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Aggregate Formation from Short Fragments of Plant DNA[1]

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ABSTRACT

Large aggregates have been observed after partial reassociation of pea (*Pisum sativum* L.) DNA preparations sheared to mean single strand fragment lengths as short as 350 nucleotides. At high DNA concentrations and conditions of salt and temperature which require only moderate precision of base pairing, aggregates pelletable by brief centrifugation account for 30 to 40% of the total DNA from peas, while calf thymus DNA reassociated under similar conditions forms less than 10% pelletable structures. In contrast to networks formed during the reassociation of long DNA fragments containing interspersed repetitive sequences, these aggregates contain a high percentage of double-stranded DNA and are enriched in repetitive sequences.

Aggregates detectable by centrifugation do not begin to appear until after extensive repetitive sequence reassociation has already occurred. The results are consistent with a model involving secondary reassociation between single-stranded regions ("hanging tails") remaining after initial duplex formation. This process would lead to formation of large multimers of the original fragments, analogous to the large hyperpolymers which have been observed in extensively reassociated prokaryotic DNA. Randomly sheared fragments containing short (about 300 base pairs) repetitive sequences interspersed with single copy DNA would not be expected to hyperpolymerize significantly under these conditions. I suggest, as a working hypothesis, that much of the repetitive sequence DNA in peas is contained in regions considerably longer than 300 base pairs.

DNA sequences which reassociate as though they are repeated several to many times are a characteristic feature of eukaryotic genomes (6, 7). This repetitive fraction appears to consist of families of related sequences whose members, while not necessarily identical, are sufficiently similar to cross-react with one another during standard reassociation experiments. Britten and Davidson (3, 4, 10) have proposed a model in which repetitive sequences interspersed among unique sequences throughout the genome are thought to play a role in regulating gene expression, and several experimental studies with animal systems have demonstrated patterns of interspersion which are consistent with this model (9-12). Information on repetitive sequence organization in plant DNA would be of particular interest, since the fraction of plant genomes made up by repetitive DNA, where known, often are much larger than in most animal genomes so far studied (2, 13).

In preliminary experiments on repetitive sequences in pea DNA, large aggregates (pelletable by centrifugation at 40,000*g* for 30 min) were observed after repetitive sequence reassociation. Aggregates frequently accounted for 30 to 40% of the DNA, even when DNA fragments as small as 350 nucleotides were used. In the present experiments, I attempted to characterize these structures and some of the parameters affecting their formation. This work was undertaken primarily for technical reasons, since aggregate formation can be a complication in many kinds of experiments. However, the ability to form large amounts of aggregates may also provide a clue to some aspects of repetitive sequence organization in plant genomes.

Aggregates or networks formed from longer DNA fragments have been reported previously in partially reassociated plant (1) and animal (8) DNA. In both cases, more than 90% of DNA (fragments in the range of 3×10^4 nucleotides, single-stranded) could be included, while 60 to 70% could be obtained with DNA sheared to approximately 1200 nucleotides.

During reassociation of long DNA fragments, several repetitive sequence regions on a given fragment may react with similar regions on several other fragments, even though the reassociated repetitive regions may be widely separated from one another. In the case where repetitive sequences are short and interspersed among unique sequences, reducing the fragment length would result in fewer and fewer fragments with more than one repetitive element, thus reducing the proportion of fragments capable of participating in networks. Observations on plant DNA were not extended to fragments smaller than 1200 nucleotides, but fragments of calf DNA sheared to about 600 nucleotides gave only 10% aggregates under the same conditions. In addition, Graham and Britten (14) recently reported that partially reassociated sea urchin DNA (sheared to an initial length of approximately 300 nucleotides) consists mainly of structures involving four or fewer of the original fragments, with only a small fraction of larger hyperpolymers. The initial observation that pea seedling DNA fragments in the range of 350 to 500 nucleotides could form aggregates involving 30 to 40% of the total DNA seemed worthy of further investigation.

