STUDY OF NUCLEIC ACIDS : DNA COMPARISON OF PHASEOLUS PLANT SPECIES

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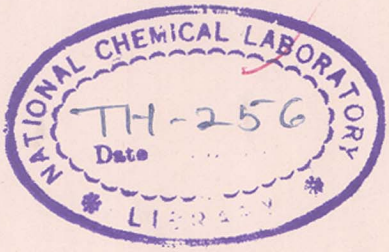

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ABSTRACT

DNAs of four diploid Phaseolus plant species namely Phaseolus aconitifolius, Phaseolus aureus, Phaseolus mungo and Phaseolus vulgaris were compared among themselves with an objective of getting a detailed information about the molecular consequences of speciation.

1. Buoyant densities and melting temperatures of the DNAs of P. aconitifolius, P. aureus and P. mungo were very similar. All the DNAs banded at a density of 1.694 g/cm^3 in neutral CsCl density gradients. The DNAs of P. aconitifolius and P. mungo clearly revealed the presence of a dense satellite with a density of 1.703 g/cm^3. The DNA melting temperatures of the above three Phaseolus species were in the range of 83.5 - 83.7°C. The melting profile of the P. vulgaris DNA was different and revealed the presence of a minor (15%) high melting component with a Tm of 93.5°C and a major (85%) low melting component with a Tm of 83.7°C.

2. Reassociation kinetics of the sonicated DNAs (an average fragment length of 550 np) of all the four Phaseolus species were studied in the Cot range of 10^{-1} to 10^4. The proportion of the fast reassociating DNA was in the range of 34.5 - 47% and the sequences present in this fraction were considered to be repetitive. The reassociation of the repeated DNA sequences was

nearly complete by Cot 10 in P. mungo, Cot 50 in P. vulgaris and Cot 100 in P. aconitifolius and P. aureus.

3. Thermal stability of the repetitive DNA fraction in three four Phaseolus species was in the range of 74.5°C to 79°C. 78.6°C The repeated nucleotide sequences therefore, revealed a base mismatching in the range of 3.7 - 7.0%.

4. Cot 1 DNA fraction in each Phaseolus species was also isolated and characterized by studying its denaturation-reassociation properties. This DNA fraction was assumed to include mainly highly repeated DNA sequences. The Tm values of the Cot 1 DNA fractions were in the range of 77.5°C to 78°C in all the Phaseolus species except in P. vulgaris. In the latter, the melting profile of the Cot 1 DNA was biphasic where the major low melting component had a Tm of 78.7°C and the minor high melting component had a Tm of 92.5°C.

5. Optical reassociation studies of the Cot 1 DNA fraction in each Phaseolus species (dew gram and green gram) revealed the occurrence of one minor fraction reassociating by Cot 10^{-2} and one major fraction reannealing in the Cot range of 10^{-2} to 1. The former component represented ~~25~~ 30 to 36% of the Cot 1 DNA and appeared to consist of 840 to 1100 nucleotide pairs while the latter was more complex and consisted of 1.2×10^5 to ~~2.1~~ 1.4 $\times 10^5$ nucleotide pairs.

6. The haploid genome size of the DNAs of the four Phaseolus species was determined from the Cot 1/2 of the respective slow reassociating DNA or unique DNA fraction

and was estimated to be in the range of 0.36 to 1.6 picograms.

7. Modes of DNA sequence organization in the nuclear genomes of P. vulgaris and P. aureus were studied. The reassociation kinetics of the DNAs of increasing fragment lengths revealed that approximately 60% of the genomes of P. vulgaris and P. aureus consisted of interspersed repeated and single copy sequences. Hyperchromicity and S_1 nuclease resistance of the reassociated, long DNA fragments further confirmed the occurrence of interspersion and showed the actual proportion of repetitive DNA as 35 - 40% in each species. These experiments also yielded the average size of interspersed repeated sequences as 1925-1980 nucleotides in P. vulgaris and 2012-2357 nucleotides in P. aureus. The fragment length of the interspersed single copy DNA sequences was estimated from a curve relating the fraction of DNA fragments binding to hydroxyapatite and the DNA fragment length and was in the range of 1150 to 1500 nucleotides in both the plant species. Approximately 35 - 40% of the single copy sequences were interspersed in this manner in P. vulgaris and P. aureus.

CHAPTER I

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Concept of repetitive DNA and satellite DNA in eukaryotes:

Nucleic acids are the molecules that store and transmit genetic information. Genes in an organism determine its pattern of chemistry and its probable life history. In most organisms, DNA plays a fundamental role in storing genetic information and RNA helps cells in transmitting and expressing the genetic information stored in DNA.

Biochemical and other kinds of studies on genetic variations in bacteria have yielded considerable understanding of the linear organization of genes and the controlling sequences. Eukaryotic genomes are enormously complex and to gain such information is rather difficult. However, new biochemical approaches to the study of DNA have enabled us to look into the properties of DNA and to study its functions. In general, techniques such as thermal denaturation, isopycnic centrifugation and DNA reassociation kinetics studies have contributed greatly to our understanding of eukaryotic genomes. In recent years, *in situ* hybridization, reassociation studies of DNAs of long fragment lengths and electrophoretic analysis of DNA fragments produced by the action of several DNA sequence specific restriction endonucleases have added significantly to our knowledge of DNA localization and DNA sequence organization in eukaryotes.

Reassociation kinetics studies of prokaryotic DNAs revealed that the Cot 1/2 was proportional to the genome size (Britten and Kohne, 1968; Cairns, 1963). Since eukaryotic genomes are several times complex than those of prokaryotes their reassociation was expected to take a very long time. However, it was not found to be the case. A fraction of an eukaryotic genome was observed to reassociate very fast. In order to explain this paradoxical behaviour of eukaryotic DNAs, the hypothesis was put forward that some nucleotide sequences were frequently repeated in eukaryotic DNAs (Britten and Kohne, 1968).

The pioneering work by Britten and Kohne (1968) on the reassociation kinetics studies of several eukaryotic genomes demonstrated the presence of repeated DNA sequences. The latter have been classified into highly and intermediately repetitive DNA fractions depending upon their complexity and number of copies present (frequency of repetition) per genome. In general, highly repetitive DNA is assumed to include all DNA sequences reannealing by Cot 1 and intermediately repetitive DNA is assumed to consist of nucleotide sequences forming duplexes in the Cot range of 1 to 100. All DNA sequences reassociating after Cot 100 are considered to be mostly unique or single copy sequences.

When the frequency of repetition of a certain nucleotide sequence is very high (Salser, 1976) and its base composition is different from the bulk DNA then the repetitive DNA bands differently from that of main band DNA

in CsCl density gradients and this band (other than main band) is termed as satellite DNA. The latter was first discovered by Kit (1961) and Sueoka (1961). In some cases, satellite DNAs have been demonstrated using Cs_2SO_4 gradients containing heavy metals like Ag^+ and Hg^{2+} (Nandi et al., 1965; Jensen and Davidson, 1966; Guille and Grisvard, 1971; Huguet and Jouanin, 1972; Filipski et al., 1973) and CsCl gradients containing antibiotics (Kersten et al., 1966; Peacock et al., 1973; Votavová and Šponar, 1975). Such satellite DNAs are called 'cryptic' satellites.

Repetitive and satellite DNA in plants:

Most of the earlier work on repetitive DNA and satellite DNA was carried out in animal genomes and only a few plant species were investigated in this respect (Britten and Kohne, 1968). The presence of families of repeated nucleotide sequences was demonstrated in a large number of evolutionarily diverged species such as bovine (Britten and Kohne, 1968), human (Saunders et al., 1972), rodents (Santiago and Rake, 1973), Xenopus (Davidson et al., 1973), chicken (Jimenez et al., 1974), Silkworm (Gage, 1974), sea urchin (Graham et al., 1974), Drosophila (Manning et al., 1975), mouse (Cech and Hearst, 1975; Ginelli et al., 1977), fish (Vladychenskaya et al., 1975), amphibians (Baldari and Amaldi, 1976) and oyster (Kamalay et al., 1976). In most cases repetitive DNA varied from 30-40% of the total genome. Likewise extensive studies

were also carried out on satellite DNA in animal genomes (reviewed by Walker, 1971; Bostock, 1971). One of the first satellite DNA studied in detail was that of mouse (Waring and Britten, 1966; Bond et al., 1967; Flamm[illegible] et al., 1967; Corneo et al., 1968). This was followed by analysis of satellites in several other animal species namely, calf (Polli et al., 1965; Yasmineh and Yunis, 1971; Filipski et al., 1973; Votovová and Šponar, 1975), Drosophila (Laird and McCarthy, 1969; Gall et al., 1971; Blumenfold et al., 1973; Gall and Atherton, 1974) human (Corneo et al., 1970; Ginelli and Corneo, 1975), Crustaeceans (Skinner and Beattie, 1974), sea urchin (Stafford and Guild, 1969), guineapig (Corneo et al., 1968; Corneo et al., 1970), kangroo rat (Fry et al., 1973), sheep and goat (Curtain et al., 1973; Forstova et al., 1979) and chicken (Ayres, 1978).

Plant genomes contain a higher proportion (40-85%) of repetitive DNA (Flavell et al., 1974; Walbot and Goldberg, 1978) as compared to that in animal genomes. Monocotyledon species belonging to the family Graminae have been observed to contain unusually high percentage of repeated DNA sequences (Bendich and McCarthy, 1970a; Mitra and Bhatia, 1973; Smith and Flavell, 1975; Ranjekar et al., 1976). In wheat, rye, barley and oat, for example, nearly 80% of the genome consists of repeated DNA sequences (Bendich and McCarthy, 1970a). In recent years, other monocotyledon species such as pearl millet (Wimpee and

Rawson, 1979) and seven Allium species (Ranjekar et al., 1978b) have been chracterized for the presence of repetitive DNA.

Reassociation kinetics studies have also been carried out in several dicotyledons and gymosperms, namely, pea (Sivolap and Bonner, 1971; Pearson et al., 1978; Murray et al., 1978), Vicia (Chooi, 1971; Straus, 1972; Gnucheva et al., 1977), Conifers (Miksche and Hotta, 1973), Anemones (Cullis and Schweizer, 1974), Artichoke (Nze-Ekekang et al., 1974), Lathyrus (Narayan and Rees, 1976), cotton (Walbot and Dure, 1976), tobacco (Zimmerman and Goldberg, 1977) four vascular plants (Bendich and Anderson, 1977), Cichorieae (Bachmann and Price, 1977), soybean (Goldberg, 1978; Gurley et al., 1979) and Umbelliferae (Kiper and Herzfeld, 1978).

Nuclear satellite DNA has been observed in a number of plant species studied so far. The first analysis of plant DNA by the buoyant density method is by Ingle et al. (1973). This work included plant species belonging to a wide spectrum of families. It was shown that satellite DNA is scattered throughout the angiosperm families in an irregular fashion. Among the Magnoliidae, it is present in Drimys but absent from Magnolia and Ranunculus. It was found in Caryophyllaceae (Beta, Spinacia, Dianthus). In the Cucurbitaceae, prominent satellites were found in Cucumis, Cucurbita, Citrullus, Bryonia and Luffa but not in Momordica. In the Cruciferae, satellite DNA was

found in Lobularia and Raphanus. In the Rutaceae, conspicous satellite peaks appeared in DNA of Citrus and Fortunella but not in Choisya or Skimma. Other families containing some genera having satellite DNA and some without it are Leguminosae, Solanaceae and Compositae. In the genus Linum it was found in L. usitatissimum but not in L. grandiflorum. None of the DNA's extracted from the ten genera of monocotyledons investigated had satellite peaks.

The next intensive work on plant DNA by isopycnic centrifugation in neutral CsCl gradients was by Beridze (1972, 1975) and Beridze et al., (1973). They identified satellites in six Phaseolus and seven Brassica species and showed a similarity of buoyant density of satellite DNAs in the species belonging to the same genus. Furthermore, the proportion of satellite DNA was shown upto 37% in Brassica and Phaseolus plant species. Some of the other plant species which showed the presence of satellite DNA in neutral CsCl gradients are carrot and Amni (Ohyama et al., 1972) cymbidium (Caspesius et al., 1975) raddish and cucumber (Kadouri et al., 1975; Ranjekar et al., 1978c)and flax and cucumber (Timmis and Ingle, 1977).

Cryptic satellite DNA components have been observed in Ag^{+}/Cs_2SO_4 and Hg^{2+}/Cs_2SO_4 gradients in a number of monocotyledons anddicotyledons (Deumling et al., 1976). Satellite DNA in Ag^{+}/Cs_2SO_4 density gradient was first observed in wheat (Huguet and Jouanin, 1972). Later

satellite DNA was also detected in Hg^{2+}/Cs_2SO_4 gradients in wheat (Ranjekar et al., 1976). In case of barley, two satellites were observed in Ag^{+}/Cs_2SO_4 density gradients and one satellite was noticed in Hg^{2+}/Cs_2SO_4 gradients (Ranjekar et al., 1976), Scilla sibirica DNA showed the presence of a satellite DNA in Ag^{+}/Cs_2SO_4 gradients with a buoyant density of 1.705 g/cm^3 in CsCl gradients (Timmis et al., 1975).

Analysis of satellite DNAs in species such as musk melon (Bendich and Anderson, 1974; Bendich and Taylor, 1977), melon (Sinclair et al., 1975), Scilla sibirica (Timmis et al., 1975), tomato (Chilton, 1975), French bean (Beridze and Bragvadze, 1976), Cymbidium (Caspesius, 1976), flax and cucumber (Timmis and Ingle, 197),barley and wheat (Ranjekar et al., 1978a) and cucumber and raddish (Ranjekar et al., 1978c) by thermal denaturation and optical reassociation kinetics measurements have revealed the presence of atleast two DNA components varying in Tm values and kinetic complexity.

Localization of satellite DNAs:

With the advent of the technique of in situ hybridization by Pardue and Gall (1969, 1970) and Pardue et al. (1970), it has been possible to study the exact localization of satellite and repetitive DNA in eukaryotic chromosomes. The first satellite DNA to be localized was that of mouse and it was found to be

exclusively in the centromeric heterochromatin of all the chromosomes except Y chromosome (Pardue and Gall, 1970; Jones, 1970). Subsequent studies in other animal species namely guinea pig (Yunis and Yasmineh, 1970), Drosophila (Jones and Robertson, 1970), fly (Eckhardt, 1970), calf (Yasmineh and Yunis, 1971; Kurnit et al., 1973), salamander (Macgregor and Kezer, 1971), Rhynchosciara hollaendera (Papaconstantinou et al., 1972; Eckhardt and Gall, 1971) and human (Jones et al., 1973, 1974) have shown the localization of satellite DNA either in centromeric heterochromatin or to wholly heterochromatic arms.

The relatively small size of chromosomes in plants containing satellite DNA and the inherent impermeability of plant cell walls to various chemicals and radioactive materials have frustrated the earlier attempts to localize satellite DNA in plant chromosomes (Timmis et al., 1975).

One of the first plant species studied for in situ localization was that of Scilla sibirica where it is shown that the satellite DNA is distributed on the heterochromatic regions of the chromosomes (Timmis et al., 1975). In situ hybridization of satellite DNA from Vicia faba shows that satellite DNA is more generally spread over the nuclei and the chromosomes (Timmis et al., 1975). Similar studies in Cymbidium revealed the location of satellite DNA preferably in the region of the heterochromatic chromocentres (Nagl and Caspesius, 1977). In Tropaeolum majus, satellite DNA is localized at the nucleolar

organizer regions (Deumling and Nagl, 1978). In rye, the highly repetitive DNA (Cot 0.02) was isolated and shown to be localized mainly within C-bands near the distal ends of most chromosome arms (Appels et al., 1978). From the above data it can be concluded that satellite DNA in both plants and animals is generally localized at the heterochromatic blocks, in the regions of centromere, nucleolar organizer and telomeres of the chromosomes (Yunis and Yasmineh, 1971). The elucidation of the relation between satellite DNA and heterochromatin permits some conjecture concerning the origin and function of this significant portion of the genome of higher organisms (Yunis and Yasmineh, 1971; Sharma, 1978). Satellite DNAs possibly have evolved in eukaryotes in response to increase in genome size and the complexity of chromosomal and nucleolar organisation of cell. The specific location of satellite DNAs indicates their possible functions such as genetic regulators, spacers and mutation accumulating loci (Sharma, 1978).

DNA - DNA hybridization:

One of the main tools for phylogenetic studies at the molecular level is DNA/DNA hybridization. Degree of hybridization and thermal stability of the hybrid DNA provide a measure of the relatedness of the two species in question. This approach has already been applied to several species of related and unrelated plants

(Bendich and Bolton, 1967) four species of Graminae (Bendich and McCarthy, 1970a; Smith and Flavell, 1974), four biotypes of wheat (Bendich and McCarthy, 1970b), six species of Vicia (Chooi. 1971), three species of Osmunda (Stein and Thompson, 1975) and four species of Atriplex (Strong and Thompson, 1975). In Graminae, the results of such DNA hybridization have supported the conventional classification whereas those from Osmunda and Atriplex have given a different picture from that obtained using conventional taxonomic approaches. DNA/DNA hybridization of repeated sequences in barley, oat, rye, and wheat have confirmed the phylogenetic relationship that has been derived from morphological analyses and have shown that the amount of repeated fractions held in common diminishes with taxonomic separation of the species (Flavell et al., 1977). Additionally, this study revealed that each species has its own type of repeated sequences which appears to have arisen after the species diverged from a common ancestor. The hypothesis that repetitive DNA arose during a short period of evolutionary time (Walker, 1971) and that single copy DNA (unique sequences) held a better palaeontological record (Kohne, 1970) than repetitive DNA has led several investigators to prefer the use of single copy DNA for phylogenetic analysis (Angerer et al., 1976; Rice and Paul, 1971; Belford and Thompson, 1976). Thus contrary to the evidence from DNA/DNA hybridization using repetitive DNA, an equivalent study involving single copy

DNA among four species of Atriplex gave support to the traditional concept of the phylogenetic relationships between them (Belford and Thompson, 1976).

Organization of eukaryotic genomes:

All eukaryotes examined so far contain repetitive and nonrepetitive DNA sequences. The nonrepetitive or unique sequences have been shown to include structural genes such as globin (Bishop and Freeman, 1973), ovalbumin (Harris et al., 1973) and fibroin (Suzuki et al., 1972). Repetitive DNA has also been well characterized in eukaryotes but its function is still not yet clear. For a better understanding of the function of repeated sequences and the molecular basis of gene regulation in higher organisms, work on the organization of eukaryotic genomes was undertaken. Discussions on gene regulation and chromosome structure have resulted in specific predictions regarding the arrangement of repetitive and nonrepetitive sequences (Callan, 1967; Thomas et al., 1970; Crick, 1971; Britten and Davidson, 1969, 1971).

Studies on the organization of many eukaryotic genomes have revealed that repetitive and nonrepetitive DNA sequences are interspersed. Extensive data on DNA sequence arrangement are available on animal genomes and only a few plant species have been studied in this respect. From these studies it is clear that two major patterns of interspersion namely, short period and long

period exist in eukaryotic genomes. The short period interspersion pattern has been found in many animal genomes as diverse as Xenopus (Davidson et al., 1973), seaurchin (Vorobev and Kosjuk, 1974; Graham et al., 1974; Lee et al., 1977), mollusc (Angerer et al., 1975), rat (Pays and Ronsse, 1975; Wu et al., 1977), marine invertebrates (Goldberg et al., 1975), housefly (Efstratiadis et al., 1976; Crain et al., 1976b), human (Ginelli and Corneo, 1976) and amphibians (Bozzoni and Beccari, 1978). This pattern also exists in a protist, the cellular slime mold (Firtel and Kindle, 1975). Short period interspersion pattern is distinguished by an alternation of single copy sequences approximately 1000 nucleotides in length and short repetitive sequences having a size in the range of 200 - 400 bases. In long period interspersion pattern the lengths of repetitive and nonrepetitive DNAs are more than 1000 and 5000 nucleotide pairs, respectively. Such patterns have been shown to exist in Drosophila melanogaster (Manning et al., 1975; Crain et al., 1976a), honeybee (Crain et al., 1976b), Chironomus tentanus (Wells et al., 1976) and mold Achlya (Hudspeth et al., 1977). Animal genomes showing short period interspersion pattern also exhibit a small proportion of long period interspersion pattern. Examples are Xenopus (Davidson et al., 1973), seaurchin (Graham et al., 1974) and mollusc (Angerer et al., 1975).

While precise information exists for the organization and expression of many diverse animal genomes (Lewin, 1975; Davidson and Britten, 1973), only little is known about plant genomes in this respect. Higher plants may offer a unique approach to the study of eukaryotic gene expression due to the fact that single cells can be programmed to differentiate into mature plants (Street, 1973). Thus the information regarding the organization in plant genomes is very important.

Plant species which have been studied for their DNA sequence organization are cotton (Walbot and Dure, 1976), wheat (Flavell and Smith, 1976), rye (Smith and Flavell, 1977), tobacco (Zimmerman and Goldberg, 1977), soybean (Goldberg, 1978; Gurley et al., 1979), pea (Murray et al., 1978), Umbelliferae (Kiper and Herzfeld, 1978) and pearl millet (Wimpee and Rawson, 1979). These limited studies indicate that a portion of repetitive sequences of both monocotyledons and dicotyledons is organized with an alternating arrangement of repetitive and single copy DNA. The interspersed single copy sequences vary in size from 200 - 800 nucleotides with interspersed single copy lengths ranging from 800 - 1800 nucleotides. Since plants often contain relatively large amounts of repetitive DNA, many repetitive regions are not contiguous with single copy DNA sequences. The organization of these repetitive sequences is either a tandem arrangement (Ingle et al., 1973; Bendich and Anderson, 1977;

Timmis et al., 1975) or a complex interspersion of unrelated families of repetitive sequences as is the case in the genomes of wheat (Flavell and Smith, 1976) and rye (Smith and Flavell, 1977).

Fold back sequences:

DNA reassociation studies have revealed a fraction of DNA which reanneals instantaneously after denaturation. The nucleotide sequences present in this fraction are called as palindromic, fold back or zero time binding (Wilson and Thomas, 1974). The occurrence of fold back sequences has been shown in animal genomes (Cech and Hearst, 1975; Davidson et al., 1973) as well as in plant genomes (Huguet et al., 1975; Bazetaux et al., 1978). They were studied in detail in wheat (Smith and Flavell, 1975), cotton (Walbot and Dure, 1976), rye (Smith and Flavell, 1977), tobacco (Zimmerman and Goldberg, 1977), soybean (Gurley et al., 1979) and pea (Murray et al., 1978).

