.
7. MCCARTHY, B. J. AND E. T. BOLTON. 1963. An approach to the measurement of genetic relatedness among organisms. Proc. Natl. Acad. Sci. US 50: 156–64.
8. REES, H. F., M. CAMERON, M. H. HAYARIKA, AND G. H. JONES. 1966. Nuclear variation between diploid angiosperms. Nature 211: 828–30.
9. SHIPP, W. S., F. J. K. KIERAS, AND R. HASELKORN. 1965. DNA associated with tobacco chloroplasts. Proc. Natl. Acad. Sci. US 54: 207–13.
10. TEWARI, K. K., W. VÖTSCH, H. R. MAHLER, AND B. MACKLER. 1966. Biochemical correlates of respiratory deficiency VI. Mitochondrial DNA. J. Mol. Biol. 20: 453–81.
11. WARING, M. AND R. J. BRITTEN. 1966. Nucleotide sequence repetition: A rapidly reassociating fraction of mouse DNA. Science 154: 791–94.
12. WARNAAR, S. O. AND J. A. COHEN. 1966. A quantitative assay for DNA-DNA hybrids using membrane filters. Biochem. Biophys. Res. Commun. 24: 554–58.