MATERIALS AND METHODS

Plant Material and DNA Preparation. DNA was prepared from combined leaves and stems of 10- to 14-day-old greenhouse-grown pea (*Pisum sativum* L. var. Alaska) seedlings. Plant material was homogenized (3 × 10 sec, high speed) in a Waring Blendor with 2 volumes of buffer A (0.5 M sucrose, 0.05 M Na_2 EDTA, 0.05 M tris-HCl [pH 8] at 25 C, containing 1% [v/v] 2-mercaptoethanol), and the homogenate was filtered through four layers of cheesecloth and 2 layers of Miracloth. Chloroplasts were lysed by adding Triton X-100 (30% [v/v] in buffer A) to a final concentration of 3%. After stirring for 5 to 10 min, the homogenate was centrifuged for 20 min at 5,000*g*. The pellet was resuspended in buffer A containing 3% Triton X-100, centrifuged as above, and then resuspended in buffer B (0.01 M EDTA, 0.01 M tris-HCl [pH 8] at 25 C plus 1% [v/v] 2-mercaptoethanol) and centrifuged for 20 min at 10,000*g*. All steps up to this point were carried out at 0 to 4 C. The final pellet was dispersed at 60 C in preheated buffer C (0.5 M NaCl, 0.01 M EDTA, 0.1 M tris-HCl [pH 8] at 25 C plus 1% [v/v] SDS). After heating for 20 min, this solution was chilled in ice and adjusted

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to 1 M $NaClO_4$ prior to at least two extractions with an equal volume of chloroform-octanol mixture (24:1, v/v). DNA was precipitated from the final aqueous phase with 2 volumes of ethanol by "spooling" on a glass rod and subjected to further purification (involving RNase and pronase digestions, additional chloroform-octanol extractions and ethanol precipitations) as previously described (17). At this point, the DNA was pure as judged by its UV spectral properties (260/280 1.85; 260/230 2.35), diphenylamine reaction, and thermal denaturation profile. Selective precipitation with cetyltrimethylammonium bromide (17), banding in CsCl-ethidium bromide gradients, or chromatography on Bio-Gel A-50m and/or hydroxyapatite failed to increase the purity of pea DNA preparations measured by these criteria. These procedures also failed to alter reassociation kinetics or aggregate formation and were therefore normally omitted. Calf thymus DNA was obtained commercially (Worthington) and purified by enzyme digestions and subsequent treatments as described for pea DNA. *Bacillus subtilis* DNA (Calbiochem) was purified by hydroxyapatite chromatography.

Shearing and Fragment Size Measurements. DNA (usually 5–10 mg) was sheared for 1 hr in a VirTis 60 homogenizer at 48 to 50,000 rpm in a medium consisting of 0.2 M Na acetate in 67% (v/v) glycerol as described by Britten *et al.* (5). A VirTis No. 16-117 flask with a nominal capacity of 100 ml was used with a single No. 16-108 blade. Sample volumes were 30 or 45 ml. Ice water was used for cooling, and the ice was renewed every 10 min. After shearing, the DNA was precipitated with 2 volumes of ethanol at −20 C overnight and recovered by centrifugation at 20,000*g* for 30 to 60 min.

Mean fragment length was routinely determined from measurements of the Tm reduction associated with decreased fragment length according to the formula given by Britten *et al.* (5), and checked by means of alkaline sucrose gradients and agarose gel (Bio-Gel A-50m) chromatography (11, 12) using radioactive marker DNAs kindly supplied by Dr. N. R. Rice. Although fragment sizes are given in the text as exact numbers of nucleotides, it should be understood that these numbers represent estimates of weight-average lengths. In alkaline sucrose gradients, the width of the distribution at half peak height was about half the peak sedimentation distance, in accord with previous observations (12).

Reassociation and Assay Conditions. Sheared DNA was dissolved in 10 mM ammonium acetate and dialyzed extensively against this buffer. After dialysis, samples were passed sequentially through columns of AG50W×8 cation exchange resin (NH_4^+ form) and Chelex-100 chelating resin (both obtained from Bio-Rad Laboratories) previously equilibrated with 10 mM ammonium acetate. This procedure was designed to remove metal ions and other trace contaminants which might accelerate DNA degradation at high temperatures.