To know more about the function of palindromic sequences, their organization in eukaryotic genome was investigated. In Xenopus (Davidson et al., 1973), seaurchin (Graham et al., 1974), Drosophila (Schmid et al., 1975), Mollusc (Angerer et al., 1975) and mouse (Cech and Hearst, 1975) these sequences were found to be interspersed with either repetitive DNA or single copy DNA and were scattered throughout the genome. Among

plants, the distribution of palindromic sequences was also somewhat similar to that in animal genomes. In wheat (Smith and Flavell, 1975),for example, the proportion of zero time binding fraction increased with increase in DNA fragment length almost certainly due to the presence of single strand tails on the fold back duplexes. These sequences were shown to be spaced throughout atleast 20% of the genome and were probably clustered in groups. In cotton (Walbot and Dure, 1976), fold back sequences occurred in a quasi clustered arrangement and these, therefore, appeared to be a general feature of DNA from eukaryotes.

Functions of repeated DNA sequences:

Repetitive DNA has been shown to be present in all the eukaryotes characterized so far. Since this DNA fraction represents upto 80% of the genome, it is reasonable to assume that it plays an important role in cellular function. Several theories have been put forward to explain the possible roles of repetitive DNA in aspects such as control of gene expression (Walker, 1971), chromosome replication, chromosome folding, and chromosome pairing (Walker, 1971). Intermediately repetitive DNA, for example, has been suggested to have a regulatory function (Britten and Davidson, 1969). Likewise mRNA for histone proteins has been shown to be transcribed from repeated sequences (Weinberg et al., 1972). The

latter have also been found to code for non-translatable RNA (Mahr and Fox, 1973; Lima de Faria, 1975) and rRNA (Price, 1976). Walker (1971, 1978) suggested that such sequences perform some mechanical role of 'house keeping" and are involved in arranging functional sequences in relation to higher order chromatin structure. Chromosomal specificity of satellite DNA sequences has been suggested to play a role in homologous chromosomal recognition in the cell (Brutlag and Peacock, 1975). Nagl (1978) has proposed that repeated sequences are important in evolution and speciation. According to him, noncoding , simple highly repeated sequences may be involved in ontogenetic differentiation and phylogenetic diversification. It was concluded that heterochromatin (satellite DNA) controls both the gross morphology of karyotypes and chromosomes and their evolution. For this reason, the genetically inert, highly repetitive DNA has been called "chromosome engineering DNA" (Nagl, 1978).

Scope of Thesis

Phaseolus Linn. is economically the most evolved and important genus of the family Leguminosae (Bailey, 1919; Hutchinson, 1948, 1959, 1964). Various species and varieties of the genus Phaseolus Linn. are classed as important "Pulses and Beans" considered to be the most nutritious with high percentage of vegetable proteins next to soybean. Some of the common and important Phaseolus species like P.mungo, Linn. P. aconitifolius, Jacq., P.lunatus Linn. P. multiflorus Willd, and P.vulgaris are cultivated as field crops or garden bean throughout India.

At the time of undertaking the present work in Phaseolus genomes no data were available except those of Beridze (1972) and Beridze and Bragvadze (1976). We therefore selected four commonly occuring Phaseolus plant species namely P. aconitifolius, P. aureus, P. mungo and P. vulgaris and have characterized their DNAs extensively to provide a detailed information of the molecular consequences of speciation. The evidences used for the DNA comparisons of these four Phaseolus species stem from (1) Buoyant density and melting temperature measurements of the total, high molecular weight DNAs, (2) Reassociation kinetics studies of the sonicated DNAs of an average length of 550 nucleotide pairs, (3) Thermal denaturation and optical reassociation

of the repetitive DNA fractions and finally (4) Determination of DNA sequence organisation. The DNAs of P. vulgaris and P. aureus have been selected for investigation of the arrangement of repeated and nonrepeated DNA sequences.

The results of the above comparisons form the basis of this thesis and are summarized in the form of four chapters that follow this Chapter. General discussion is the last chapter of the thesis.

CHAPTER II

BUOYANT DENSITY AND THERMAL DENATURATION STUDIES OF PHASEOLUS DNAs

BUOYANT DENSITY AND THERMAL DENATURATION STUDIES OF PHASEOLUS DNAs

INTRODUCTION

Thermal denaturation and isopycnic centrifugation in neutral CsCl gradients are two important basic techniques used for the characterization of DNA. Thermal denaturation studies give a valuable information concerning the GC content and the base composition heterogeneity of DNA. Likewise, neutral CsCl equilibrium centrifugation yields the buoyant density of a given DNA preparation and also tells us whether a satellite DNA component is present or not. Furthermore, GC content can be estimated from the buoyant density value.

Phaseolus vulgaris is one of the first plant species where a discrete satellite DNA component with a buoyant density of 1.703 g/cm^3 was discovered by Beridze et al. (1967). Later, Beridze (1972) studied the DNAs of six Phaseolus species by analytical equilibrium ultracentrifugation for the presence of satellite DNA. Likewise, the DNA melting properties of Phaseolus vulgaris and Phaseolus multiflorus were reported by Beridze et al. (1967) and Quetier and Guille (1968) respectively. No other data on the molecular characterization of the genomes of Phaseolus species were known when we started our work in the middle of 1976. We selected four Phaseolus plant species namely Phaseolus mungo (black gram), Phaseolus aconitifolius

(dew gram), Phaseolus vulgaris (French bean) and Phaseolus aureus (green gram) and carried out a comparative study of their DNA properties. All these four plant species are diploid in character and have 22 chromosomes. This Chapter describes the physical characteristics of the DNAs of these four species.

MATERIALS AND METHODS

All the chemicals used throughout our work were of analytical reagent (AR) grade or GR grade obtained either from BDH, Sarabhai Chemicals or E.Merck. Certain chemicals such as RNAase, Pronase, PIPES, Tris were obtained from Sigma Chemical Corporation, USA. λ DNA, T_4 DNA, S_1 nuclease, ECoRI and molecular weight markers were secured from Boehringer Mannehiem, West Germany.

Seeds of black gram (variety: BR10), dew gram (variety: PLMO 130), French bean (variety: Bountiful) and green gram (variety: China Moong) were obtained locally.

Treatment and germination of seeds:

Microorganisms are known to be associated with a variety of plants including those of Phaseolus (Lewis and Crotty, 1977; Dunleavy and Urs, 1973; Lange, 1966; Mundt and Hinkle, 1976). It is not known whether these microorganisms should always be regarded as contaminants or whether associations may have a deeper biological significance. To effectively reduce the level of bacterial

contamination without altering the germination of seeds, the latter were treated as follows. Seeds were thoroughly washed first with soap water and then with distilled water and were allowed to remain in alcohol (70%) for 10 min. After this treatment, they were washed and soaked in 1% chlorine water for 5 min and washed again to remove all traces of chlorine (Mascarenhas et al., 1976). These seeds were soaked in distilled water for 3-4 hours. The swollen seeds were then kept for germination on wet cotton in the dark. After five days, shoots (5-6 inches in length) were harvested about one inch above the surface to minimize bacterial contamination. These shoots were immediately frozen in liquid nitrogen and stored at -20°C till further use.

DNA extraction and isolation:

The DNA extraction procedure is a combination of that of Marmur (1961) and Ranjekar et al. (1976). The frozen Phaseolus shoots were homogenized in Buffer I (0.5 M sucrose, 0.05 M tris, 0.05 M maleic acid and 0.003 M $CaCl_2$, pH 6.0) containing 0.1% triton (X-100) using a Remi blender at high speed for 60 seconds. The homogenate was passed through four layers of guaze cloth and the filtrate centrifuged at 1000 g for 15 min. The crude nuclear pellet was washed twice with Buffer I and once with Buffer II (1.0 M sucrose, 0.05 M tris, 0.05 M maleic acid and 0.003 M $CaCl_2$, pH 6.0) containing 0.1% triton (X-100). Each

washing involved suspension of the pellet in the specific buffer followed by centrifugation at 1000 g for 15 min. The pellet was finally washed with Buffer I. The washed pellet was suspended in saline-EDTA (0.15 M NaCl plus 0.1 M sodium salt of EDTA, pH 8.0) solution and the DNA was extracted according to Marmur's procedure with slight modifications. The crude DNA dissolved in SSC (0.15 M NaCl and 0.015 M trisodium citrate pH 7.0, abbreviated as SSC) was incubated with pretreated RNAase (RNAase was preheated at 80°C for 10 min. to denature any DNAase present) for one hour. After the RNAase treatment the DNA was incubated for two hours with pretreated pronase (200 µg/ml). At the end of pronase treatment, sodium chloride concentration was increased to 2.5 M and the DNA solution was deproteinized with chloroform-isoamyl alcohol (24:1 v/v) mixture. The aqueous layer was extracted and the DNA precipitated with 1.5 volumes of chilled alcohol. At least three to four deproteinizations were necessary for obtaining pure DNA preparations. From 1000 g of shoots, the yield of pure DNA was approximately 2.5mg.

All the Phaseolus DNA preparations were routinely analyzed for their content in DNA, RNA and protein by diphenylamine assay (Burton, 1968), orcinol assay (Volkin and Cohn, 1954) and Lowry's assay (Lowry et al., 1951) respectively. Only those DNA preparations with RNA content of less than 1% and protein content of less than 1-2% and 230/260 optical density ratio of less than 0.45 were used for further experiments.

Analytical ultracentrifugation:

DNA solution (0.4 ml) containing 2 to 5 µg of plant DNA and 0.5 µg of marker DNA (Micrococcus lysodeikticus) was adjusted to a density of 1.710 g/cm^3 by addition of crystalline CsCl (E. Merck, suprapure). The ultracentrifugation was carried out using an aluminium an-G .. 6 cell rotor, 12 mm double sector cells and a L2 65B Beckman preparatory ultracentrifuge equipped with Beckman ultraviolet scanner and a multiplexer. The run was performed at 40,000 rpm and at 20°C for 40-80 hours. Ultraviolet scans were taken at 265 nm at a slit width of 0.1 mm. A scanning speed of 1.74 cm/min was used with a chart speed of 24.5 cm/min, so as to give an enlargement of 14 fold along the abscissa. In neutral CsCl gradients, densities were calculated from the position of a marker having a known density of 1.731 g/cm^3.

Thermal denaturation:

Thermal denaturation of various DNAs were carried out in Gilford 250 spectrophotometer equipped with thermoprogrammer (Model 2527), analog multiplexer (Model 6046) and automatic reference compensator (Ranjekar et al., 1976). Native DNA samples dialyzed with 0.12 M sodium phosphate buffer (PB), pH 6.8, were used for studying their melting properties. Approximately, 0.2 ml of the DNA solution (25 to 50 µg/ml) was added to the thermal cuvettes and the temperature of the solution was raised at a rate of

1°C/min upto 98°C. The absorbance changes at 260 nm were continuously recorded during the heating process. From the absorbance change, the hyperchromicity of the DNA sample was calculated using the formula:

$$H = \frac{A_{260}(98°C) - A_{260}(25°C)}{A_{260}(98°C)}$$

where H is the hyperchromicity and A_{260} is the absorbance at 260 nm. The total hyperchromicity was normalized to 100% and the graph of percent hyperchromicity versus temperature was plotted. E.coli and calf thymus DNAs were used as standards for all the thermal denaturation studies.

RESULTS

Buoyant density:

The nuclear DNAs from three Phaseolus species namely, black gram, dew gram and green gram were centrifuged to equilibrium in neutral CsCl gradients to estimate the buoyant density and to assess the presence of a satellite DNA component. The DNAs of all the three Phaseolus species banded as a single narrow peak with a buoyant density of 1.694 g/cm^3 (Fig. 1). A satellite DNA component was clearly present at a buoyant density of 1.703 g/cm^3 in black gram and dew gram. In green gram DNA, however, there was only a slight indication for the presence of a satellite component. The GC content was estimated from the buoyant density of each DNA and was found to be of the order of 34.7%.

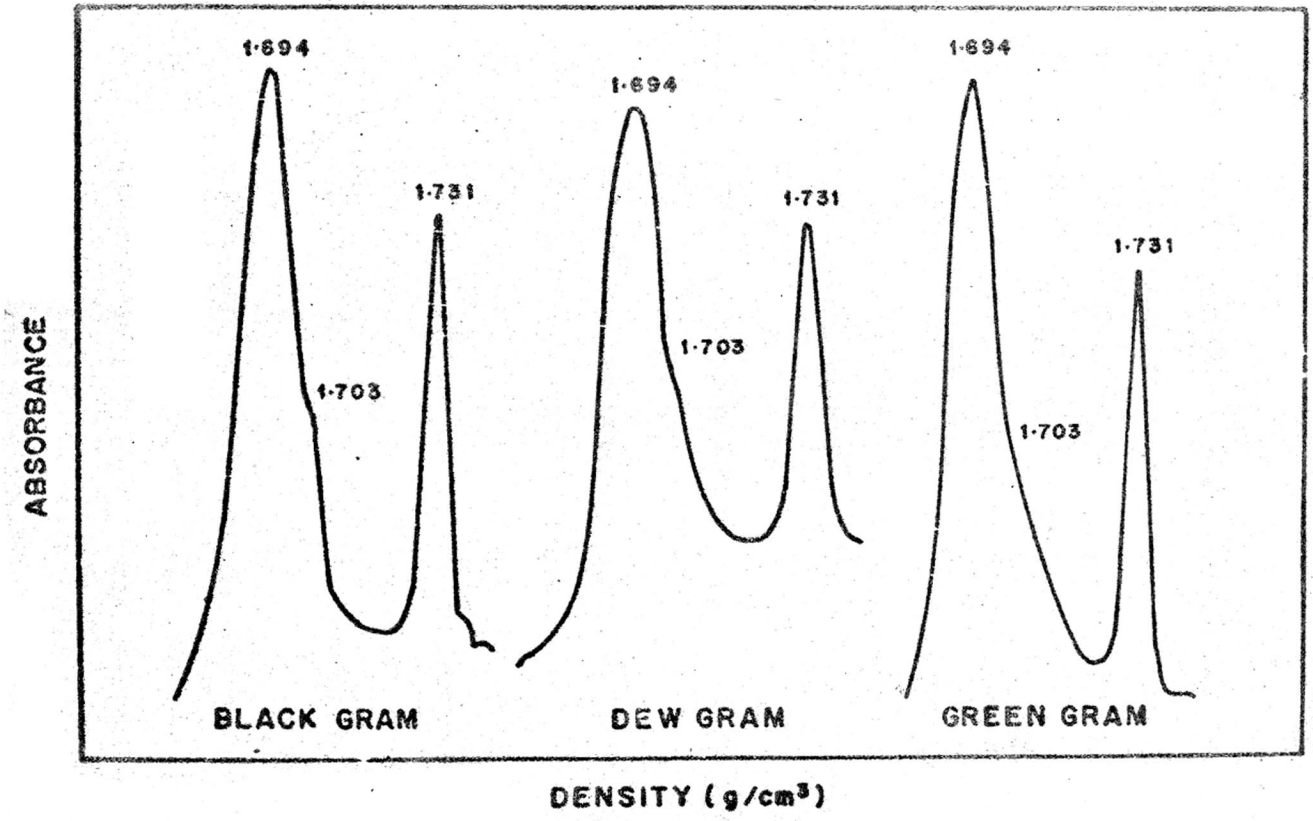

FIG. 1

Analytical ultracentrifugation of black gram, dew gram and green gram total DNA in neutral CsCl gradients using Micrococcus lysodeikticus DNA, with a buoyant density of 1.731 g/cm^3, as marker.

Melting properties:

The DNAs of the four Phaseolus species were next analyzed for their thermal denaturation behaviour. The thermal denaturation profiles of black gram, dew gram and green gram DNAs were very similar and their Tms were in the range of 83.5°C to 83.7°C (Figs. 2,3,5). The melting curve of French bean DNA (Fig. 4) was clearly biphasic showing one minor high melting and one major low melting DNA fraction. The former DNA fraction represented 15% of the total DNA and its Tm was 93.5°C while the latter fraction accounted for 85% of the total DNA and had a Tm of 83.7°C. The DNA hyperchromicity of the four Phaseolus species was in the range of 0.24 - 0.26.

To reveal molecular heterogeneity, the melting curve of each DNA was differentiated (Figs. 2-5). In black gram, two major components, one at 81°C and the other at 85°C and atleast four minor components in the temperature range of 88 - 96°C were observed. The derived graph of dew gram revealed three major peaks at 82°C, 84°C and 86°C respectively, a small peak each at 78°C and 92°C and a shoulder at 80°C and 88°C. In French bean one main peak at 84°C, four minor peaks in the temperature range of 77 - 94°C and one shoulder at 81°C were apparent. In green gram, one major peak at 84°C, three small peaks at 87°C, 89°C and 92°C and a shoulder at 82°C and 90°C were evident. All the differential patterns of the DNAs were found to be reproducible in a minimum of four experimental replicates.

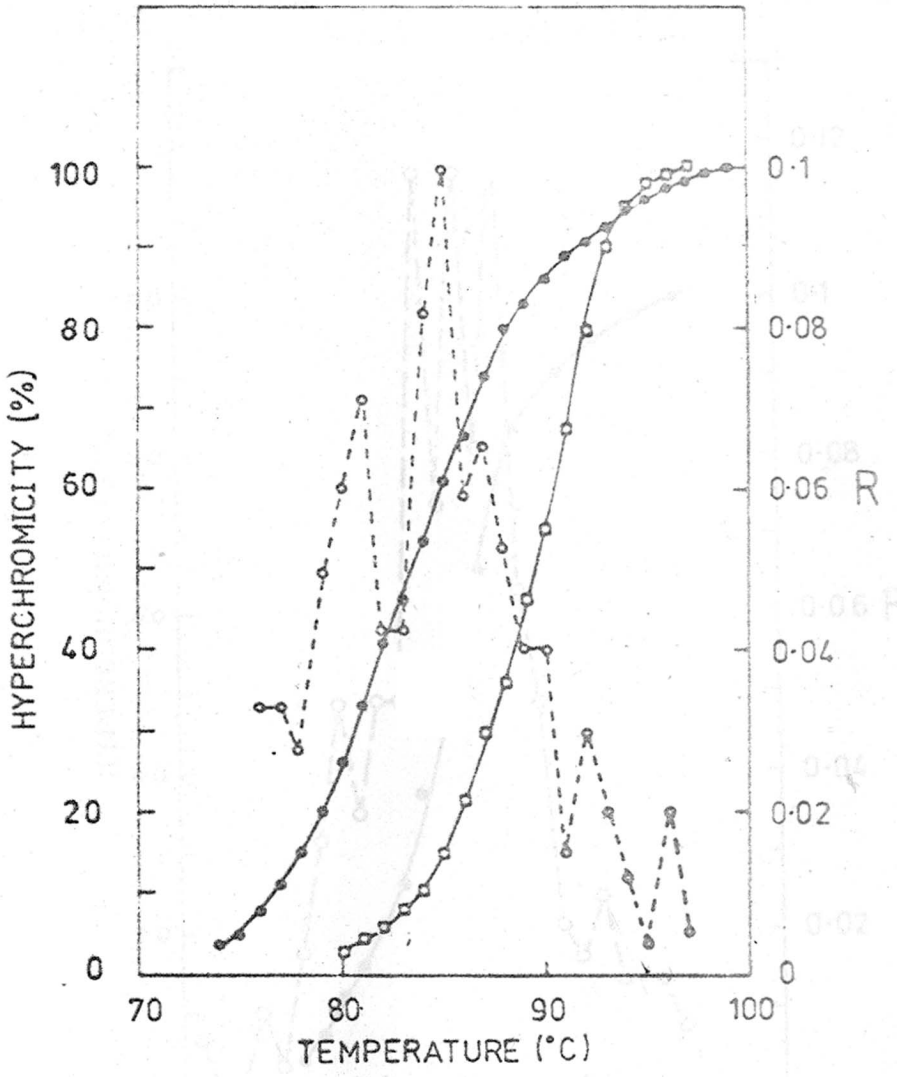

FIG. 2

Melting profile of native unsonicated black gram DNA (●——●) and its differential curve (o——o). The differential curve is obtained by using the formula:

$$R = \frac{A_{260}(t_2) - A_{260}(t_1)}{t_2 - t_1}$$

where $A_{260}(t_2)$ and $A_{260}(t_1)$ are the absorbance at temperatures t_2 and t_1.

F.coli DNA (□——□) was used as standard. ($T_m = 90°C$)

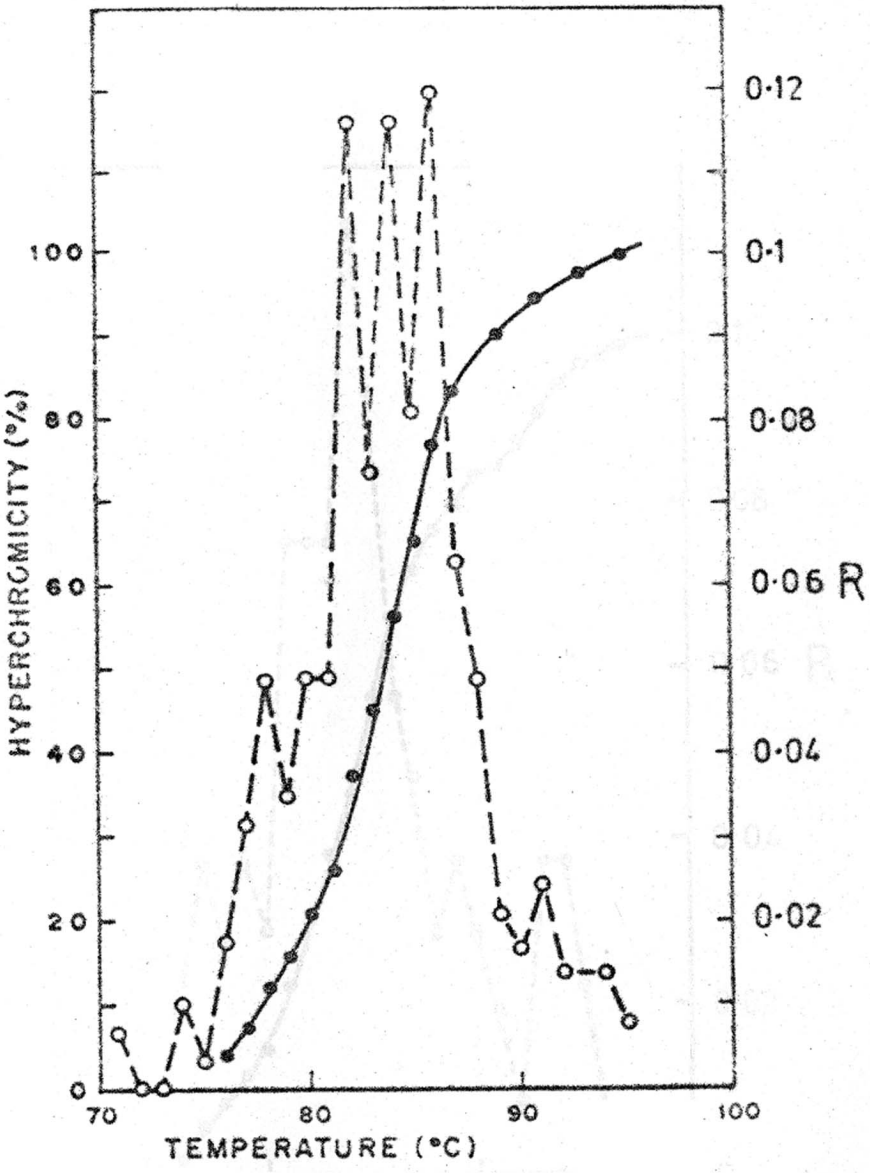

FIG. 3

Melting profile of native unsonicated dew gram DNA (●——●) and its differential curve (o——o).