All experiments were conducted using SP buffer (0.6 or 1 M NaCl, 0.01 M PIPES (piperazine-N-N-bis(2-ethanesulfonic acid), pH 6.7) with or without formamide (Fisher certified grade). Before use, each batch of buffer was treated with 0.1 g/ml Chelex-100 for 30 min, the pH was readjusted as necessary, and the resin was removed by filtration. Appropriate aliquots of DNA were concentrated by lyophilization and dissolved in reassociation buffer to give the indicated final concentrations. Aliquots were sealed in capillary tubes, denatured by heating for 5 min at 110 C (in the absence of formamide) or at 80 C (when formamide was included), and then incubated as indicated. When reassociation kinetics were measured, the data are plotted as a function of C_0t (DNA concentration in moles of nucleotides/l multiplied by time of incubation in seconds) (5).

In most experiments, reactions were terminated by chilling the capillary tubes in ice water, followed by dilution into 0.12 M Na phosphate buffer (pH 6.8) and heating to 60 C for 5 to 10 min. After the heating step, diluted samples were chilled in ice and centrifuged for 30 min at 20,000 rpm in the SS-34 rotor of a Sorvall RC-2B centrifuge at 4 C. After removing the supernatant, the pellet was washed by resuspension and centrifugation in fresh buffer. In order to obtain a homogeneous solution for absorbance measurements, the pellet was solubilized by brief sonication as described below. Sonication was found to have no effect on the absorbance of supernatant DNA.

Experiments were carried out in an attempt to determine whether or not the chilling steps either before or after dilution were essential for detection of aggregates. Chilling solutions of denatured DNA to 0 C at the usual concentrations of 8 to 12 mg/ml did not result in aggregate formation. Direct dilution of reassociated DNA with reassociation buffer at the reassociation temperature (criterion dilution) gave results equivalent to those obtained with the standard assay. Good yields of aggregates were also obtained when the entire incubation and assay procedure, including centrifugation, was carried out at 20 C using 0.6 M SP buffer containing 70% formamide (in which the Tm of native DNA was 45 C). When concentrated samples of reassociated DNA were chilled without a subsequent heating step, the yield of pelletable aggregates was about 1.5 times higher than with the standard assay. The basis for this difference is not understood, although it may be inferred that whatever intermolecular interactions are involved must be of low thermal stability.

Hydroxyapatite fractionation was carried out according to standard procedures (5). Typically, aliquots containing 40 to 50 μg of DNA in 0.12 M Na phosphate buffer at 60 C were applied to 0.25 g of hydroxyapatite (Bio-Gel HTP, Bio-Rad Laboratories) equilibrated with the same buffer at 60 C. Single strands were removed by washing at 60 C with the same buffer. DNA bound to the column was eluted by raising the temperature to 97 C or by washing with 0.4 M Na phosphate buffer.

Since aggregates do not pass through hydroxyapatite at 60 C (and sometimes have a tendency to block the columns) it was necessary to solubilize aggregate DNA before salt fractionation. A Branson S-125 sonifier equipped with a microtip was used for this purpose. Samples were chilled in ice water and sonicated for 10 sec at a setting of 4 (about half maximum output). To facilitate comparison, this treatment was routinely applied to both supernatant and aggregate fractions. Sonication under these conditions did not affect the absorbance or optical melting profile of reassociated supernatant DNA. However, even brief sonication may release single-stranded DNA from partially reassociated duplexes, or produce short pieces of double-stranded DNA too small to bind to hydroxyapatite. Thus measurements of hydroxyapatite binding in sonicated preparations underestimate the fraction of the original fragments which contained double-stranded regions.

Optical Reassociation. Reassociation of the DNA in separated aggregate and supernatant fractions was carried out in 1 M SP buffer containing 50% formamide. Appropriate aliquots of each sample were heat denatured and reassociated at 37 C in a Gilford recording spectrophotometer equipped with a thermal programming device. Absorbance was monitored at 270 nm, since formamide absorbs significantly at shorter wavelengths. Similarly sheared *B. subtilis* DNA was run simultaneously as a reference DNA with known reassociation kinetics and a base composition close to that of pea DNA.

RESULTS

Table I shows that pea DNA sheared to about 500 nucleotides can form over 30% aggregates on partial reassociation, while calf DNA sheared and incubated in the same fashion forms less than 7% pelletable structures. Although 500 nucleotide fragments were used for most experiments reported, about 30 to 35% aggregates could be formed from pea DNA fragments about 350 nucleotides in length (Fig. 1).

Table I. *Comparison of Aggregate Formation with Pea and Calf DNA*

DNA sheared to 500 nucleotides was heat-denatured and incubated in SP buffer at 70 C for 1 hr at 12 mg/ml. Aggregate and supernatant fractions were separated following dilution into 0.12 M NaPB at 60 C. For comparison, undenatured aliquots of the same preparations were similarly diluted and fractionated.

Sample	% Pelletable
Native pea DNA	3.1
Native calf DNA	1.8
Reassociated pea DNA	34.5
Reassociated calf DNA	6.7

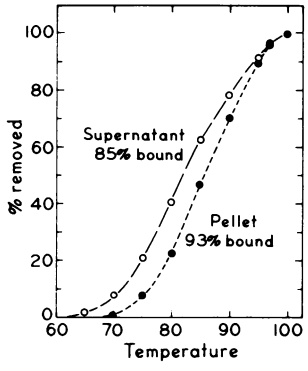

FIG. 1. Hydroxyapatite thermal elution profiles of aggregate and supernatant fractions. DNA sheared to 350 nucleotides was denatured and incubated in SP buffer at 70 C for 1 hr at 9.4 mg/ml. Aggregate and supernatant fractions were separated as usual, preheated to 60 C in 0.12 M Na phosphate buffer and applied to hydroxyapatite equilibrated with the same buffer at the same temperature. After elution at 60 C, the temperature of the column was increased in 5 C increments, eluting with 0.12 M Na phosphate at each temperature. Cumulative per cent elution (of material initially bound at 60 C) is plotted as a function of temperature. Initial retention was 85% for supernatant DNA and 93% for the aggregate fraction. Aggregates accounted for 35% of the total DNA.

As expected from its particulate nature, the aggregate fraction of pea DNA cannot be eluted from hydroxyapatite columns with phosphate buffer at 60 C. Although elution can be accomplished at elevated temperatures (Fig. 1), thermal stability measurements based on hydroxyapatite elution profiles may be misleading. Comparing Figures 1 and 2, it may be seen that the midpoint of the elution profile for the aggregate fraction exceeds that of the supernatant by 4 C, while the optical melting profiles in Figure 2 show very little difference in Tm between the two fractions. Although the mean thermal stability of the base-paired regions in aggregate DNA is approximately the same as that of the supernatant, somewhat higher temperatures are required to dissociate the aggregates sufficiently to permit them to pass through hydroxyapatite.

As shown in Figure 2A, quite large hyperchromic shifts (24% in this case) may be observed in aggregate preparations which have been suspended in buffer without further treatment. In experiments where aggregates were isolated by a procedure involving more extensive centrifugation, apparent hyperchromicities of up to 50% were obtained. These high apparent hyperchromicity values are probably related to the particulate nature of DNA in the aggregate pellets. As thermal dissociation occurs, the particles presumably assume a more open configuration in which more of the DNA is available to absorb light from the measuring beam. Brief sonication prior to melting completely solubilized aggregate DNA (as measured by the standard centrifugation assay or by elution from hydroxyapatite at 60 C), but had little or no effect on melting profiles obtained for the supernatant fraction. Figure 2B shows that the hyperchromicity of solubilized aggregate DNA is still quite high. The observed value of 19 to 20% is approximately 70% of that for native DNA, indicating that about 70% of the bases present are paired (11).