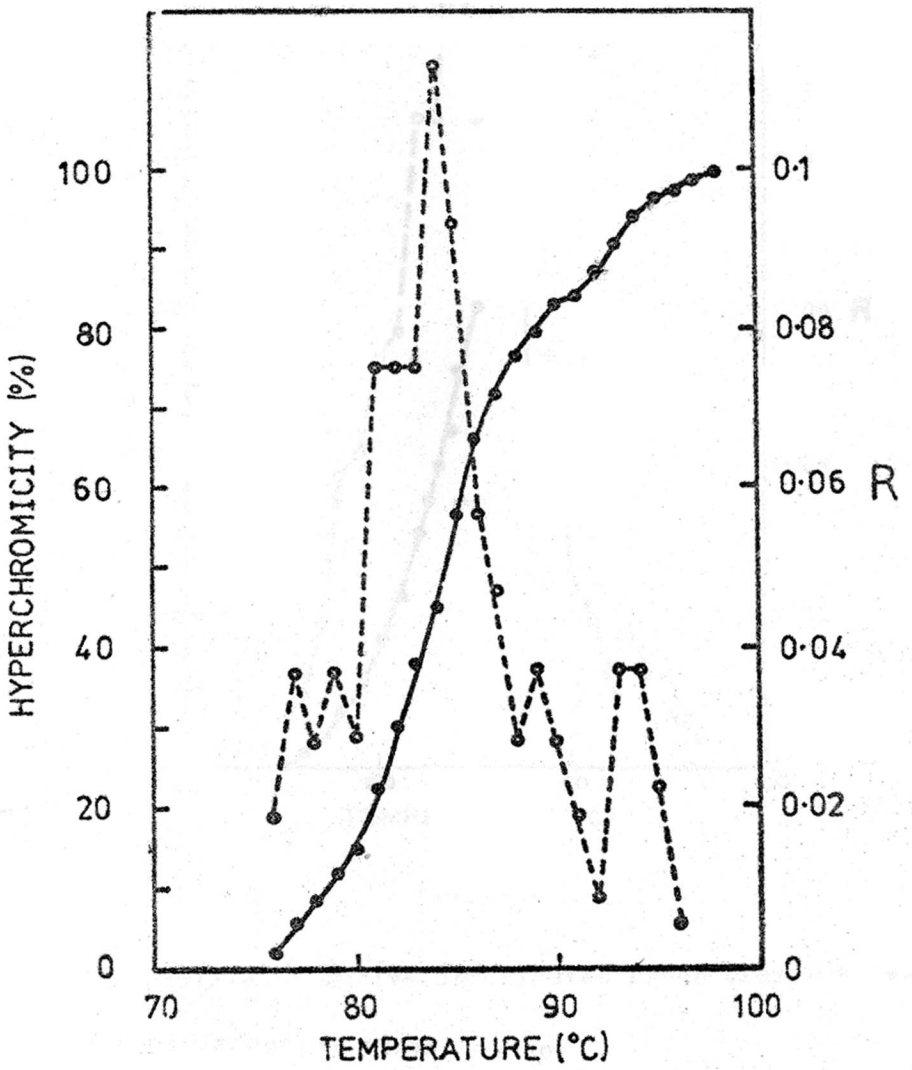

FIG.4

Melting profile of native unsonicated French bean DNA (●—●) and its differential curve (o—o).

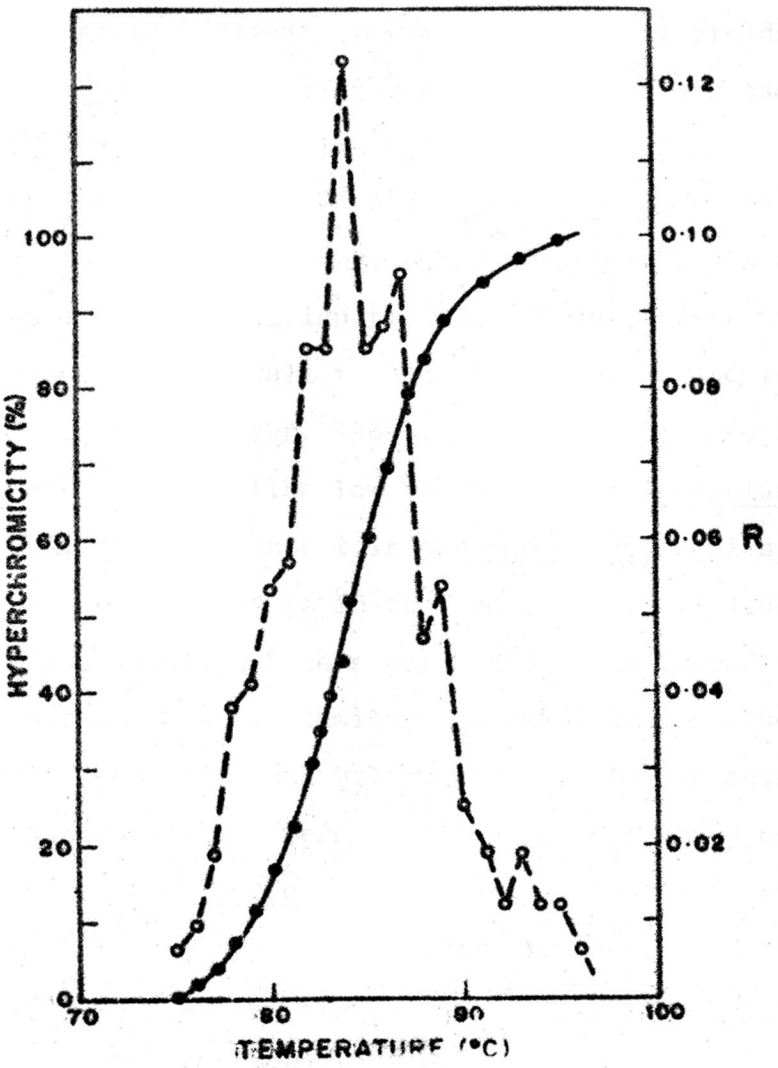

FIG. 5

Melting profile of native unsonicated green gram DNA (●——●) and its differential curve (o---o)

DISCUSSION

In general, obtaining DNA in sufficient yields from the four Phaseolus species was a very difficult task. We tried different extraction procedures including those of Bendich and Bolton (1967) and Flavell et al. (1974). However, all the methods gave low DNA yields. In plants, the two important difficulties in DNA extraction are resistance of cell wall towards its breakage and very high DNAase activity (Stern, 1968). These factors may be responsible for getting low DNA yields in Phaseolus. In addition, the DNA content in our Phaseolus species appears to be rather low and is in the range of 0.5 to 1.85 pg (Ayonoadu, 1974). These species have thus proved to be poor sources of DNA. Similar observations are also reported by Bendich and Bolton (1967). Due to this problem, numerous extractions were required to be carried out in the case of each Phaseolus species. In fact the DNA yields in the case of black gram and dew gram were so poor that we were unable to get enough DNA for carrying out the DNA sequence interspersion studies.

The DNA preparations of Phaseolus plant species were isolated from nuclear pellets instead of total tissue homogenates, thus minimising the possibility of contamination by chloroplast or mitochondrial DNAs. Furthermore, precautions were taken against bacterial contamination by carrying out surface sterilization of seeds prior to germination and by using only the shoots

for DNA extraction. Since bacterial DNAs are generally known to band in the range of 1.710 to 1.720 g/cm^3 and as we do not see any peaks in this range in the CsCl centrifugation patterns of our DNA preparations (Bendich, 1972) bacterial contamination appears to be very insignificant. It can, therefore, be concluded that satellite fraction observed in black gram and dew gram are of mainly nuclear origin.

Before the present work was undertaken, DNAs of at least seven Phaseolus species were characterized by isopycnic neutral CsCl centrifugation (Beridze, 1972; Chen, 1971). Phaseolus vulgaris (French bean) DNA, for example, revealed a main band DNA and satellite DNA at buoyant densities of 1.694 and 1.703 g/cm^3 respectively. In this species, satellite DNA represented 30% of the total DNA. Beridze (1972) later characterized the DNAs of three American and three Asian species of Phaseolus and showed the presence of satellite DNA to be more (upto 30%) in the former than that in the latter. Our centrifugation data on the DNAs of three Phaseolus species compare well with that reported by Beridze (1972). For example, the buoyant density values of the main band and satellite DNAs in dew gram and black gram are identical to those reported for other Phaseolus species. Moreover, black gram and dew gram are Asian species and the proportion of satellite DNA is 5-10% of the total DNA as estimated from their ultraviolet scans. This is in keeping with the trend

described by Beridze (1972) that the proportion of satellite DNA in Asian species is less as compared to those in American species. About green gram satellite DNA, conflicting reports are available. According to Beridze (1972) there is no satellite DNA in green gram. In our work, there is only a slight indication for the presence of a satellite DNA in green gram. Ingle et al. (1973) have reported that satellite DNA in green gram accounts for 5% of the total DNA.

The Tm values of black gram, dew gram and green gram DNAs are remarkably very similar. The French bean DNA has a melting profile which is strikingly different from those of the other three Phaseolus species. According to earlier work by Beridze et al. (1967), French bean DNA was shown to consist of two components with Tms of 85°C and 95°C. Our melting data of French bean DNA are broadly similar to these results except that the Tm values of the low melting and the high melting DNA component are slightly lower and are 83.7°C and 93.5°C respectively.

If GC content is estimated from the buoyant density of the main band DNA in each Phaseolus species and compared to that derived from the Tm value of the total DNA in black gram, dew gram and green gram and of the low melting fraction in French bean, a close similarity between the two is observed (Table 1). This indicates an absence of 5 methyl-cytosine in the main band DNA of all the four Phaseolus plant species. 5 methyl-cytosine, if present, is known to lower the buoyant density value of a DNA

TABLE 1

Buoyant density and thermal denaturation of four Phaseolus species

Species	Buoyant density (g/cm^3)		Melting temperature (Tm °C)		GC^a (%)	GC^b (%)
	Main band	Satellite band				
Black gram	1.694	1.703		83.6	34.7	34.6
Dew gram	1.694	1.703		83.5	34.7	34.4
French bean	1.694*	1.703*	Major (85%)	83.7	34.7	34.9
			Minor (15%)	93.5		
Green gram	1.694	1.703 (?)		83.7	34.7	34.9

[a]GC value obtained from buoyant density value of main band using the formula of Mandel and Marmur (1968):

$$GC = \frac{\rho - 1.660}{0.098} \times 100$$

[b]GC value obtained from Tm value using the formula of Mandel *et al.* (1968):

$$GC = (Tm - 69.4)\ 2.44$$

*These data are taken from the published report by Beridze (1972).

(Kirk, 1967).

The main band and the satellite DNA components in all the four species of Phaseolus have similar buoyant densities. Likewise, except French bean, all the other three Phaseolus plant species have similar Tm values. Such data are consistent with other studies where these two parameters do not seem to differ much among species within the same genus (Beridze et al., 1973; Beridze, 1975; Dutta et al., 1976; Levings III et al., 1976; Ranjekar et al., 1978b; Subramanyam and Azad, 1978). The similarity of the two parameter in different species of Phaseolus presuambly reflects the similarity of base composition of their DNAs. However, buoyant density and Tm measurements yield only a limited information about the Phaseolus genome characteristics. We have, therefore, undertaken the measurement of reassociation kinetics of all the four Phaseolus species. This constitutes the next Chapter of the thesis.

CHAPTER III

REASSOCIATION KINETICS OF PHASEOLUS DNAs

REASSOCIATION KINETICS OF PHASEOLUS DNAs

INTRODUCTION

Reassociation kinetics measurements of DNAs of a large number of eukaryotic species have shown that a fraction of their genome reassociates very rapidly and consists of repeated DNA sequences. Furthermore, from the Cot 1/2 of non-repetitive DNA fraction it has been possible to arrive at the haploid genome size estimate. Among various techniques, hydroxyapatite column chromatography offers a very useful method for measurement of DNA reassociation kinetics. Using this method, it is possible to construct a Cot curve as well as to isolate different repetitive DNA fractions. No data on the DNA reassociation kinetics measurements of the Phaseolus plant species were available. We, therefore, carried out these studies to assess the proportion of repetitive DNA and to estimate the haploid genome size of the four Phaseolus species.

MATERIALS AND METHODS

Hydroxyapatite preparation:

Hydroxyapatite was prepared essentially according to the procedure described by Tiselius et al.(1956). Two litres of 0.5 M solution of $CaCl_2$ and two litres of 0.5 M solution of Na_2HPO_4 were mixed at a flow rate of 120 drops per minute. The precipitate was washed four to five times

with distilled water. To the precipitate, distilled water was added to a final volume of four litres and to this was added 100 ml of 40% (w/v) NaOH solution under constant slow stirring. This mixture was allowed to boil for one hour. The precipitate was washed with distilled water till the pH of the mixture was 7. Sodium phosphate buffer (pH 6.8) was added to a final concentration of 0.012 M and the solution was heated until it just started to boil. After decantation, 0.012 M phosphate buffer was added to the precipitate and it was allowed to boil for 5 min. Another boiling with 0.012 M PB was carried out for 15 min. The hydroxyapatite was stored at 4°C with a few drops of chloroform.

DNA extraction:

DNA was extracted from shoots as described in Materials and Methods in Chapter II.

DNA fragmentation and sizing:

For reassociation studies, fragment size of DNA is very important. DNA was fragmented by sonication as follows: Approximately 30 ml of DNA solution was sonicated for a total period of 3 min using Biosonic III apparatus fitted with a half inch probe. Prior to sonication, N_2 gas was bubbled through the DNA solution for 5 - 10 min. During the sonication treatment the beaker containing the DNA solution was kept immersed in an ice bath and care was taken to see that the solution was not heated.

The average size of the DNA fragments after sonication was determined by sedimentation through neutral sucrose gradients in a Beckman preparatory ultracentrifuge. Approximately 0.1 ml of DNA solution (500 ug/ml) was layered on sucrose gradients (5 - 25%) and centrifuged at a speed of 34,000 rpm for 18 hours (Van der Schans, 1969). From the sedimentation patterns, average 'S' values and average molecular weights of the DNA fragments were calculated according to McEwan (1967) and Doty et al. (1958) respectively.

DNA reassociation studies:

The slurry of hydroxyapatite was poured into a double-jacketed column (15 x 2.5 cm) to a bed volume of 15 ml. The column was equilibrated with 0.12 M PB (pH 6.8) and brought to 62°C by connecting it to a constant temperature water bath equipped with a circulating pump.

Reassociation of DNA was carried out essentially by the method of Britten et al. (1974). Sonicated DNA was dialyzed against 0.12 M PB and the final DNA concentration determined by absorbance at 260 nm. A small aliquot of each DNA in 0.12 M PB was run through hydroxyapatite column operating at 62°C to determine what percent (if any) was incapable of binding at this temperature and this value was corrected for in subsequent calculations. The DNA solution was denatured by heating in a waterbath at 100°C for 10 min and then incubated at 62°C for a desired Cot value. A Cot

value of one is equivalent to incubation of a DNA solution of concentration 83 ug/ml (approximately 2 O.D.) for 1 hour (Britten and Kohne, 1968). The incubated DNA was transferred to the hydroxyapatite column maintained at 62°C. After adsorption of the DNA solution at the top of the column, 0.12 M PB (62°C) was passed through in order to elute the DNA which had not undergone reassociation (single stranded or denatured DNA). The reassociated double stranded DNA was then eluted with 0.4 M PB (pH 6.8). The percentage of reassociation was calculated using the formula:

$$\text{Percent reassociation} = \frac{B}{A + B} \times 100$$

where A = micrograms of DNA eluted with 0.12 M PB

B = micrograms of DNA eluted with 0.4 M PB

RESULTS

Cot curve of Phaseolus DNAs:

The reassociation kinetics of the sonicated DNAs (550 np) of four Phaseolus plant species were studied over a wide Cot range of 10^{-1} to 10^{4}. The reassociation kinetics of sonicated E. coli DNA were also determined under identical conditions. As illustrated in Figs. 6-9, the reassociation of all the four DNA preparations extended over a broad Cot range of at least five decades. The reassociation of E.coli DNA on the other hand took place in a rather narrow

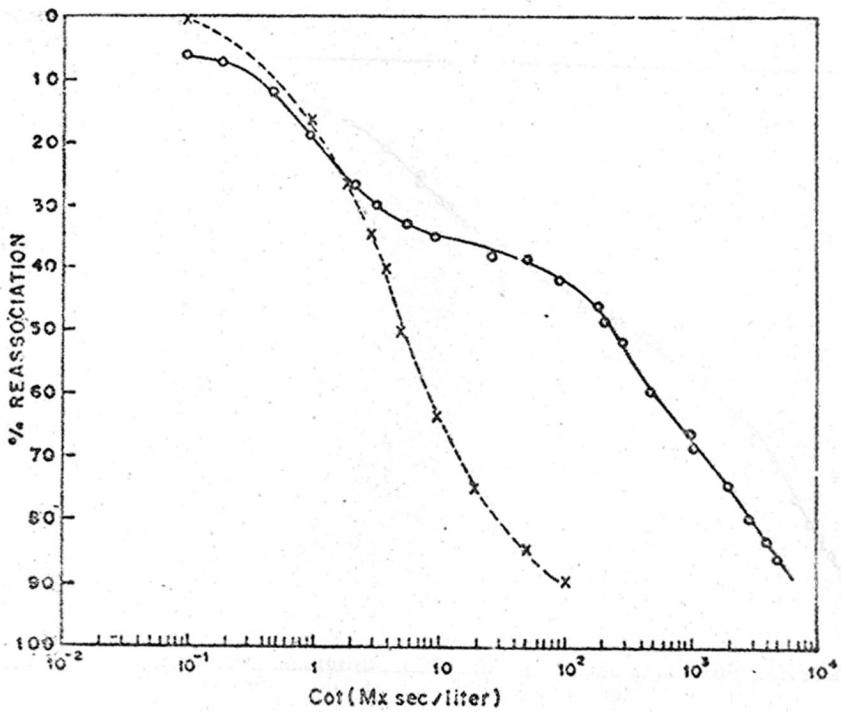

FIG. 6

Reassociation kinetics of black gram sonicated (o—o DNA (550 np) with E.coli DNA (550 np) as (x--x) standard, by hydroxyapatite column chromatography.

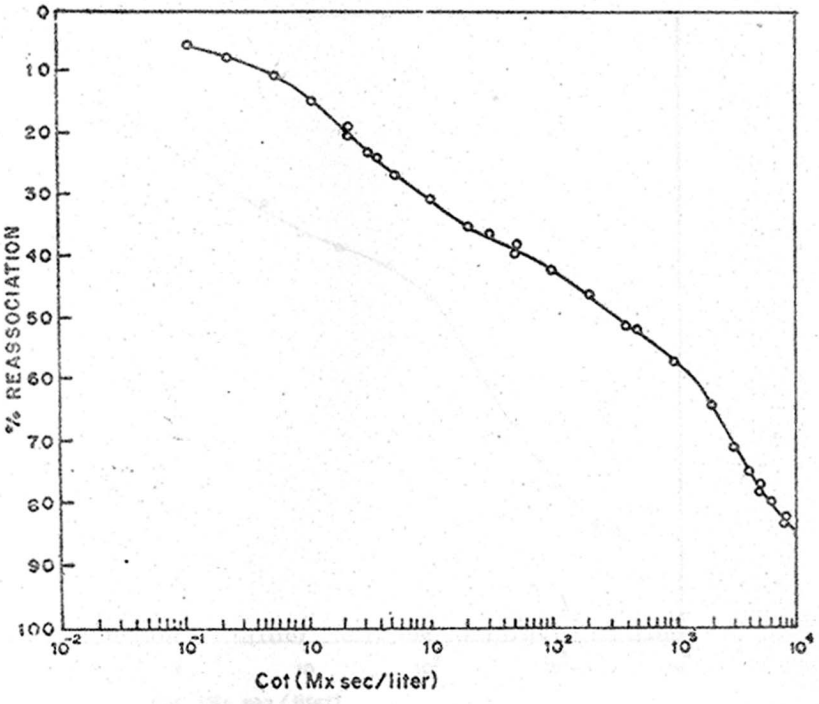

FIG. 7

Reassociation kinetics of dew gram sonicated DNA (550 np) by hydroxyapatite column chromatography.

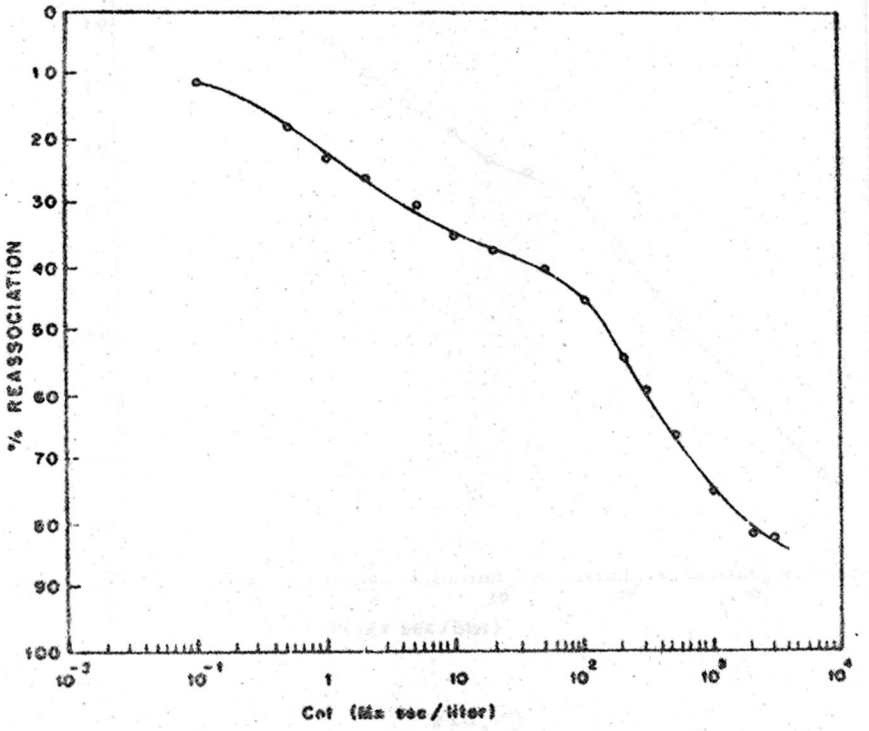

FIG. 8

Reassociation kinetics of French bean sonicated DNA (550 np) by hydroxyapatite column chromatography.