Effect of Reassociation Temperature. Since higher incubation temperatures require more base pairing for stable duplex formation, I examined the effect of varying temperature on aggregate formation and overall reassociation. DNA was incubated at various temperatures to a C_0t of 100 and separated into aggregate and supernatant fractions. Each fraction was then sonicated and fractionated on hydroxyapatite. Figure 3 shows that aggregate formation was much more sensitive to incubation temperature than the percentage duplex in either fraction (as measured by hydroxyapatite binding). Per cent binding to hydroxyapatite declined slightly with increasing temperature, in approximate agreement with observations on other plant DNAs (2), while

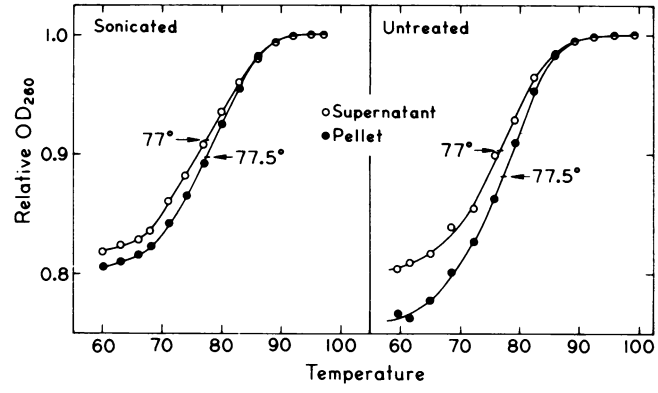

FIG. 2. Optical melting profiles of aggregate and supernatant DNA. Aliquots of DNA sheared to 500 nucleotides were incubated in SP buffer at 70 C for 1 hr at 12 mg/ml, and the fractions were separated as usual. Suitable aliquots of each fraction (in 0.12 M Na phosphate buffer) were melted in the spectrophotometer. In A, both supernatant and pellet fractions were sonicated briefly prior to melting in order to solubilize the aggregates, while in B the pellet was resuspended by vortexing. Native pea DNA fragments in this solvent had a Tm of 86 C.

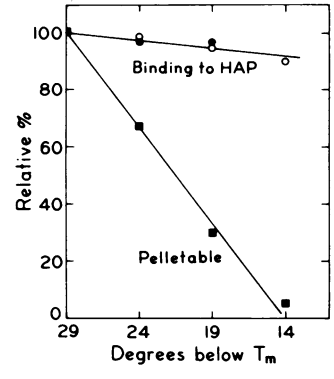

FIG. 3. Differential effect of reassociation temperature on formation of aggregates and total duplex. DNA sheared to 500 nucleotides was incubated for 1 hr at 8.4 mg/ml in SP buffer containing 50% formamide at temperatures between 35 and 50 C. Aliquots were diluted into the same buffer at the reaction temperature and fractions separated by centrifugation at 4 C. Each fraction was then dialyzed to 0.12 M Na phosphate buffer, sonicated, and applied to hydroxyapatite at 60 C. Reassociation temperatures are given as degrees below the Tm of native DNA, which was 64 C in the formamide buffer. Data are expressed relative to values obtained by reassociation at Tm-29 C (40% aggregate, with 68% binding to hydroxyapatite in the supernatant and 91% in the aggregate fraction). Binding data: supernatant (○); aggregate fraction (●).

the aggregate fraction was reduced practically to zero at 14 C below the Tm of native DNA.

In order to minimize thermal strand scission during these experiments, reassociation was carried out at relatively low temperatures in buffer containing 50% formamide. The data in Figure 3 were obtained at temperatures between 35 and 50 C, with dramatic reduction in aggregate formation being observed even at 40 C. Results similar to those of Figure 3 were also obtained when the incubation time was shortened from 1 hr to 20 min. Alkaline sucrose gradient profiles for DNA fragments incubated at 50 C for 2 hr were not significantly different from those for control samples. The strong temperature dependence of aggregate formation therefore does not appear to result from accelerated strand scission at higher temperatures. Instead, higher temperatures seem to preferentially inhibit secondary reassociation reactions leading to formation of large aggregates.

Kinetics of Aggregate Formation. At a given DNA concentration, aggregate formation follows kinetics very similar to those of an ideal second order reaction (Fig. 4, top). The apparent rate constant decreases at lower concentrations (a 5-fold decrease in concentration resulting in an approximately 2-fold rate reduction in the experiment of Fig. 4A). The reaction is kinetically complex rather than truly second order. In experiments involving even lower concentrations of DNA, very few aggregates formed during incubations to C_0t values approximating those of Figure 4, top. For example, at about 0.7 mg/ml only about 10% aggregate was observed after incubation to a C_0t of 200. This value is much less than would be predicted from Figure 4A, and it appears that the rate of aggregate formation is a nonlinear function of DNA concentration, increasing rapidly above about 1 mg/ml. Incubation of pea DNA fragments in formamide buffer for periods up to 24 hr (corresponding to the longest times used in these experiments) failed to alter their sedimentation profile in alkaline sucrose gradients, even when a slightly higher temperature (40 C as against 35 C) was used. It is unlikely that the observed concentration dependence of aggregate formation results from strand scission during the longer incubations at low DNA concentrations.

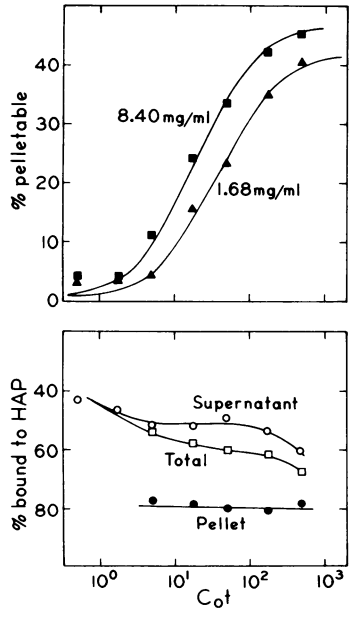

FIG. 4. Kinetics of aggregate and total duplex formation. DNA sheared to 500 nucleotides was reassociated in SP buffer containing 50% formamide for various times at 35 C (29 C below the Tm of native DNA). At each time, aliquots were removed, and the aggregate and supernatant fractions separated with the standard procedures. Each fraction was then sonicated and applied to hydroxyapatite as described in Fig. 3. In the upper portion of the figure the solid lines depict theoretical second order kinetics for final values of 47.5% aggregate at 8.4 mg/ml and 42.5% at 1.68 mg/ml. In the lower portion, total duplex (as hydroxyapatite binding) is plotted against C_0t for total DNA and the separated aggregate and supernatant fractions. Results for the two DNA concentrations were very similar, and data is presented for the high concentration series only.

Figure 4B shows the extent of duplex formation, assayed by hydroxyapatite chromatography at 60 C in the same samples used in Figure 4A. Comparison of Figure 4, A and B, shows that most aggregates appear only after a large majority of the fragments containing repetitive sequences have formed at least one double-stranded region capable of binding to hydroxyapatite. Thus, even though aggregates contain mainly repetitive DNA (see below and Fig. 5), their formation occurs more slowly than the initial bimolecular reassociation of these same sequences. This observation suggests a reaction sequence involving several intermediates before final formation of structures large enough to be detected in the standard centrifugation assay. The concentration dependence mentioned above would also be consistent with a complex reaction of this type. During the intermediate stages, it seems likely that single-stranded regions remaining after initial reassociation interact with similar regions on other fragments, leading eventually to formation of large multimeric structures.

Reassociation of Isolated Aggregate and Supernatant DNA. In order to determine the type of DNA sequences involved in aggregates, the reassociation kinetics of isolated aggregate and supernatant DNA were followed optically, using low DNA concentrations (approximately 75 μg/ml) to minimize aggregation during the measurements. The two fractions were separated after reassociation of 350 nucleotide fragments at 37 C in 1 M SP buffer containing 50% formamide by dilution and centrifugation in the same buffer; 33% of the DNA was recovered in the pellet. Aliquots were then heat-denatured and reassociated in the spectrophotometer, together with a sample of similarly sheared *Bacillus subtilis* DNA in the same buffer. The hypochromic shift corresponding to 100% reassociation was estimated by fitting a theoretical second order kinetic curve to the *B. subtilis* data, a procedure designed to compensate for the presence of a small fraction of single-stranded regions or "hanging tails" (6, 16) in reassociated duplexes.