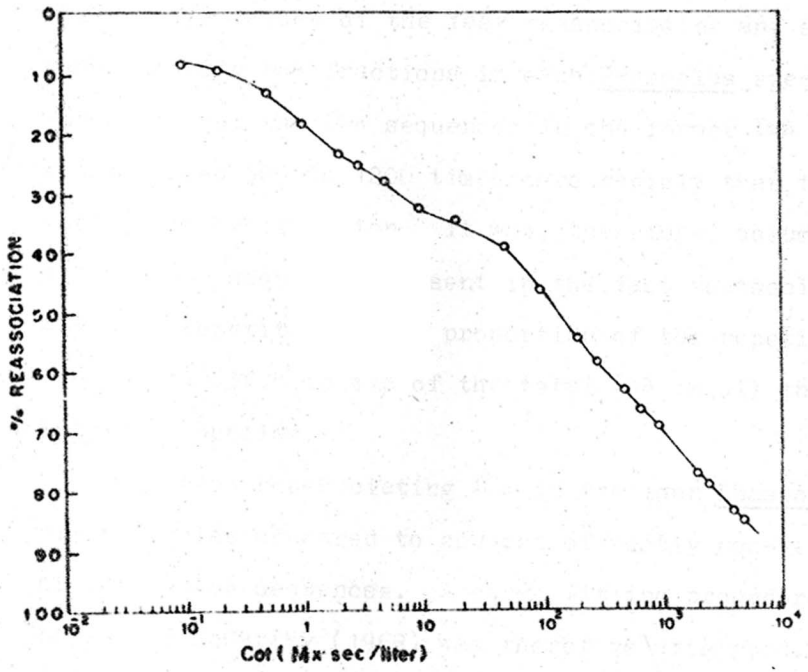

FIG. 9

Reassociation kinetics of green gram sonicated DNA (550 np) by hydroxyapatite column chromatography.

Cot range of 10^{-1} to 10^{2}. The Cot curves of all the four Phaseolus DNAs were found to consist of two main fractions: fast reassociating and slow reassociating. The fast reassociating DNA appears to include those sequences forming DNA duplexes by Cot 10 in black gram, Cot 50 in French bean and Cot 100 in dew gram and green gram. The comparison of the Cot 1/2 values of the fast reassociating and slow reassociating DNA fractions in each Phaseolus species revealed that the DNA sequences in the former DNA fraction reassociated 500 to 1200 times more rapidly than that of the latter DNA fraction. It was, therefore, assumed that all the DNA sequences present in the fast reassociating DNA were repetitive. The proportion of the repetitive DNA is thus 34.5 to 47% of the total DNA in all the four Phaseolus species.

The slow reassociating DNA in the four Phaseolus plant species appeared to consist of mostly non-repeated or unique DNA sequences. A curve fitting procedure of Laird and McCarthy (1968) was therefore attempted. In this procedure, the proportion of the slow reassociating DNA was normalized to 100% and the experimental Cot curve representing the above fraction was replotted maintaining its original Cot 1/2. An ideal second order curve with a Cot 1/2 of the slow reassociating DNA fraction was also plotted using the following formula:

$$\frac{C}{Co} = \frac{1}{1 + K_2 \, Cot}$$

where $\frac{C}{Co}$ is the fraction of the denatured DNA and K_2 is the reciprocal of the Cot 1/2. As revealed from Figs. 10-13, a close fit between the ideal Cot curve and that of the experimental Cot curve representing slow reassociating DNA was obtained only after assuming that the Cot 1/2 of the slow reassociating DNA was 1.0×10^3 in black gram, 3×10^3 in dew gram, 6.8×10^2 in French bean and 1.3×10^3 in green gram. The proportion of single copy DNA in Phaseolus is thus in the range of 53 - 65.5% in each species.

The fast reassociating DNA fraction which mainly consists of repeated DNA sequences was further classified into two subfractions, namely rapidly reassociating and intermediately reassociating. The proportion, the Cot 1/2 value, the frequency of repetition and the kinetic complexity of these two subfractions along with that of slow reassociating DNA fraction are given in Table 2.

Haploid genome size in Phaseolus:

It is known that the Cot 1/2 of a DNA is directly proportional to genome size assuming that all the DNA sequences are nonrepetitive in character. After determining, the proportion and the Cot 1/2 of the single copy or unique DNA sequences in the four Phaseolus plant species, we carried out the genome size determination. For this purpose, the Cot 1/2 of the unique DNA was compared with that of E.coli DNA. The latter is known to consist of non-repeated DNA sequences, has a kinetic complexity of 4.5×10^6 np

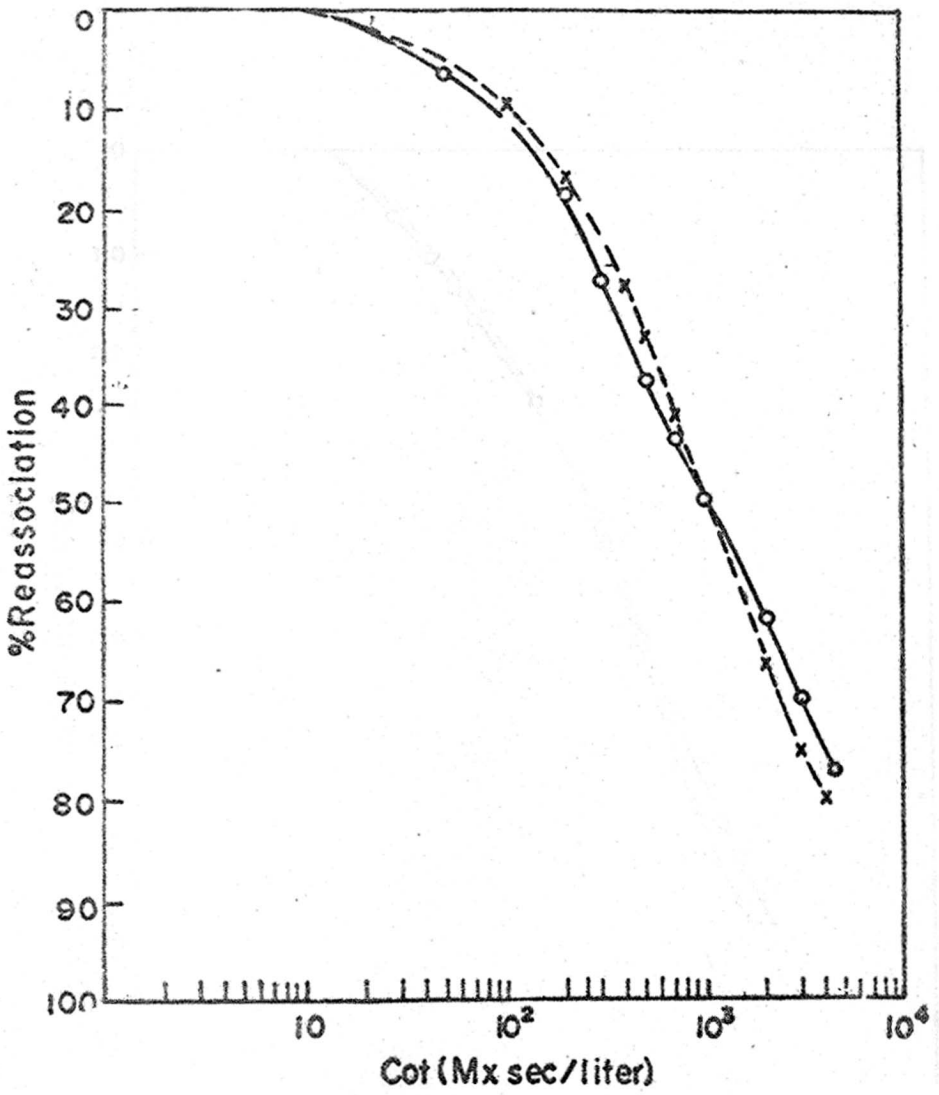

FIG. 10

Ideal Cot curve (x--x) and the experimental Cot curve (o—o) of the slow reassociating DNA in black gram. The ideal Cot curve was obtained using the formula:

$$\frac{C}{Co} = \frac{1}{1 + K_2\ Cot}$$

where $\frac{C}{Co}$ = fraction of denatured DNA

K_2 = reciprocal of Cot 1/2.

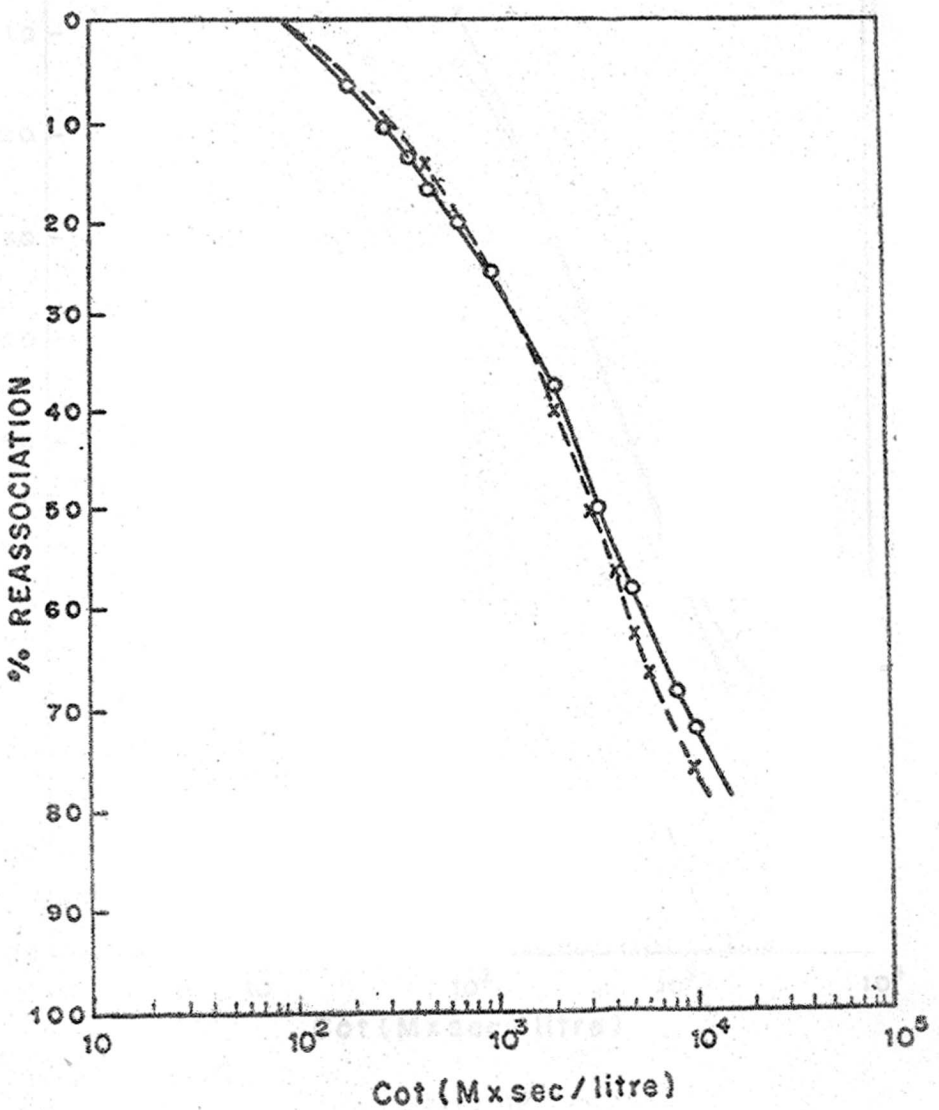

FIG. 11

Ideal Cot curve (x—x) and the experimental Cot curve (o—o) of the slow reassociating DNA in dew gram. The ideal Cot curve was obtained as described in the legend of Fig. 10.

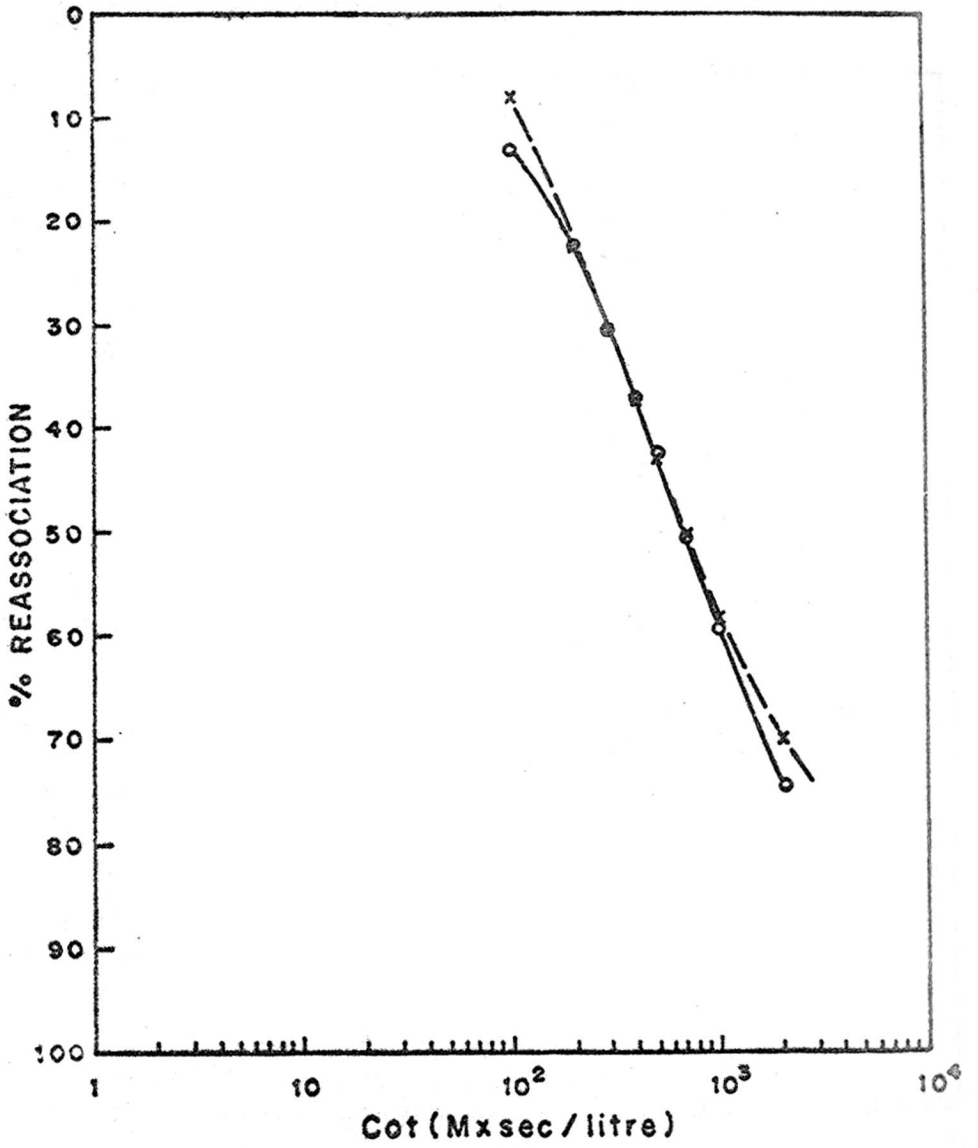

FIG. 12

Ideal Cot curve (×—×) and the experimental Cot curve (o—o) of the slow reassociating DNA in French bean. The ideal Cot curve was obtained as described in the legend of Fig. 10.

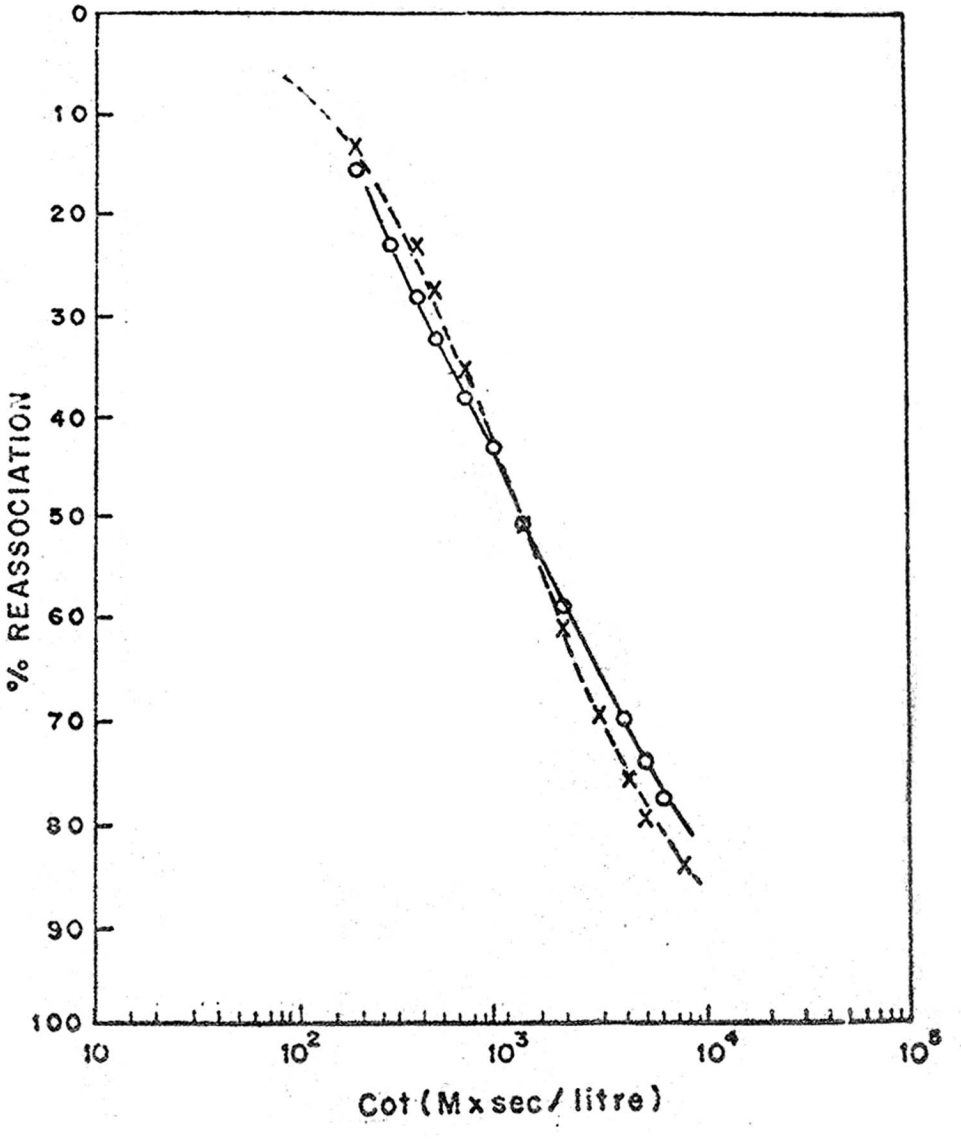

FIG. 13

Ideal Cot curve (×—×) and the experimental Cot curve (o—o) of the slow reassociating DNA in green gram. The ideal Cot curve was obtained as described in the legend of Fig. 10.

TABLE 2

Kinetic complexity of Phaseolus DNAs

Species	DNA fraction	Percent[a] of the Total DNA	Cot 1/2[a] observed	Cot 1/2[b] pure	Frequency[c] of repetition	Kinetic complexity[d]	
						Daltons	Nucleotide pairs
Black gram	Rapidly reassociating (Cot 1)	18	0.3	5.4×10^{-2}	3333	2.9×10^{7}	4.4×10^{4}
	Intermediately reassociating (Cot 1 - 10)	16.5	2.3	3.8×10^{-1}	434.8	2.0×10^{8}	3.1×10^{5}
	Slow reassociating (Cot > 10)	65.5	1.0×10^{3}	6.5×10^{2}	1	3.5×10^{11}	0.53×10^{9} (0.57 pg)
Dew gram	Rapidly reassociating (Cot 1)	14	0.2	2.8×10^{-2}	15,000	1.5×10^{7}	2.3×10^{4}
	Intermediately reassociating (Cot 1 - 100)	27	6.5	1.8	461.5	9.7×10^{8}	1.5×10^{5}
	Slow reassociating (Cot > 100)	59	3.0×10^{3}	1.77×10^{3}	1	9.5×10^{11}	1.44×10^{9} (1.6 pg)

Contd...

Species	DNA fraction	Percent[a] of the Total DNA	Cot 1/2[a] observed	Cot 1/2[b] pure	Frequency[c] of repetition	Kinetic complexity[d]	
						Daltons	Nucleotide pairs
French bean	Rapidly reassociating (Cot 1)	22.7	0.11	2.5×10^{-2}	6181.8	1.3×10^{7}	1.9×10^{4}
	Intermediately reassociating (Cot 1 - 50)	17.3	6.0	1.0	113.3	5.4×10^{8}	8.1×10^{5}
	Slow reassociating (Cot > 50)	60	6.8×10^{2}	4.08×10^{2}	1	2.2×10^{11}	0.33×10^{9} (.36 pg)
Green gram	Rapidly reassociating (Cot 1)	19	0.2	3.8×10^{-2}	6500	2.0×10^{7}	3.1×10^{4}
	Intermediately reassociating (Cot 1 - 100)	28	12.0	3.4	108.3	1.8×10^{9}	2.7×10^{5}
	Slow reassociating (Cot > 100)	53	1.3×10^{3}	6.89×10^{2}	1	3.7×10^{11}	0.56×10^{9} (0.6 pg)

[a] Obtained from Figs. 6-9.

[b] Cot 1/2 (pure) = Cot 1/2 (observed) x fraction of the total DNA.

[c] Frequency of repetition = Cot 1/2 slow reassociating DNA/Cot 1/2 of rapidly reassociating DNA.

[d] Estimated by using E.coli DNA as standard which has a known complexity of 4.5×10^{6} np and a Cot 1/2 of 5.2.

Several reassociation experiments (atleast 8-10) were carried out at each Cot value and each value in Table 2 and Figs. 6-9 is an average reassociation value.

(Cairns, 1963) and is shown to have a Cot 1/2 of 5.2 under our experimental conditions. The Cot 1/2 of the slow reassociating DNA in all the Phaseolus species was corrected for repeated DNA sequences, prior to comparing with that of E.coli DNA. Table 2 describes the genome size estimates in all the four Phaseolus plant species.

DISCUSSION

The DNA reassociation kinetics of the four Phaseolus species have provided an estimate of repetitive DNA which comprises 34 - 47% of the total DNA. Several dicotyledons such as cotton (Walbot and Dure, 1976), pea (Pearson et al., 1978; Murray et al., 1978), tobacco (Zimmerman and Goldberg, 1977), Cichorieae (Bachmannand Price, 1977), broad beans (Gnucheva et al., 1977), soybean (Goldberg, 1978; Gurley et al., 1979), Lathyrus (Narayan and Rees, 1976), Artichoke (Nze-Ekekang et al., 1974) and Umbelliferae (Kiper and Herzfeld, 1978) have been studied for their content in repetitive DNA. In most of these species the proportion of repetitive DNA is in the range of 50 - 70%. In cotton and Artichoke, however, the repetitive DNA content is lower and represents 40 - 45% of the total genome. The four Phaseolus plant species resemble cotton and Artichoke genomes in their repetitive DNA values. In fact black gram appears to have the lowest repetitive DNA content (34.5%) reported so far by hydroxyapatite measurements.

The classification of the fast reassociating DNA into

two subfractions is according to the method of Britten and Kohne (1968). Thus, DNA sequences reassociating by Cot 1 are rapidly reassociating and presumably consist of highly repetitive sequences. Similarly DNA sequences forming duplexes in the Cot range of 1 - 100 are intermediately reassociating and probably include intermediately repetitive DNA sequences. This method of grouping total repetitive DNA into two subfractions, though rather crude, enables us to get some detail information about the repeated sequences.

The proportions of rapidly reassociating and intermediately reassociating DNA fractions in all the four Phaseolus species appear to vary from each other and thus are species specific. In dew gram and French bean, for example, the rapidly reassociating DNA represents 14% and 22.7% of the total genome respectively. Likewise, the proportion of intermediately reassociating fraction varies in a wide range of 16.5 - 28%. The rapidly reassociating DNA is likely to include a small percentage of zero time binding fraction. From the data in Figs. 6-9, it is not possible to say anything definitely about the occurrence and the proportion of zero time binding fraction in Phaseolus genomes. It is well known that repetitive DNA consists of several families of repeated sequences differing in their frequency of repetition and complexity. Hence the values of the frequency of repetition and the kinetic complexity of the rapidly reassociating and the intermediately reassociating DNA fractions given in Table 2 should be

considered as average estimates.

The haploid genome size of the four Phaseolus plant species is estimated from their respective Cot 1/2 of the slow reassociating DNA fraction and is found to vary in the range of 3.3×10^8 np - 1.44×10^9 np or 0.36 pg to 1.6 pg. We have not carried out the cytophotometric DNA content determination in these Phaseolus species. However, such data are available in the case of French bean and green gram genomes. In French bean, Ayonoadu (1974) has shown the haploid DNA content as 1.85 pg. This estimate is five times more than the kinetic estimate of ours. In green gram, our estimate of haploid DNA content (0.6 pg) compares reasonably well to that of Murray (Wimpee and Rawson, 1979). Relationship between analytical DNA content and genome size estimate is not straightforward in plants. In tobacco (Zimmerman and Goldberg, 1977), for example, the DNA content value (1.65 pg) obtained from reassociation analysis differs significantly from that of analytical DNA content (10 pg). Similar discrepancies have also been observed in other plant species such as cotton (Walbot and Dure, 1976), pea (Pearson et al., 1978) and broad bean (Gnucheva et al., 1977). There can be a number of explanations for such discrepancies in the genome size estimates. One explanation is that truly unique sequences are not present in these species. For example, the genome of Vicia faba (Gnucheva et al., 1977) contains 60 identical copies of the so-called unique DNA sequences whereas

Vicia Sativa contains four identical copies (Gnucheva et al., 1977). Certain inaccuracies in the Cot 1/2 determination of unique DNA may also lead to erroneous estimation of genome size. According to Kemp and Merlo (1975) and Merlo and Kemp (1976), factors capable of accelerating the rate of DNA reassociation may be present in plant DNA preparations. The genome size estimates of the four Phaseolus species can, therefore, be considered as approximate ones and further confirmations through extensive cytophotometric determinations are required.

CHAPTER IV

DENATURATION-REASSOCIATION PROPERTIES OF REPETITIVE DNA IN PHASEOLUS

DENATURATION-REASSOCIATION PROPERTIES OF REPETITIVE DNA IN PHASEOLUS

INTRODUCTION

In order to understand the functions of repetitive DNA sequences, it is necessary to isolate them and study their various properties such as thermal denaturation behaviour, kinetic complexity, in situ localization, restriction endonuclease mapping and primary sequencing. The repetitive DNA of many animal species has already been analyzed in this manner (Human: Britten, 1968a, Saunders et al., 1972; Calf: Britten and Smith, 1969, Votavová et al., 1970; Microtus: Yasmineh and Yunis, 1971; Xenopus: Davidson and Hough, 1971; Amphibians: Straus, 1971; Drosophila: Laird and McCarthy, 1969; Wu et al., 1972; Nassaria: Davidson et al., 1971). Relatively less information is available on the characterization of plant repetitive DNAs. Thornburg and Siegel (1972) for example, have shown the presence of two or more classes of repetitive components in four plant species by isopycnic centrifugation of their repetitive DNAs in neutral CsCl gradients. Likewise, Nze-Ekekang et al. (1974) have revealed the occurrence of two rather homogenous fractions with an average complexity of 2×10^8 and 10^9 daltons respectively in Jerusalem Artichoke repetitive DNA. Smith and Flavell (1975) isolated a very rapidly reassociating fraction and a heterogenous intermediately reannealing fraction from wheat genome and

characterized them by studying their denaturation reassociation properties. Ranjekar et al. (1974 , 1976, 1978b) isolated rapidly and intermediately reassociating DNA fractions from rye, wheat, barley and seven Allium species and characterized them by isopycnic centrifugation in neutral CsCl gradients and by thermal denaturation properties. Recently Bachmann and Price (1977) have reported the analysis of repetitive DNA in Cichorieae and have demonstrated the presence of two distinct fractions of repetitive DNA in eleven plant species. In tobacco (Zimmerman and Goldberg, 1977), three discrete repetitive DNA fractions have been described.

In the case of the present four Phaseolus species, no information was reported about the properties of repeated DNA sequences except for some data on their satellite DNA. From the DNA reassociation kinetics studies of the four Phaseolus species described in the previous Chapter, their content in repetitive DNA is estimated to be 34 - 47% of the total DNA. In this Chapter, we describe the isolation of repetitive DNA in each of the four Phaseolus species and its characterization by studying its thermal denaturation-reassociation properties. We have also isolated the Cot 1 DNA fraction which is assumed to include the highly repetitive DNA sequences in each species and have similarly characterized it.

MATERIALS AND METHODS

DNA isolation:

DNA was isolated and purified from the four Phaseolus plant species as described in Materials and Methods of Chapter II.

Isolation of repetitive DNA:

Total DNA was sonicated to an average length of 550 np. Sonication and sizing of DNA is as described in Chapter III. For isolation of a repetitive DNA fraction, the sonicated DNAs in 0.12 M PB (pH 6.8) were denatured and were incubated to Cot values of 10 in black gram, 50 in French bean and 100 in dew gram and green gram. The reassociated DNA was separated from the unreassociated single strand DNA by hydroxyapatite column chromotography as described in Materials and Methods of Chapter III. The reassociated DNA fractions were dialyzed against 0.12 M PB prior to their characterization.

Thermal denaturation:

The melting studies of various DNA samples in 0.12 M PB were carried out in Gilford 250 spectrophotometer as described in detail in Chapter II.

Optical reassociation:

DNA samples in 0.12 M PB were added to thermal cuvettes and the absorbance at 260 nm at 25°C was recorded.

DNA concentration (10 - 40 ug/ml) was such that after melting, the absorbance still remained within the linear region of the spectrophotometer. The temperature of the DNA was raised to 98°C at a rate of 1°C/min. The DNA samples were maintained at this temperature for 4-5 min. to ensure complete denaturation. The temperature of the cuvette was next quickly lowered to 62°C by circulation of water. This lowering process took about 60 - 90 seconds. The changes in the absorbance of the DNA solutions were continuously recorded with time. For the determination of zero time or the start of reassociation, the midpoint (80°C) between 98°C and 62°C was taken (Britten et al., 1974).

The total change in absorbance from 25°C to 98°C was taken as 100% hyperchromicity. From the decrease in absorbance (hypochromicity), percentage reassociation was calculated by using the following formula at various Cot values:

$$\% \text{ reassociation} = \frac{A_{98} - A_{62}(t_1)}{A_{98} - A_{25}} \times 100$$

where A_{62} (t_1) is the absorbance (260 nm) at 62°C at different times. A_{98} and A_{25} are the absorbance (260 nm) at 98 and 25°C respectively.

Collapse hypochromicity was calculated from the instantaneous loss in absorbance (260 nm) which was observed when total denatured DNA was cooled from 98°C to 62°C (incubation temperature).

Bacterophage T_4 DNA was used as standard in all the optical reassociation experiments.

RESULTS

Thermal denaturation:

From Chapter III, it is clear that the repetitive DNA accounts for 34 to 47% of the total DNA in all the four Phaseolus species. Likewise, the highly repetitive DNA (Cot 1 DNA) represents 14 to 22.7% of the total DNA.

In black gram the Tm of repetitive DNA was 74.5°C (Fig. 14) while that of Cot 1 DNA was 77.8°C. In the case of dew gram, the melting curves of the repetitive and the Cot 1 DNA fractions were similar and the Tms were 76.8°C and 77.5°C respectively (Fig. 15). In French bean, the repetitive DNA exhibited a monophasic melting curve with a Tm of 79°C. The Cot 1 DNA, however, had a melting curve which was clearly biphasic (Fig. 16) indicating the presence of two components. The major low meling DNA fraction which comprised 75% of the Cot 1 DNA had a Tm of 78.7°C and the minor (25%) high melting DNA fraction had a Tm of 92.5°C (Fig. 16). The melting curve of the repetitive DNA in green gram exhibited a monophasic curve with a Tm of 76.8°C. The Cot 1 DNA had also a smooth thermal denaturation profile and had a Tm of 78°C (Fig. 17).

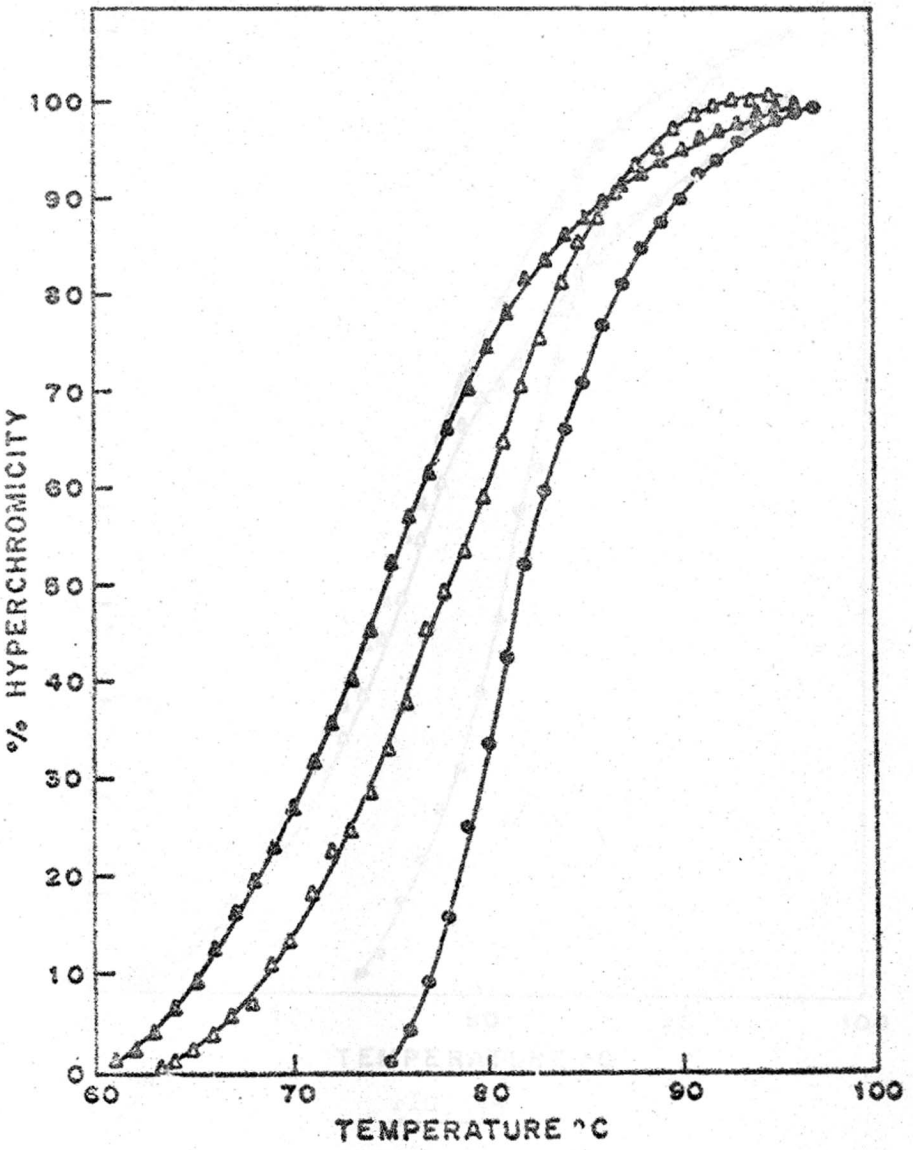

FIG. 14

Thermal denaturation profiles of the repetitive and the Cot 1 DNA in black gram, along with that of the sonicated (550 np) total black gram DNA. Repetitive DNA (▲——▲), Cot 1 DNA (△——△) and sonicated total DNA (●——●). All the experimental conditions are given in Materials and Methods.

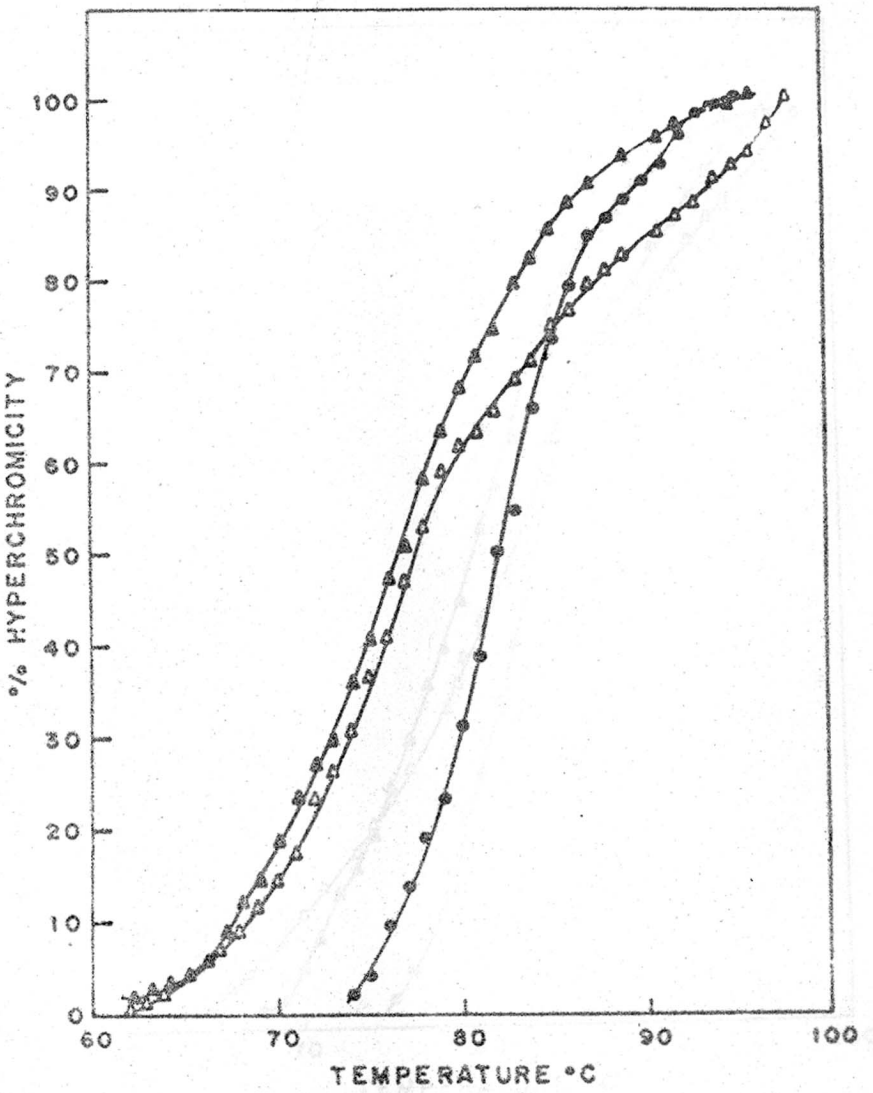

FIG. 15

Thermal denaturation profiles of the repetitive (▲—▲), and the Cot 1 DNA (△—△) in dew gram along with that of the sonicated (550 np) total dew gram DNA(● — ●).

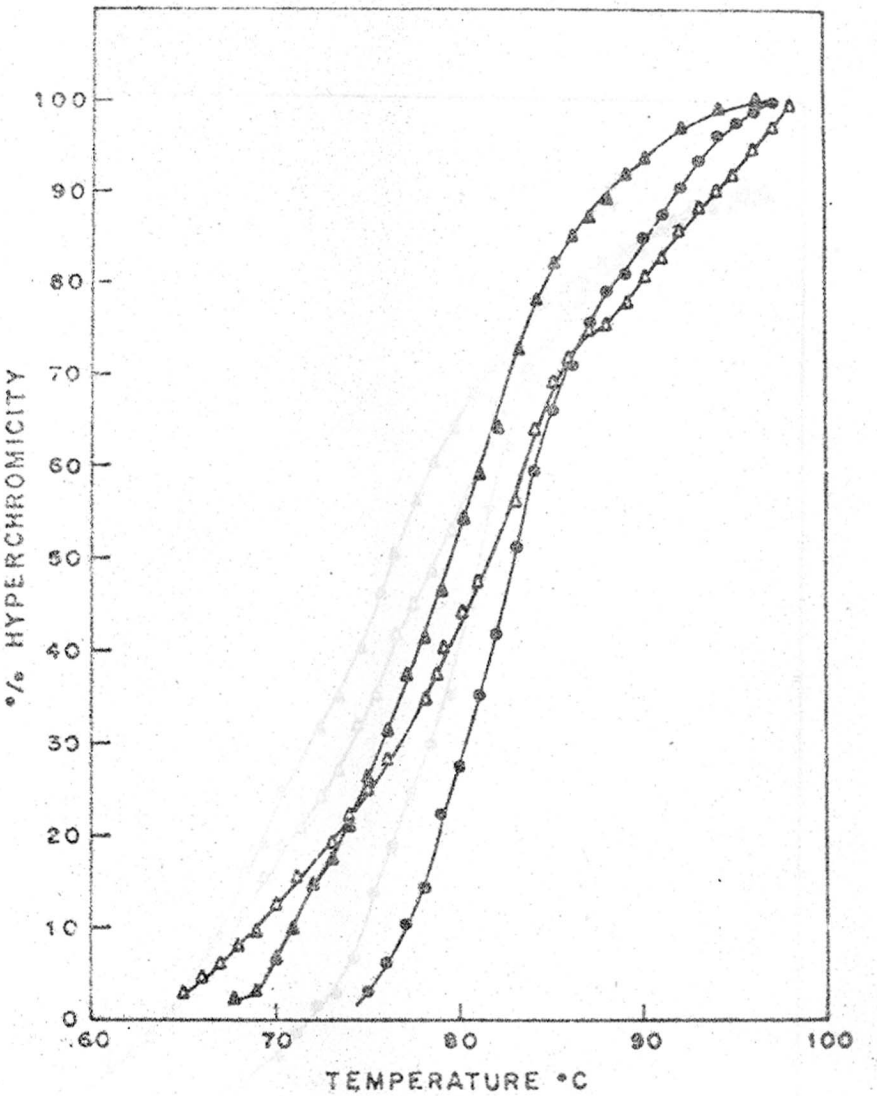

FIG. 16

Thermal denaturation profiles of the repetitive and the Cot 1 DNA in French bean, along with that of the sonicated (550 np) total French bean DNA. Repetitive DNA (▲—▲), Cot 1 DNA (△—△) and sonicated total DNA (●—●).