If the aggregate fraction were merely a random sample of total DNA, the reassociation kinetics of the two fractions should be identical. Figure 5 shows that aggregate DNA in fact reassociates more rapidly and extensively than supernatant DNA, indicating a substantial enrichment of repetitive sequences in the aggregate fraction. The aggregate data can be described as the sum of two second order reactions, as indicated by the lines in the figure. Together, these two components would account for 85% of the DNA in this fraction. The major (65%) component of aggregate DNA reassociates more rapidly than the most rapid component (50%) in the supernatant. The curves shown are for second order reactions with $C_0t_{0.5}$ = 0.17 and 0.86, respectively. If these components were present as pure fractions, undiluted with other sequences, the corresponding values would become 0.11 (0.17 × 0.65) and 0.43 (0.86 × 0.50). Thus the aggregate component would reassociate almost four times as fast as the most rapidly reassociating sequences in the supernatant.

Aggregates in DNA from Other Plants. Aggregates of short DNA fragments have been detected by centrifugation in partially reassociated DNA from three species of the fern genus *Osmunda* (observations by D. Stein in this laboratory) and from oats (*Avena sativa*). Indirect evidence, based on incomplete salt elution of reassociated DNA from hydroxyapatite, suggests that similar structures may also form in DNA from beans (*Phaseolus vulgaris*), tobacco (*Nicotiana tabacum*), and duckweed (*Lemna gibba*). Aggregate formation from short DNA fragments may therefore be a common phenomenon in a wide variety of vascular plants.

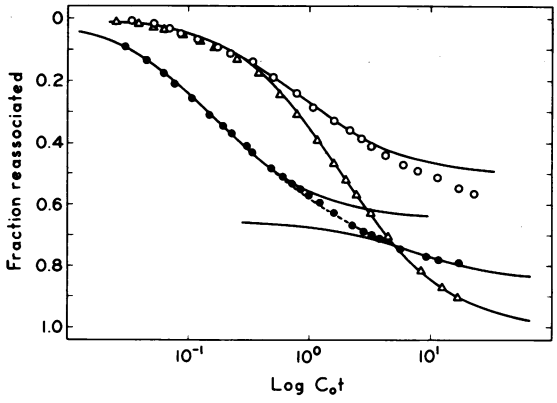

FIG. 5. Optical reassociation of separated aggregate (●), supernatant (○) and *Bacillus subtilis* (△) DNA. Aggregate and supernatant fractions were prepared from pea DNA sheared to 350 nucleotides and reassociated at (10.2 mg/ml, 2 hr) 37 C in 1 M SP buffer containing 50% formamide. The two fractions were isolated and appropriately diluted in the same buffer, heat denatured, and reassociated in a Gilford recording spectrophotometer at 37 C. Similarly sheared *B. subtilis* DNA was reassociated simultaneously in the same buffer. The data have been corrected for the approximately 3% "collapse" hypochromicity measured with *B. subtilis* DNA and normalized so that the *B. subtilis* points fall on a theoretical curve for 100% reaction. Theoretical curves fitted to the pea DNA data by a graphical method (——); sum of the two theoretical components for aggregate DNA (- - -).

DISCUSSION

Technical Implications. The presence of aggregates can create problems in experiments involving hydroxyapatite column fractionation. If phosphate elution procedures are used, the aggregate fraction will remain on the column, undetected except as an apparent loss of DNA. If elution at high temperature is used to recover bound DNA, the entire aggregate fraction will be scored as double-stranded along with nonaggregated duplexes. Since aggregates of pea DNA isolated by centrifugation appear to contain few completely single-stranded fragments, this procedure can be recommended for routine assays. However, the thermal elution profile of aggregate DNA is anomalous (compare Figs. 1 and 2) and may lead to erroneous estimates of duplex thermal stability.