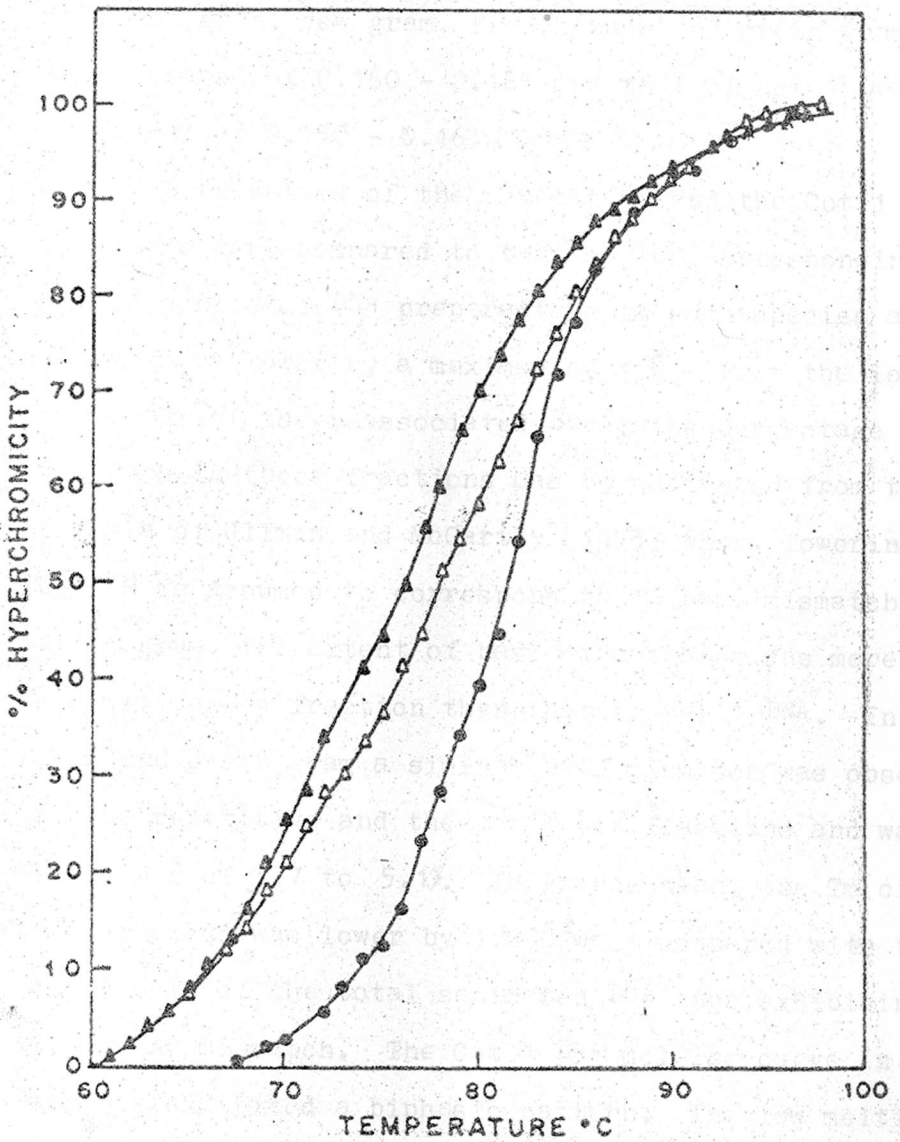

FIG. 17

Thermal denaturation profiles of the repetitive (▲—▲), and the Cot 1 DNA (△—△) in green gram along with that of the sonicated (550 np) total green gram DNA (●—●).

Hyperchromicity and base mismatch of repetitive DNA fractions:

The hyperchromicity of the repetitive DNA fractions in black gram, dew gram, French bean and green gram was in the range of 0.160 - 0.165 and that of Cot 1 DNA was in the range of 0.150 - 0.163 (Table 3).

The Tm values of the repetitive and the Cot 1 DNA fractions were compared to that of the corresponding native sonicated DNA preparations in each species and were found to be lower by a maximum of 7°C. From the lowering in the Tm of the reassociated DNAs, the percentage base mismatch in these fractions can be estimated from the formula of Ullman and McCarthy (1973) where lowering in Tm by 1°C is assumed to correspond to 1% base mismatch. In black gram, the extent of base mismatching was more in repetitive DNA fraction than that in Cot 1 DNA. In dew gram and green gram a similar base mismatch was observed in the repetitive and the Cot 1 DNA fractions and was of the order of 3.7 to 5.1%. In French bean, the Tm of Cot 50 DNA fraction was lower by 4.2°C when compared with the average Tm of the total sonicated DNA thus exhibiting a 4.2% base mismatch. The Cot 1 DNA melting curve in this species exhibited a biphasic pattern. The low melting major component had a Tm of 78.7°C which was lower by 3°C when compared to the low melting component of the total sonicated DNA thus exhibiting a base mismatch of the order of 3%. The high melting minor component of the Cot 1 DNA

TABLE 3

Thermal denaturation properties

DNA		Tm °C	Hyperchromicity[a] (H)	Base mismatch[b] (%)
Black gram				
Total sonicated (550 np)		81.5	0.236	-
Repetitive (Cot 10)		74.5	0.160	7.0
Highly repetitive (Cot 1)		77.8	0.150	3.7
Dew gram				
Total sonicated (550 np)		81.9	0.234	-
Repetitive (Cot 100)		76.8	0.165	5.1
Highly repetitive (Cot 1)		77.5	0.163	4.4
French bean				
Total sonicated (550 np)	Major (85%)	81.7	0.248	-
	Minor (15%)	92.5		
	Average	83.2		
Repetitive (Cot 50)		79.0	0.162	4.2
Highly repetitive (Cot 1)	Major (75%)	78.7	0.153	3.0
	Minor (25%)	92.5		
Green gram				
Total sonicated (550 np)		81.7	0.238	-
Repetitive (Cot 100)		76.8	0.163	4.9
Highly repetitive (Cot 10)		78.0	0.154	3.7

[a] Hyperchromicity (H) is calculated as described in Chapter II.

[b] Base mismatch is calculated by the method of Ullman and McCarthy, 1973.

had the same Tm as that of the high melting component of the total sonicated DNA, thus showing no base mismatch in this component.(Table 3).

Optical reassociation of Cot 1 DNA fraction:

During reassociation kinetic studies of total Phaseolus DNA by hydroxyapatite column chromatography, we have carried out very few experiments at Cot values less than 1. We, therefore, do not know about the kinetic heterogeneity of the nucleotide sequences reannealing by Cot 1. In order to get some information about the number of kinetic components present in Cot 1 DNA fraction, the optical reassociation of the latter was studied along with that of total sonicated DNA (550 np) in dew gram, French bean and green gram. The Cot 1 DNA fraction in each of the three Phaseolus species underwent reassociation in the range of 80 - 85% by Cot 1 whereas 18 - 29% of the total sonicated DNA formed duplexes under similar Cot conditions (Figs. 18-20). The Cot 1 optical reassociation value of the total DNA compared well with that of hydroxyapatite in each Phaseolus species. In French bean (Fig. 19) for example the sonicated DNA exhibited the reassociation of the order of 22.7% by hydroxyapatite chromatography and 24% by optical reassociation. From the Cot curves of the three Cot 1 DNA fractions (Figs. 18-20) there appeared to be one minor fraction reassociating by approximately Cot 10^{-2} and a major fraction reannealing in the Cot range of 10^{-2} to 1. The proportion of the minor fraction varied between 25-36%

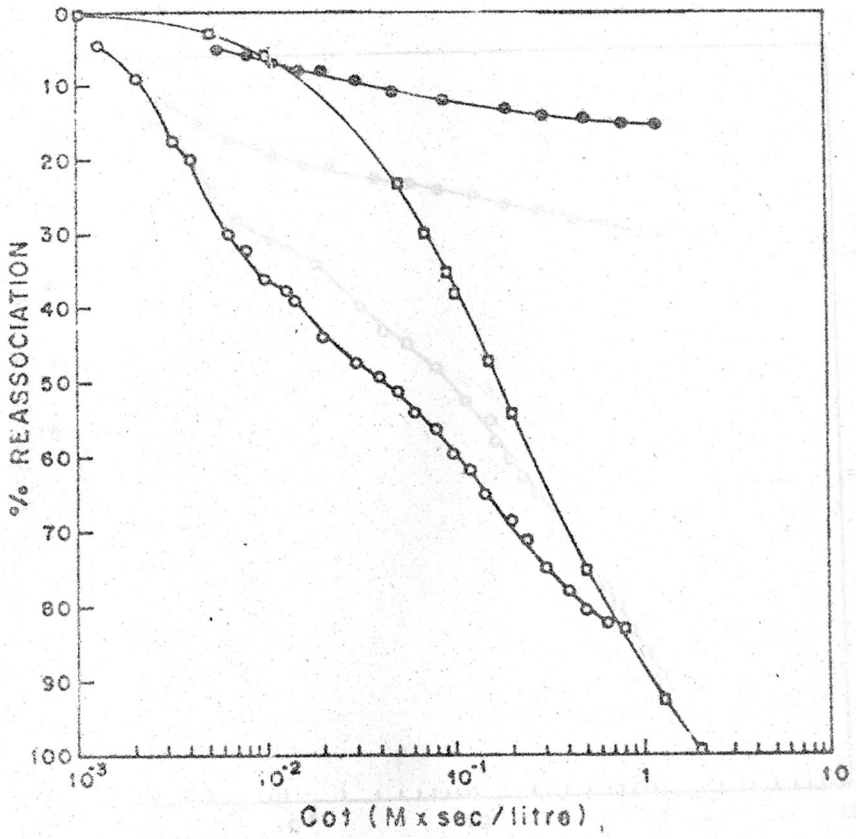

FIG. 19

Optical reassociation curves of total sonicated DNA (●——●) and Cot 1 fraction (o——o) of dew gram along with that of sonicated T_4 DNA (□——□).

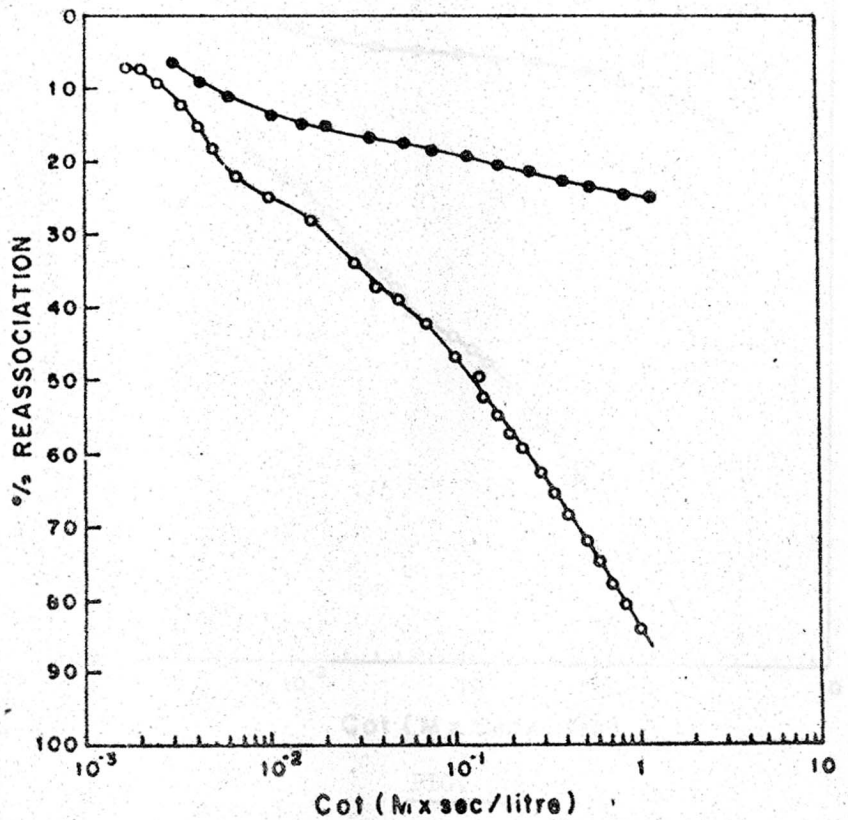

FIG. 19

Optical reassociation curves of total sonicated DNA (●——●) and Cot 1 fraction (o——o) of French bean.

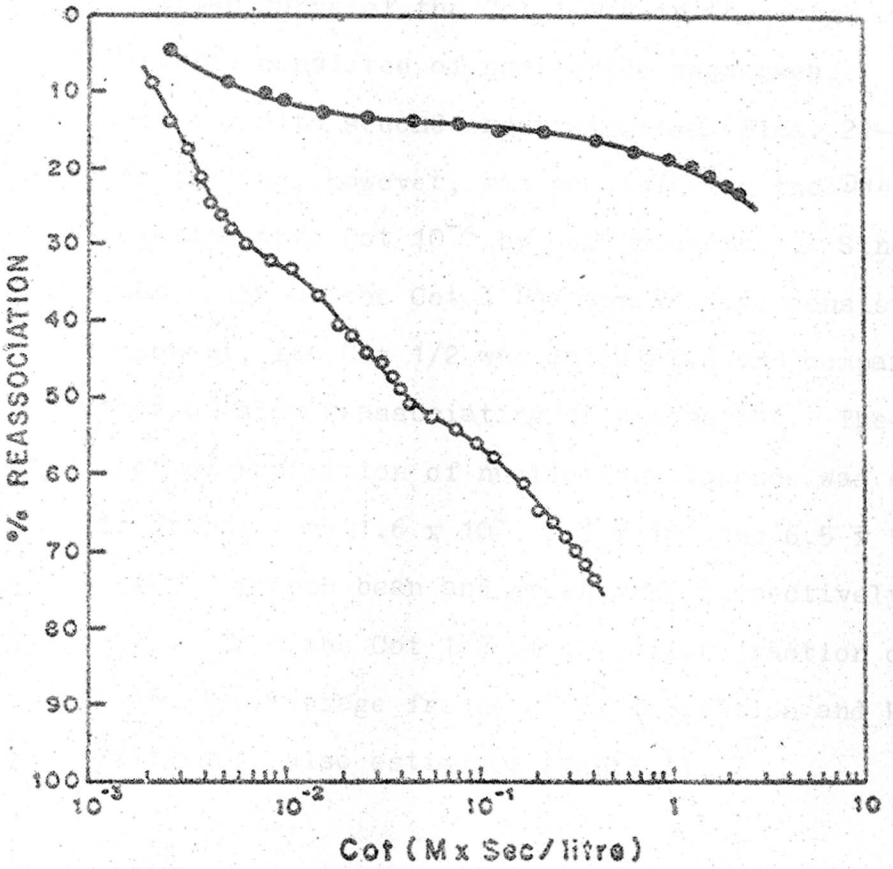

FIG. 20

Optical reassociation curves of total sonicated DNA (●——●) and Cot 1 fraction (o——o) of green gram.

of the total Cot 1 DNA. In order to determine the number of components reannealing with second order kinetics, the reassociation curve of the Cot 1 DNA in each species was analyzed by the curve fitting procedure of Laird and McCarthy (1969). It was observed that the portion of the reassociation curve of the Cot 1 DNA in the range of 10^{-2} to 1 actually consisted of nucleotide sequences reassociating with second order kinetics (Figs. 21-23). No curve fitting, however, was possible for the DNA reassociating upto Cot 10^{-2} by our procedures. Since nearly 64 - 75% of the Cot 1 DNA appeared to consist of one component, its Cot 1/2 was calculated and compared with that of slow reassociating or unique DNA. The frequency of repetition of nucleotide sequence was estimated in this fraction as 1.6×10^4, 2.3×10^3 and 6.5×10^3 in dew gram, French bean and green gram respectively (Table 4). From the Cot 1/2 of the minor fraction of the Cot 1 DNA, the average frequency of repetition and kinetic complexity were also estimated (Table 4).

DISCUSSION

Among plants, satellite DNAs have been identified and characterized in a number of plant species (Bendich and Anderson, 1974; Chilton, 1975; Timmis et al., 1975; Beridze and Bragvadze, 1976; Sinclair et al., 1975; Caspesius, 1976; Bendich and Taylor, 1977; Timmis and Ingle, 1977; Ranjekar et al., 1978a, 1978c). These

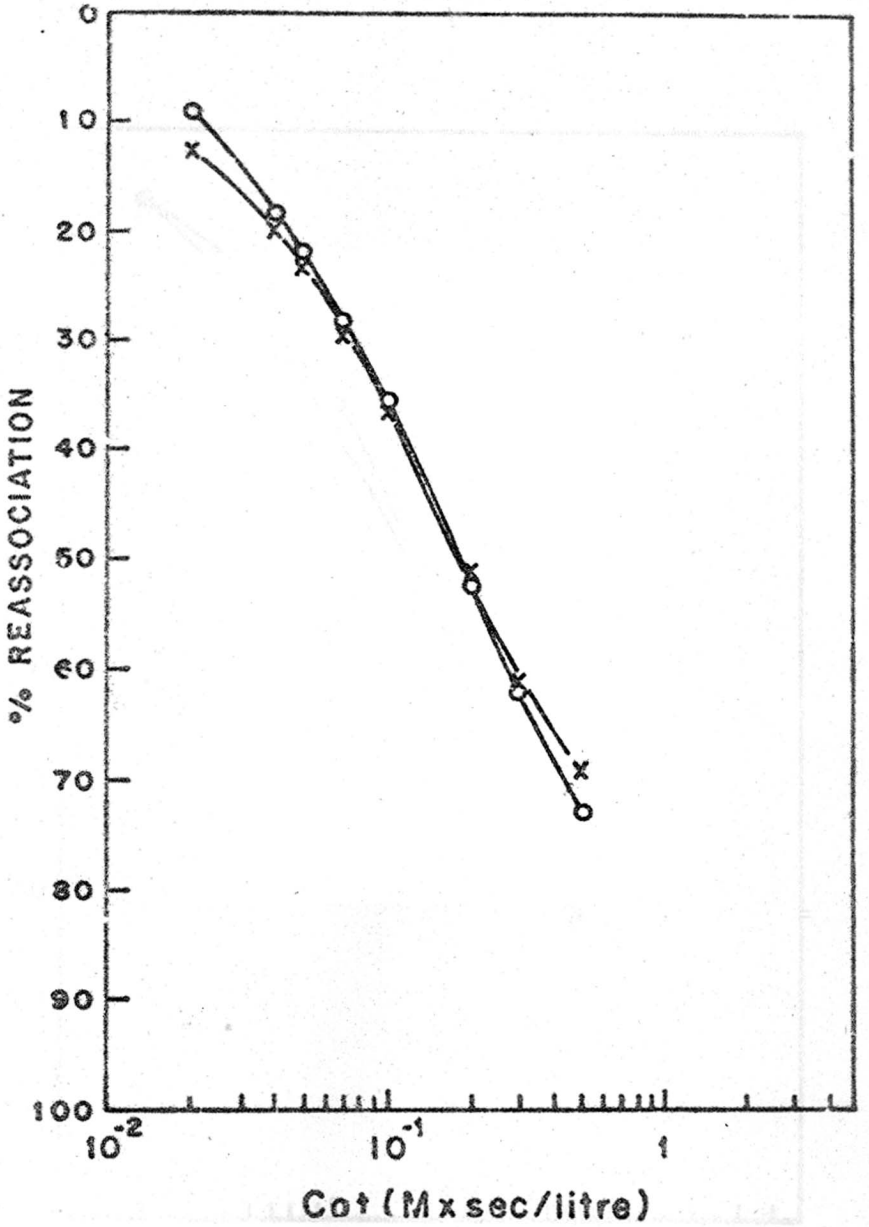

Fig. 21

Ideal Cot curve (x——x) and the experimental Cot curve (o——o) of the major component of the Cot 1 DNA of dew gram. The ideal curve is obtained using the equation:

$$\frac{C}{Co} = \frac{1}{1 + K_2 \, Cot}$$

where $\frac{C}{Co}$ = fraction of denatured DNA.

K_2 = reciprocal of Cot 1/2 of the major component of Cot 1 DNA fraction.

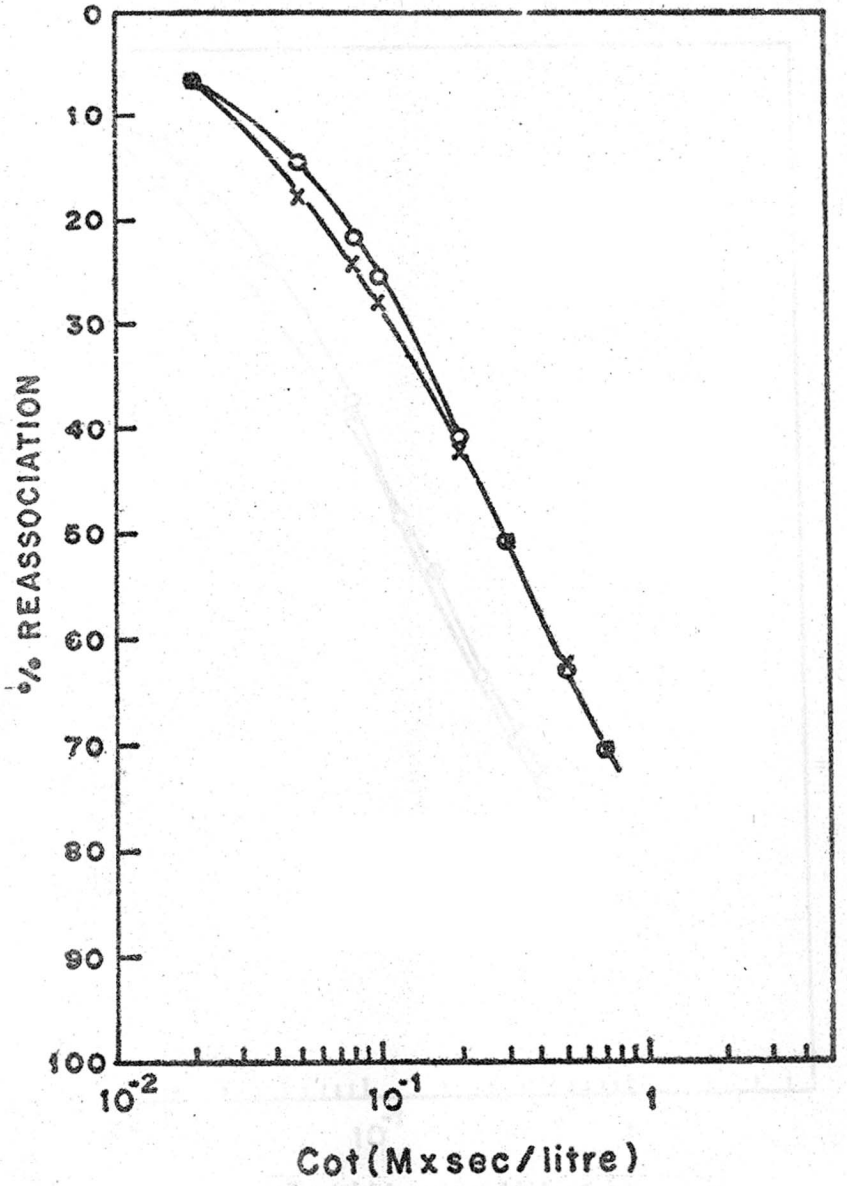

FIG. 22

Ideal Cot curve (x---x) and the experimental Cot curve (o---o) of the major component of the Cot 1 DNA of French bean DNA. The ideal Cot curve was obtained as described in the legend of Fig. 21.

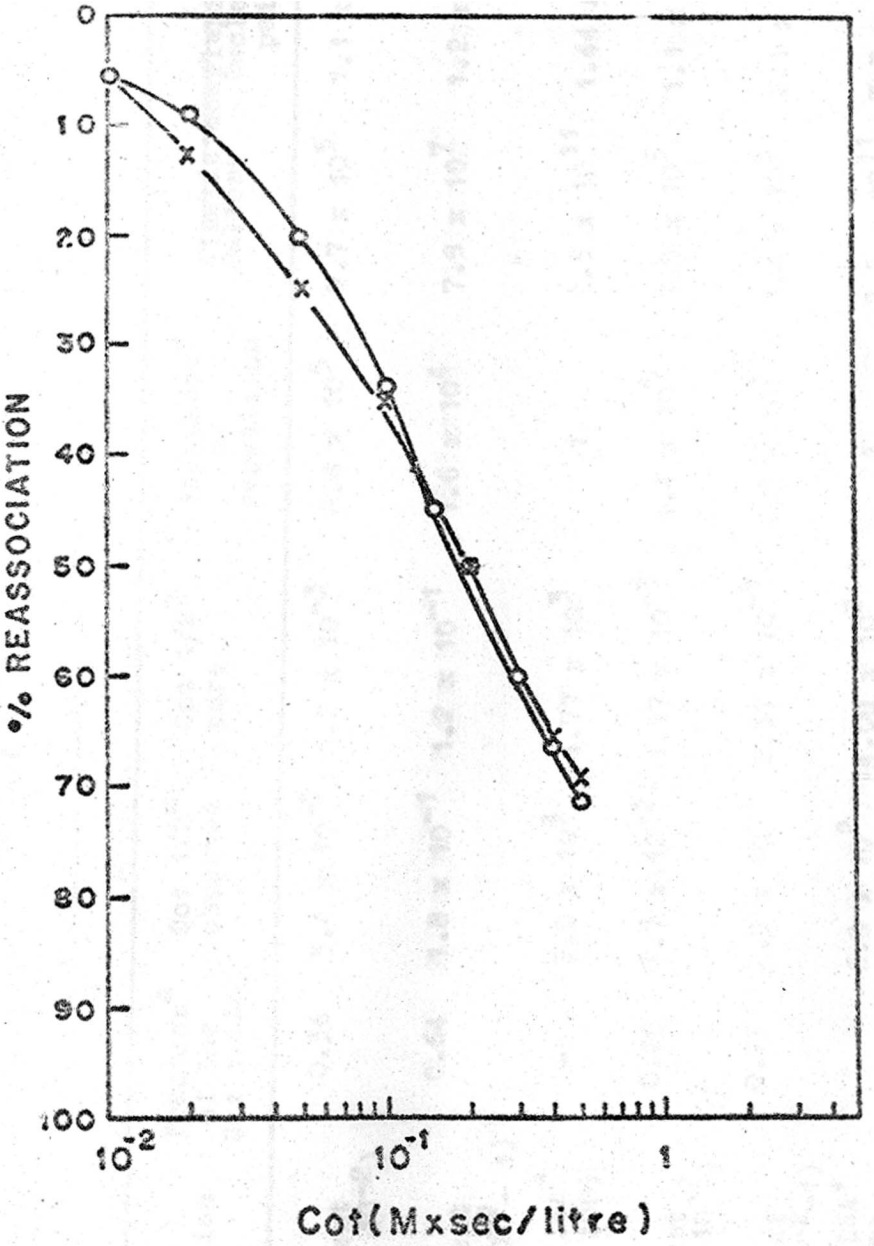

FIG. 23

Ideal Cot curve (x---x) and the experimental Cot curve (o——o) of the major component of the Cot 1 DNA of green gram DNA. The ideal Cot curve was obtained as described in the legend of Fig. 21.

TABLE 4

Kinetic complexity of Cot 1 DNA in Phaseolus

Species	DNA fraction	Fraction[a] of the Cot 1 DNA	Cot 1/2[a] observed	Cot 1/2[b] pure	Frequency[c] of repetition	Kinetic complexity[d] Daltons	Nucleotide pairs
Dew gram	Minor component (Cot $< 10^{-2}$)	0.36	3.4×10^{-3}	1.2×10^{-3}	8.8×10^{5}	7.7×10^{5}	1.1×10^{3}
	Major component (Cot 10^{-2} - 1)	0.64	1.8×10^{-1}	1.2×10^{-1}	1.6×10^{4}	7.8×10^{7}	1.2×10^{5}
	Unique DNA* (Cot $>$ 100)	-	3.0×10^{3}	1.77×10^{3}	1	9.5×10^{11}	1.44×10^{9}
French bean	Minor component (Cot $< 10^{-2}$)	0.25	4.7×10^{-3}	1.17×10^{-3}	1.4×10^{5}	7.5×10^{5}	1.1×10^{3}
	Major component (Cot 10^{-2}-1)	0.75	2.9×10^{-1}	2.37×10^{-1}	2.3×10^{3}	1.4×10^{8}	2.1×10^{5}
	Unique DNA* (Cot $>$ 50)	-	6.8×10^{2}	4.08×10^{2}	1	2.2×10^{11}	3.3×10^{8}

Contd...

Species	DNA fraction	Fraction[a] of the Cot 1 DNA	Cot 1/2[a] observed	Cot 1/2[b] pure	Frequency[c] of repetition	Kinetic complexity	
						Daltons	Nucleotide pairs
Green gram	Minor component (Cot < 7×10^{-3})	0.30	2.9×10^{3}	8.7×10^{-4}	4.5×10^{5}	5.6×10^{5}	8.4×10^{2}
	Major component (Cot 7×10^{-3} -1)	0.70	2.0×10^{-1}	1.4×10^{-1}	6.5×10^{3}	9.1×10^{7}	1.4×10^{5}
	Unique DNA* (Cot > 100)	-	1.3×10^{3}	6.89×10^{2}	1	3.7×10^{11}	0.56×10^{9}
T_4 DNA		1.0	0.17	0.17	-	1.1×10^{8}	1.6×10^{5}

[a] Obtained from Figs. 2 18-20.

[b] Cot 1/2 (pure) = Cot 1/2 (observed) x fraction of the Cot 1 DNA

[c] Frequency of repetition = Cot 1/2 of unique DNA/Cot 1/2 of minor or major component

[d] Calculated by the comparison of the rate of reassociation of each fraction of Phaseolus DNA to that of T_4 DNA.

*The kinetic data of unique DNA is as in Table 2 (Chapter III) and is given in this Table for comparison.

The values represented in Table 3 are average values from atleast 5 different experiments.

satellite DNAs have been shown to be heterogenous consisting of at least two components differing from each other in their thermal denaturation properties and kinetic complexity. Comparatively less is known in plants about the properties of repetitive DNA fractions. The thermal denaturation data of the repetitive DNA fraction in all the Phaseolus species except black gram have revealed that this fraction consists of repeated DNA sequences with an average base mismatch of 3 - 5%. In general it is known that the degree of base mismatch in a given DNA fraction gives an indication of the extent of sequence divergence. In other words, the DNA sequence divergence will be more if there is a substantial base mismatch in the DNA duplexes. In plants only a limited data is reported on the thermal stability of repetitive DNA duplexes. In rye, wheat, barley and tobacco (Ranjekar et al., 1974, 1976; Smith and Flavell, 1975; Zimmerman and Goldberg, 1977) for example upto 15% base mismatch is observed in repetitive duplexes indicating a considerable degree of sequence divergence. The repetitive DNA fraction in Phaseolus is rather unique in consisting of nucleotide sequences with rather low percent base mismatch and, therefore, less sequence divergence.

The optical reassociation data have enabled us to get some information about kinetic heterogeneity of DNA sequences reannealing by Cot 1. The lowest Cot value at which the percentage reassociation is measured during

optical reassociation studies is of the order of 10^{-3}. At this Cot approximately 9% of the Cot 1 DNA forms duplexes. We have determined the collapse hypochromicity as 3% caused by increased secondary structure when denatured DNA is cooled from 98°C to 62°C. The collapse hypochromicity was also calculated using the formula of Bendich and Anderson (1977):

$$\% \text{ collapse} = a + (t_{25} - ti)\ (0.0078\ GC + b)$$

where $a = 12.6$ for 0.14 M PB

$t_{25} = tm - 25°C$

ti = incubation temperature

GC = percentage GC value obtained from Tm

$b = 0.31$ for 0.14 M PB

From the above calculation we get % collapse hypochromicity as 11%. The hyperchromicity of total Phaseolus DNA is about 28%. So the collapse hypochromicity is (28% x 11%)/100 = 3.1%. This value of 3.1% is similar to the experimental value of 3%. If we substract the collapse hypochromicity value from the percent reassociation of 9% at Cot 10^{-3} the remaining 5% would most probably represent mainly the highly repetitive DNA sequences. The curve fitting procedures have revealed that approximately 70% of the Cot 1 DNA fraction reannealing in the Cot range of 10^{-2} to 1.0 follows a second order kinetics indicating the presence of one discrete frequency class of DNA sequences. The frequency of repetition of this

DNA fraction is in the range of $2.3 \times 10^3 - 1.6 \times 10^4$. Relatively discrete frequency components which can be physically isolated and shown to reassociate essentially by second order kinetics do exist in several (but not all) animal genomes (Britten and Davidson, 1971; Hough and Davidson, 1972). Of the higher plant genomes so far analyzed in this way only tobacco has been shown to contain a large fraction of repetitive DNA clearly belonging to three discrete frequency class. In Phaseolus approximately 64 - 75% of the Cot 1 DNA probably consists of one discrete frequency class of DNA sequences and 25 - 36% of the Cot 1 DNA is highly heterogenous revealing the presence of several discrete DNA families.

CHAPTER V

DNA SEQUENCE ORGANIZATION IN FRENCH BEAN AND GREEN GRAM

DNA SEQUENCE ORGANIZATION IN FRENCH BEAN AND GREEN GRAM

INTRODUCTION

One of the approaches to understand the functions of repeated DNA sequences is to investigate their arrangement in the chromosomes with respect to the nonrepeated or unique DNA sequences. Such studies have been carried out in a number of animal species (Davidson et al., 1973; Graham et al., 1974; Angerer et al., 1975; Goldberg et al., 1975; Manning et al., 1975; Schmid et al., 1975; Crain et al., 1976a, 1976b; Efstratiadis et al., 1976; Ginelli and Corneo, 1976; Wells et al., 1976; Wu et al., 1977; Bozzoni and Beccari, 1978) and in only a few plant species (Flavell and Smith, 1976; Walbot and Dure, 1976; Smith and Flavell, 1977; Zimmerman and Goldberg, 1977; Goldberg, 1978; Kiper and Herzfeld, 1978; Murray et al., 1978; Gurley et al., 1979; Wimpee and Rawson, 1979) and have revealed the occurrence of two main patterns of DNA sequence interspersion namely short range and long range. At this stage, however, it will be premature to say that short range and long range interspersions are the only two patterns of DNA sequence arrangement. In fact, recently a pattern intermediate between the above two patterns of DNA sequence interspersion has been described (Arthur and Straus, 1978). It is, therefore, necessary to scan the interspersion patterns of a large number of more eukaryotic species. In this Chapter we describe the DNA sequence organization of French bean and green gram.

MATERIALS AND METHODS

DNA isolation:

DNA was isolated as described in Materials and Methods of Chapter II.

DNA fragmentation and sizing:

DNA fragments of an average length of 550 np were obtained by sonication using Biosonic III as described in Chapter III. DNA was fragmented to an average length of 1100 np by sonication using Biosonic III equipped with 1/4 inch probe for one min at 20 setting. Larger DNA fragment (3200 np) were obtained by homogenising the DNA solution in Sorvall Omnimixer (Model No. 17106) for 6 min. at 25,000 rpm.

DNAs of different fragment lengths were sized in neutral sucrose gradients as described in Chapter III. The estimated molecular weights of the DNA fragments were confirmed by carrying out agarose gel electrophoresis using EcoRI and Hind III digest of λDNA as molecular weight markers.

Agarose gel electrophoresis:

These experiments were carried out as described by Thomas and Davis (1975) with slight modifications.

Agarose solution was prepared by dissolving agarose (1.4% w/v) in Tris/Borate buffer (0.089 M Tris, 0.089 M boric acid, pH 8.5 and 2.5 mM Na_2 EDTA) and heated in a

waterbath at 90°C. The hot agarose solution (60 - 80°C) was poured into glass tubes (15 cm x 0.6 cm internal diameter) at room temperature. The gels were allowed to set and were stored in cold (4°C) till further use.

Digestion of DNA with EcoRI was also according to Thomas and Davis (1975). Partial cleavage reactions of λ DNA were carried out by incubating the endonuclease EcoRI with the DNA in 0.1 M Tris (pH 7.5) and 10^{-4} M EDTA at 0°C for 1 min. The partial cleavage reaction mixture was then warmed to 25°C and the reaction initiated by the addition of 0.1 vol 0.1 M $MgSO_4$. The reaction was conducted at 37°C for one hour and was stopped by increasing the EDTA concentration to 0.02 M.

This λ DNA digest was then applied to agarose gels and the fragments were separated by carrying out electrophoresis at 50 v for 5 hours. After completion of electrophoresis the gels were stained overnight with tolueidine blue (4 mg/ml) and then destained in distilled water till DNA bands were visible. These gels were scanned in Gilford 250 spectrophotometer at 546 nm. We also used Hind III digest of λ DNA (Boehringer Mannheim Gm bH) as a molecular weight marker. Prior to electrophoresis of DNA samples, sucrose was added to a final concentration of 10%.

DNA reassociation kinetics:

All the details such as preparation of hydroxyapatite columns, DNA denaturation and reassociation, separation of

single stranded and double stranded DNA and estimation of percent reassociation at a given Cot value are as reported in Chapter III.

Thermal denaturation and optical reassociation:

These experiments were carried out in Gilford Spectrophotometer 250 equipped with thermoprogrammer, analog multiplexer and reference compensator using DNA solutions (25 to 50 ug/ml) in 0.12 M PB, pH 6.8 as described in Chapters II and IV.

Single strand collapse was determined by melting nonrepetitive Phaseolus DNA in 0.12 M PB (pH 6.8). This value was observed to be 3% for the two Phaseolus DNAs.

S_1 nuclease digestion:

Native DNAs of fragment size of 5500 np in French bean and 5750 np in green gram were dialyzed against 0.18 M NaCl plus 0.006 M PIPES buffer, pH 6.8. The DNA samples were denatured in boiling waterbath for 10 min and allowed to reassociate to a specific Cot value. After reassociation to specific Cots, the DNA samples were adjusted to 25 mM Sodium acetate (pH 4.5), 0.1 mM $ZnSO_4$, 25 mM 2-mercaptoethanol (Goldberg et al., 1975; Britten et al., 1976). 10 units of S_1 nuclease (Boehringer) per µg of DNA were also added and the samples incubated for one hour at 37°C. The reaction was terminated by adjusting the samples to 0.12 M PB (pH 6.8) and cooling the samples to 4°C. S_1 nuclease resistant

duplexes were then separated by hydroxyapatite column chromatography. These samples were dialysed against 0.12 M PB and their Tm and hyperchromicity values were determined.

RESULTS

Reassociation kinetics of DNAs of different fragment lengths:

From Chapter III, we know that the approximate proportion of repetitive DNA is 40% in French bean and 47% in green gram. A reliable method to determine the presence of interspersion of repeated and nonrepeated DNA sequences in a given genome is to compare the extent of binding to hydroxyapatite of DNAs of long fragments of known lengths under identical Cot conditions (Davidson et al., 1973; Graham et al., 1974; Goldberg et al., 1975). An increase in binding to hydroxyapatite as a function of DNA fragment lengths is considered to be an indication of the occurrence of interspersion of repetitive and nonrepetitive DNA sequences (Davidson et al., 1973; Graham et al., 1974). We have studied the reassociation kinetics of the DNAs of French bean and green gram of four different fragment lengths in the range of 550 np to 5750 np (Figs. 24,25). Unlabelled DNAs were used throughout the studies and the reassociation experiments were carried out in the Cot range of 10^{-1} to 10^{2}. In this Cot range, almost all repetitive DNA sequences and very few single copy sequences are assumed to reassociate. From Figs. 24, 25 it is observed that in both the plant species, percent reassociation (at any Cot) increases

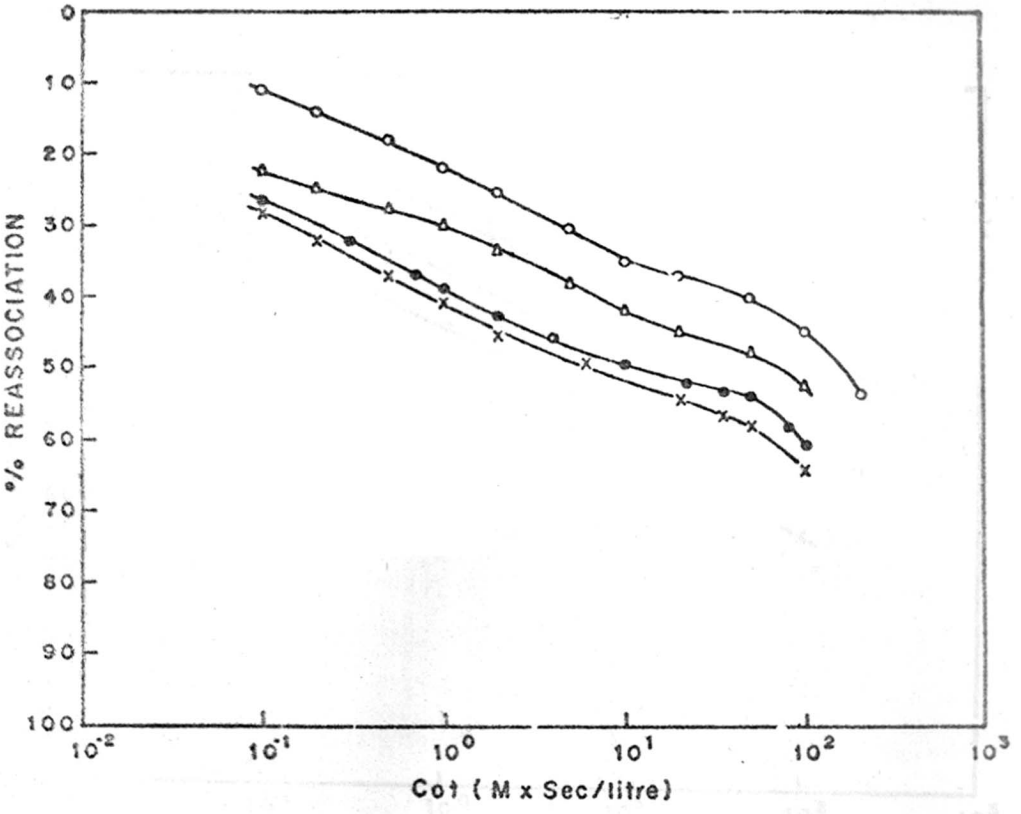

FIG. 24

Reassociation kinetics of DNAs of long fragment lengths. The DNAs of four different fragment lengths of French bean were obtained as described in Materials and Methods and their reassociation was studied in the Cot range of 10^{-1} to 10^{2}. Reassociation curves of French bean DNA (o—o) 550 np, (Δ—Δ) 1100 np, (•—•) 3100 np and (x—x) 5500 np.

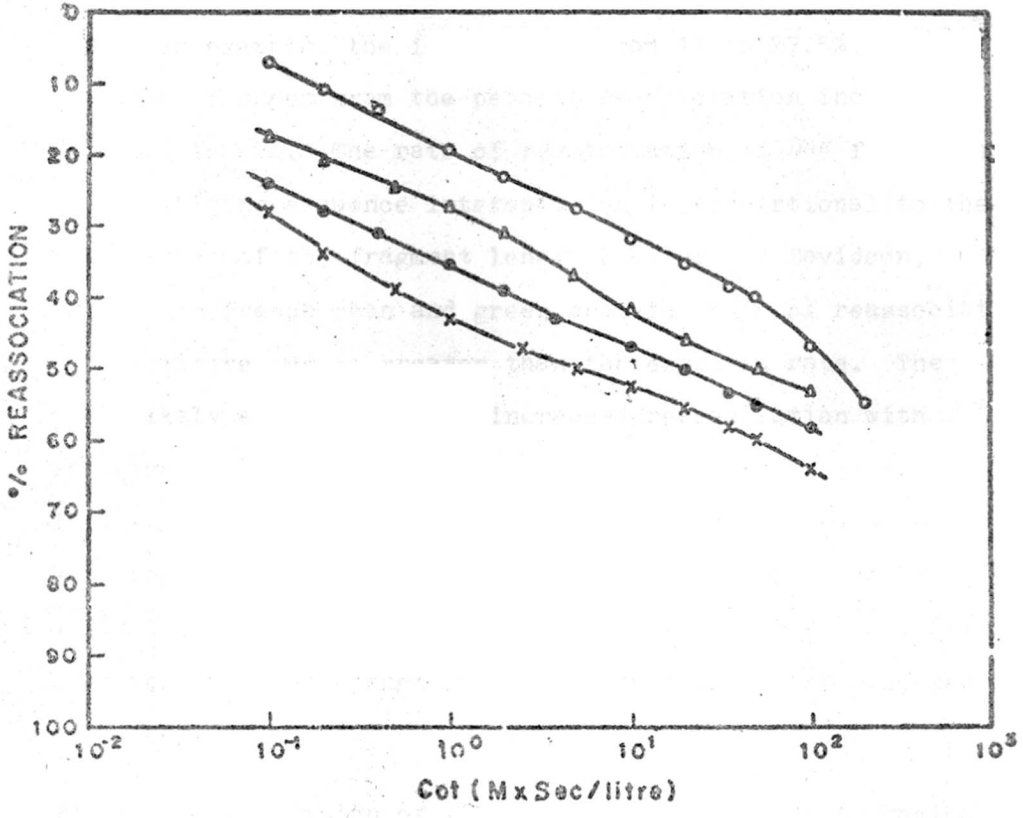

FIG. 25

Reassociation kiretics of DNAs of long fragment lengths. The DNAs of four different fragment lengths of green gram were obtained as described in Materials and Methods and their reassociation was studied in the Cot range of 10^{-1} to 10^{2}. Reassociation curves of green gram DNA (o—o) 550 np, (Δ—Δ) 1100 np, (•—•) 3100 np and (×—×) 5750 np.

with increase in fragment length of DNA. In case of French bean, the percent DNA bound to hydroxyapatite at Cot 50, increases from 40 to 58%. In green gram, the percent increase at Cot 100 is from 47 to 63%. There is also a substantial increase in reassociation at Cot 0.1. In French bean, for example, the increase is from 11 to 27.5% and in the case of green gram the percent reassociation increases from 7.5 to 27%. The rate of reassociation of DNA fragments not exhibiting sequence interspersion is proportional to the square-root of the fragment length (Wetmur and Davidson, 1968). In French bean and green gram the rate of reassociation of repetitive DNA is greater than the expected rate. The most likely explanation for increased reassociation with increase in DNA fragment size is due to the presence of single copy DNA sequences in an unreassociated form along with repetitive duplexes. Thus it appears that about 58% of the total genome of French bean (length 5500 np) and 63% of green gram DNA (length 5750 np) consists of interspersed sequences.