Interference in assay procedures may be eliminated by removing or solubilizing the aggregates, subject to the qualification that aggregates do not represent a random sample of the total DNA and must somehow be included in the final analysis. Also, in many cases, conditions may be chosen so as to minimize aggregate formation, *e.g.* by using low DNA concentrations, high temperatures, or (presumably) sufficiently small fragments. Such conditions are not always desirable, however, as in experiments in which fragment length is varied over a wide range or when attempting to measure low level sequence homology. In cases like these, it may be possible to separate aggregate and supernatant fractions prior to the actual experiment. Aggregate DNA can then be reassociated at low concentrations where secondary aggregation does not occur. Similarly, one or more classes of repetitive DNA can be removed by preparative hydroxyapatite fractionation and treated separately.

On the positive side, deliberate aggregate formation may be useful in certain situations where attention is focused on repetitive sequences. For example, Bendich (1, and personal communication) has used aggregates of somewhat larger fragments in experiments concerned with evolution of repetitive sequences in plant DNA. Aggregate formation from short fragments of pea DNA permits rapid isolation of a class of fragments which appear to be derived from regions of DNA containing a high concentration of repetitive sequences (see below). These special properties of aggregate DNA may be useful in further studies of repetitive sequence organization in higher plants.

Implications for Sequence Organization. Aggregate formation appears to be similar to formation of hyperpolymers late in the reassociation of randomly sheared simple (prokaryotic) DNA, where single-stranded regions remaining after initial reassociation may pair with similar regions on other fragments to produce large multimeric structures (5). Hyperpolymers would be expected to be extensively base-paired and to form more slowly than simple bimolecular duplexes. Similarly, aggregates contain a high percentage of base pairs, judging by the hyperchromicity of solubilized aggregate DNA (Fig. 2), and their formation proceeds more slowly than initial reassociation of the sequences involved (Figs. 4 and 5). The dependence of the apparent rate constant for aggregate formation on the concentration of DNA in the reaction mixture is consistent with a complex reaction involving several intermediates, as would be expected in hyperpolymer formation. The differential sensitivity of aggregate formation to temperature (Fig. 3) can be explained by assuming that, with the short fragments used, secondary duplexes required for hyperpolymer formation are short enough that their thermal stability is reduced, or that secondary duplexes contain more mismatched base pairs.

The most extensive studies of repetitive sequence organization so far reported have been carried out with *Xenopus* (9, 11, 12) and sea urchin (11, 15) DNA. In these organisms, the majority of repetitive sequences are short (300-350 nucleotides) and interspersed among longer regions of nonrepetitive DNA, while a minority class is contained in long (>1500 nucleotides) repetitive regions. This general pattern of sequence organization has been reported (9, and references cited therein) to hold for a wide variety of animals, although the relative amounts of repetitive DNA in the two classes may vary. Using sea urchin DNA, Graham and Britten (14) have reported that fragments derived from long repetitive regions can be induced to form hyperpolymers, while fragments derived from regions containing the short, interspersed repetitive sequences do not. This is consistent with expectation, since many fragments derived from the long regions would contain exclusively repetitive sequence, and, due to random shearing, initial reassociation of these fragments would be expected to leave single-stranded regions capable of further reassociation. In contrast, most fragments derived from regions of the DNA containing short, interspersed repetitive elements would probably not contain enough repetitive DNA to permit extensive secondary duplex formation.

By analogy, it may be argued that the extensive formation of aggregates from pea DNA fragments indicates the presence of long regions of repetitive DNA in the pea genome, and it would seem likely that similar regions exist in the genomes of other plant species whose DNA has been shown to form aggregates. It would not be surprising to find long repetitive regions in plant DNA, due to the high concentration of repetitive sequences in many plant genomes (13). Although it may be inferred that these regions must be significantly longer than the 350 to 500 nucleotide DNA fragments used in the present experiments, it is not possible at present to determine their length, or to decide whether they are composed of a single long repeating unit rather than several short ones. In addition, the presence of long repetitive regions does not rule out the possibility that short, interspersed repetitive sequences also exist.

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