Optical reassociation of DNAs of different fragment lengths:

Optical reassociation of DNA is supposed to be a true measure of the extent of DNA duplex formation. The DNA fragment size is not expected to influence the extent of reassociation as is the case of measurement of reassociation by hydroxyapatite. We, therefore, carried out the optical reassociation studies of total DNAs of different fragment

lengths upto a Cot value of 1 (Table 5). The percent reassociation thus obtained are very similar for all the DNA preparations with different fragment size. In French bean, the optical reassociation at Cot 1 is of the order of 25.5% in the case of DNA with a fragment size of 5500 np. This value is similar to that obtained with the DNA of 550 np. It is therefore, clear that the increase in percent reassociation with increase in fragment size as noticed in the hydroxyapatite measurements is mainly due to the presence of single strand tails in repetitive duplexes.

Hyperchromicity and S_1 nuclease studies of reassociated repetitive DNA fragments:

Since hyperchromicity of a given DNA fraction is nearly proportional to its duplex content (Davidson et al., 1973), the melting behaviour of repetitive DNA fractions of different fragment lengths were studied to estimate the fraction of nucleotides paired. French bean and green gram DNAs of different fragment size were allowed to reassociate till Cot 50 and Cot 100 respectively. The double stranded fragments were separated on hydroxyapatite and optical melting behaviour of these different size reassociated fragments were studied. As observed from Figs. 26,27 the hyperchromicity of reassociated duplexes decreases with increasing fragment length whereas the melting temperature increases slightly (2 to 4°C) with fragment length. The decrease in hyperchromicity with increase in fragment size

TABLE 5

Percent reassociation measured by hydroxyapatite and optical methods

DNA fragment length (np)	Hydroxyapatite reassociation Cot 1 (%)	Optical reassociation Cot 1 (%)
French bean		
550	22.7	25.4
1100	30.2	25.5
3100	38.9	-
5500	41.1	26.6
Green gram		
550	19.0	22.5
1100	27.1	22.4
3200	34.7	-
5750	41.2	24.1

The values obtained above are from a minimum of four experimental replicates.

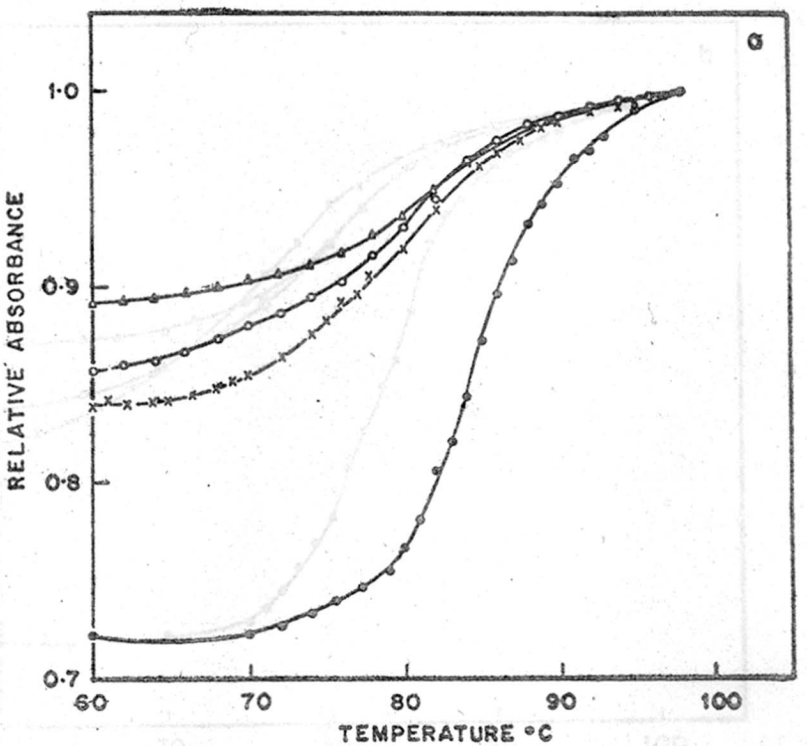

FIG. 26

Thermal denaturation profiles of French bean Cot 50 DNAs obtained from DNAs of various fragment lengths. The melting profile of total DNA was also studied for comparison. The graph of relative absorbance (A_{260} (T_1)/A_{260} (98°C)) versus temperature was plotted where $A_{260}(T_1)$ is the absorbance at the corresponding temperature. (×——×) 550 np, (○——○) 1100 np, (△——△) 5500 np and (●——●) total DNA.

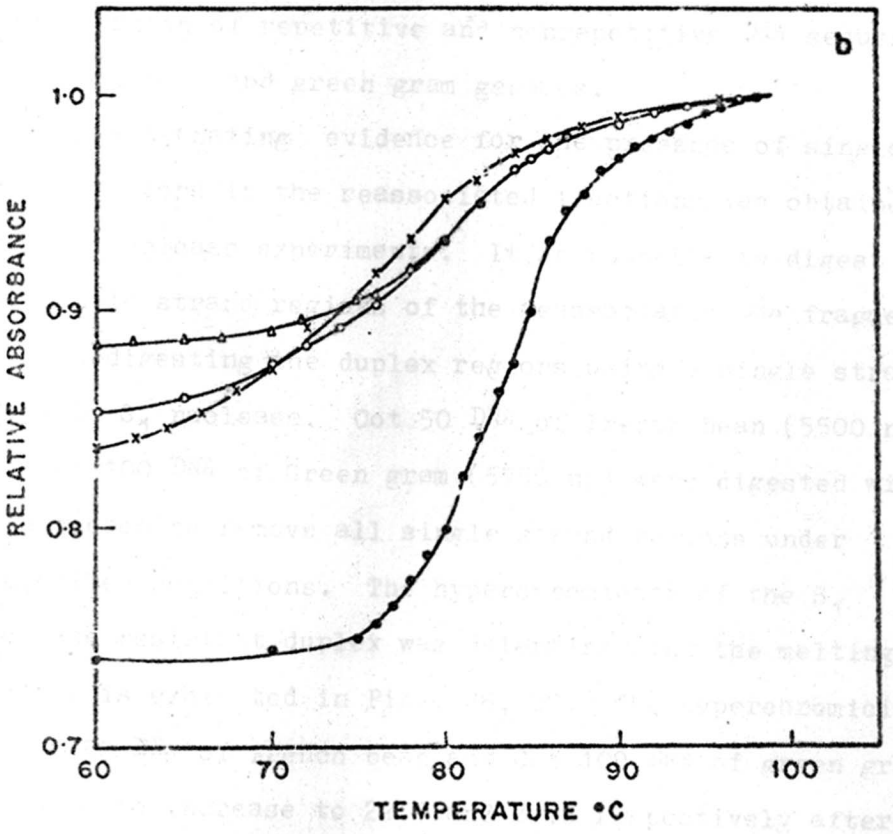

FIG. 27

Thermal denaturation profiles of green gram Cot 50 DNAs obtained from DNAs of various fragment lengths. The melting profile of total DNA was also studied for comparison. (×—×) 550 np, (○—○) 1100 np, (△—△) 5750 np and (●—●) total DNA.

can be attributed to the increasing presence of single strand regions in the reassociated fragments. These single strand regions are mainly unique sequences and they contribute very little (about 3%) to DNA hyperchromicity. This is thus an additional evidence to the existence of interspersion of repetitive and nonrepetitive DNA sequences in French bean and green gram genomes.

An alternating evidence for the presence of single strand regions in the reassociated fractions was obtained from S_1 nuclease experiments. It is possible to digest the single strand regions of the reassociated DNA fragments without digesting the duplex regions using a single strand specific S_1 nuclease. Cot 50 DNA of French bean (5500 np) and Cot 100 DNA of Green gram (5750 np) were digested with S_1 nuclease to remove all single strand regions under controlled conditions. The hyperchromicity of the S_1 nuclease resistant duplex was determined and the melting profile is exhibited in Figs. 28, 29. The hyperchromicity of Cot 50 DNA of French bean and Cot 100 DNA of green gram is found to increase to 22.7 and 21.8 respectively after S_1 nuclease treatment. The sonicated French bean DNA has an average Tm of 83.2°C and a hyperchromicity of 24.3%. The S_1 nuclease resistant Cot 50 DNA fraction has a Tm of 79°C and thus has a base mismatch of 4%. Due to the base mismatch, the hyperchromicity of this DNA fraction is expected to be (24.3 x .96) 23.5% (Davidson et al., 1973). The latter estimated value compares well with the experimental value

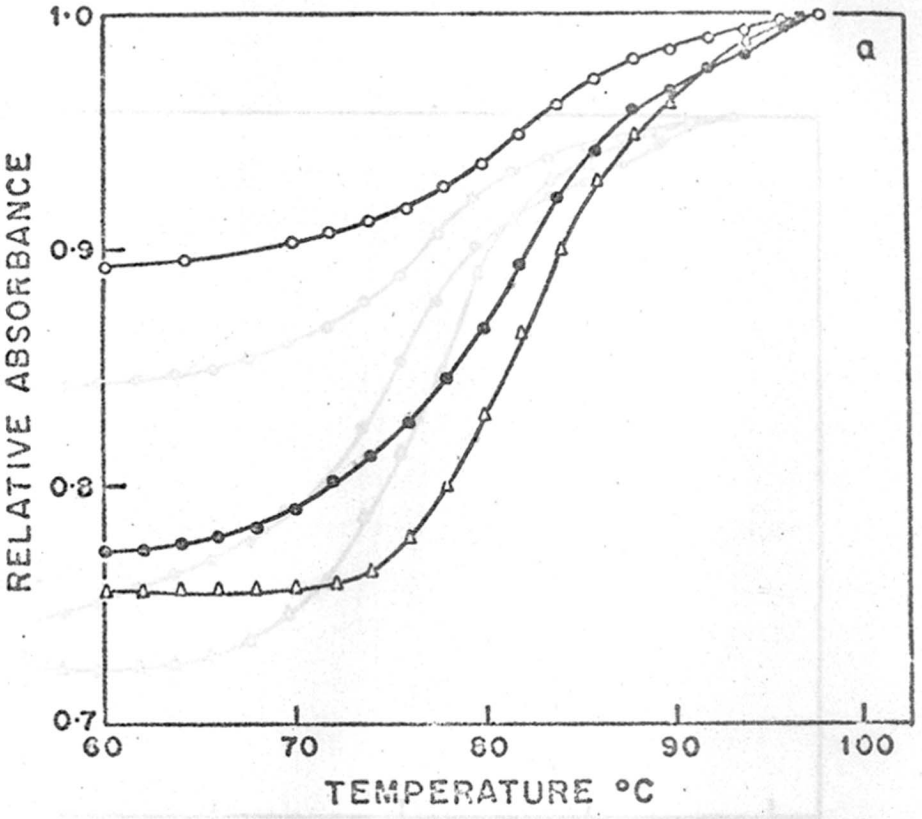

FIG. 28

Thermal denaturation curves of French bean Cot 50 DNA before and after S_1 nuclease treatment along with that of native sonicated DNA.
(o—o) before S_1 nuclease treatment,
(●—●) after S_1 nuclease treatment.and
(△—△) native sonicated DNA (550 np).

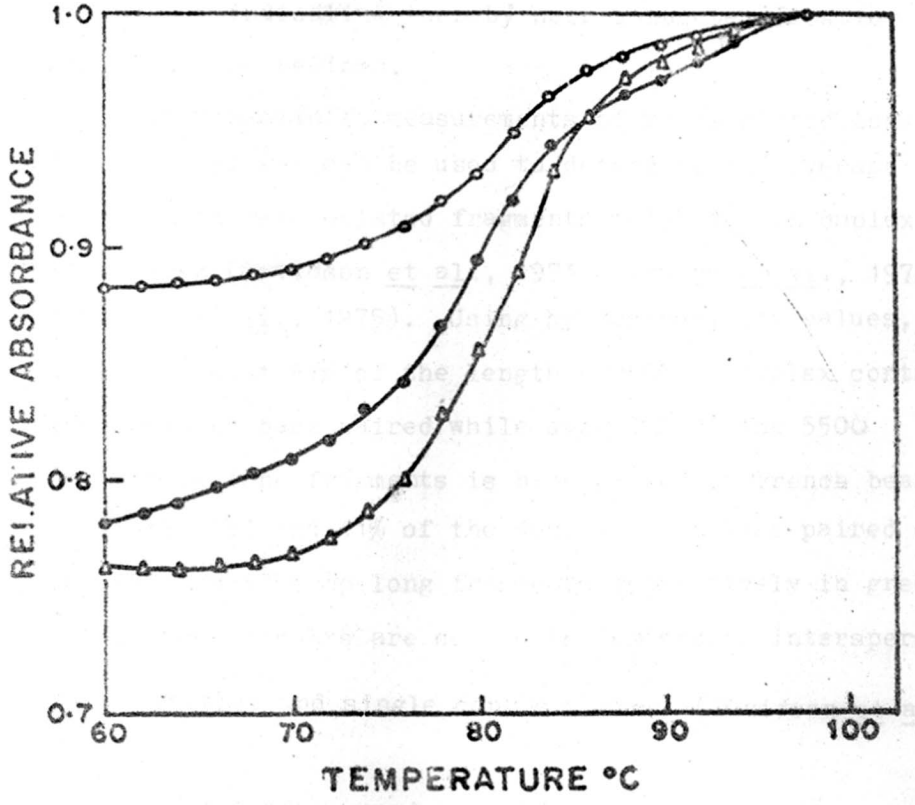

FIG. 29

Thermal denaturation curves of green gram Cot 50 DNA before and after S_1 nuclease treatment along with that of native sonicated DNA.

(o—o) before S_1 nuclease treatment,

(•—•) after S_1 nuclease treatment and

(Δ—Δ) native sonicated DNA (550 np).

of 22.7%. Similarly in the case of green gram, there is a close agreement between the expected (22.6%) and the experimental (21.8%) hyperchromicity values. The hyperchromicity of the S_1 resistant duplexes is 94% of that of the total sonicated DNA in each French bean and green gram indicating thereby near complete digestion of single strand regions.

Hyperchromicity measurements of reassociated long fragments of DNA can be used to determine the average fraction of reassociated fragments which are in duplex structures (Davidson et al., 1973; Graham et al., 1974; Goldberg et al., 1975). Using hyperchromicity values, we calculate that 61% of the length of 550 np duplex containing fragments is base paired while only 35% of the 5500 nucleotide long fragments is base paired in French bean. Similarly 64% and 41% of the duplexes are base paired of the 550 and 5750 np long fragments respectively in green gram. Such results are due to the extensive interspersion of repetitive and single copy sequences (Davidson et al., 1973).

S_1 nuclease experiments can also be used to provide an independent estimate of the fraction of nucleotides in hydroxyapatite bindable fraction which are in duplex form. Approximately 36% of 5500 np long hydroxyapatite bindable fraction at Cot 50 in the case of French bean is S_1 nuclease resistant and hence in duplex form. This estimate compares reasonably well with the estimated value of 35%

obtained from hyperchromicity measurements. Likewise in green gram 35% of 5750 np long hydroxyapatite bindable fraction at Cot 100 are in duplex structures. This is in agreement to some extent to the hyperchromicity measurements (41%) at this length.

The hyperchromicity and S_1 nuclease experiments also provide us an estimate of the actual fraction of single copy DNA in French bean and green gram. Since we assume that all the repetitive DNA sequences reassociate by Cot 50 in French bean and Cot 100 in green gram, the fraction of hydroxyapatite bindable 5500 np & 5750 np fragments which are in duplex form most probably represent the percent of repetitive sequences. The proportion of single copy DNA will, therefore, be 64-65% in French bean and 59-65% in green gram. These values are in close agreement to those obtained from the reassociation kinetics experiments of 550 np long DNA.

The average size of the repetitive DNA sequences which reassociate within 5500 np fragment in French bean and 5750 np fragment in green gram can be computed from the data in Table 6. Since 35-36% of hydroxyapatite 5500 np long fragments are in duplex in French bean, the size of repetitive sequences will be in the range of 1925 to 1930 nucleotides. Similarly in green gram, the length of repetitive sequences is estimated to be in the range of 2012 to 2357 nucleotides. It is, however, possible that all the interspersed repetitive DNA stretches may not be

TABLE 6

Hyperchromicity versus length of DNA containing reassociated repetitive duplexes

Species					
French bean	1. Fragment length (np)	550	1100	3100	5500
	2. Fraction bound to HA at Cot 50[a]	0.4	0.48	0.54	0.58
	3. Hyperchromicity[b]	0.162	0.143	0.121	0.108
	4. Tm °C[c]	79.0	80.0	81.1	81.7
	5. Duplex content (D)[d]	0.61	0.52	0.41	0.35
	6. Average duplex length[e]	335	572	1271	1925
	7. From Duplex content from S_1 nuclease[f]	-	-	-	0.36
Green gram	1. Fragment length (np)	550	1100	3200	5750
	2. Fraction bound to HA at Cot 100[a]	0.47	0.53	0.58	0.63
	3. Hyperchromicity[b]	0.163	0.148	0.127	0.116
	4. Tm °C[c]	76.8	79.0	80.2	80.5
	5. Duplex content[d]	0.64	0.57	0.46	0.41
	6. Average duplex length[e]	352	627	1472	2357
	7. Duplex content from S_1 nuclease[f]	-	-	-	0.35

Contd..

[a]Obtained from Figs. 24 and 25.

[b]Obtained from Figs. 26, 27. Hyperchromicity (H) was calculated using the formula:

$$H = \frac{A_{260}(98°C) - A_{260}(60°C)}{A_{260}(98°C)}$$

[c]Obtained from Figs. 26, 27.

[d]The average duplex content (D) of bound fragments was estimated using the formula:

$$D = \frac{H - \text{single strand collapse}}{H_{(\text{native sonicated DNA})} - \text{single strand collapse}}$$

The hyperchromicity of the denatured single copy DNA was determined in order to obtain a value for single strand collapse and was of the order of 3%.

[e]The average length of duplex region is the product of the duplex content (D) and the fragment length (np) of DNA.

[f]Duplex content of S_1 nuclease treated repetitive DNA fragments was obtained as the fraction of duplex DNA after S_1 in case of French bean and Cot 100 DNA in case of green gram. All the experimental details are described in Materials and Methods.

The values obtained above are an average of atleast four different experimental values.

of the above estimated lengths. A direct estimate of the size distribution of repetitive DNA sequences was obtained by agarose gel electrophoresis. Long fragments of French bean (5500np) and green gram (5750 np) were reassociated to Cot 50 and Cot 100 respectively and were digested with S_1 nuclease under controlled conditions. The S_1 nuclease resistant repetitive duplexes were analysed by agarose gel electrophoresis. From Fig. 30, it is seen that only one sharp peak was observed at a fragment length of 1545 nucleotides in French bean and 1900 nucleotides in green gram.

Spacing of repetitive segments:

The hydroxyapatite and the optical reassociation data of the total DNAs of different fragment size as well as the hyperchromicity measurements and the S_1 nuclease resistance of the reassociated duplexes of different fragment size have shown that the repetitive and nonrepetitive sequences are interspersed in French bean and green gram. Furthermore, these studies have revealed the proportion and sequence length of the repetitive DNA segments. The next question which arises is about the length of single copy sequences which are present adjacent to the repetitive DNA segments. A direct estimate of the length of the interspersed single copy sequences can be obtained from a curve relating the fraction of DNA fragments binding to hydroxyapatite and the fragment length (Davidson et al., 1973; Graham et al.,

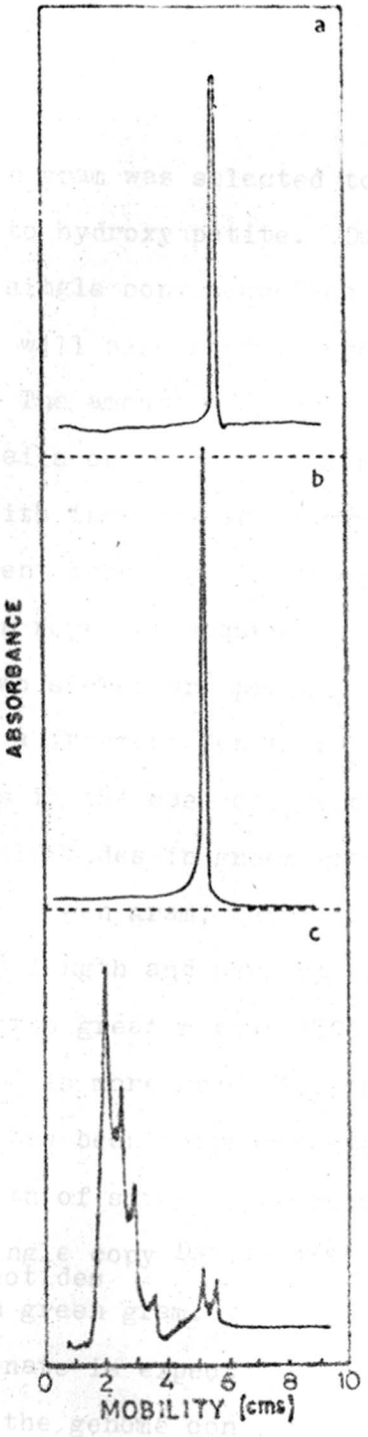

FIG. 30

Size distribution of French bean (a) and green gram (b) repetitive DNA duplexes. DNA fragments, 5500 np in length of French bean and 5750 np in green gram were reassociated to Cot 50 and Cot 100 respectively and were treated with S_1 nuclease and isolated as described in Materials and Methods. The repetitive duplexes were sized by Agarose gel electrophoresis, using Hind III digest of lambda

1974). As mentioned earlier, Cot 50 in French bean and Cot 100 in green gram was selected to measure the percent of DNA binding to hydroxyapatite. Due to interspersion of repetitive and single copy sequences a certain amount of single copy DNA will also bind to hydroxyapatite at Cot 50 or at Cot 100. The amount of single copy DNA present as single strand tails on reassociated repetitive DNA duplexes will increase with increase in fragment size. This will be true to a fragment length which is equal to the average distance between repeated sequences. From Fig. 31 it is observed that two slopes are present and the change in slope occurs at a fragment length of approximately 1200 to 1500 nucleotides in the case of French bean and between 1150 to 1400 nucleotides in green gram. Upto 1300 in French bean and 1250 in green gram, there is a linear relationship between fragment length and percent binding to hydroxyapatite. At fragment lengths greater than 1300 nucleotides, the increase in slope is more gradual. The position of the change in slope has been interpreted as indicating the approximate length of single copy sequences. Therefore, the length of single copy DNA is 1350 nucleotide in French bean and 1300/nucleotides in green gram. Extrapolation of the curve back to the ordinate is expected to provide an estimate of the fraction of the genome consisting of repetitive DNA sequences. This value is 0.325 in French bean and 0.415 in green gram and is comparable to the repetitive DNA content value of 0.35 and 0.41 respectively derived from the hyperchromicity measurement.

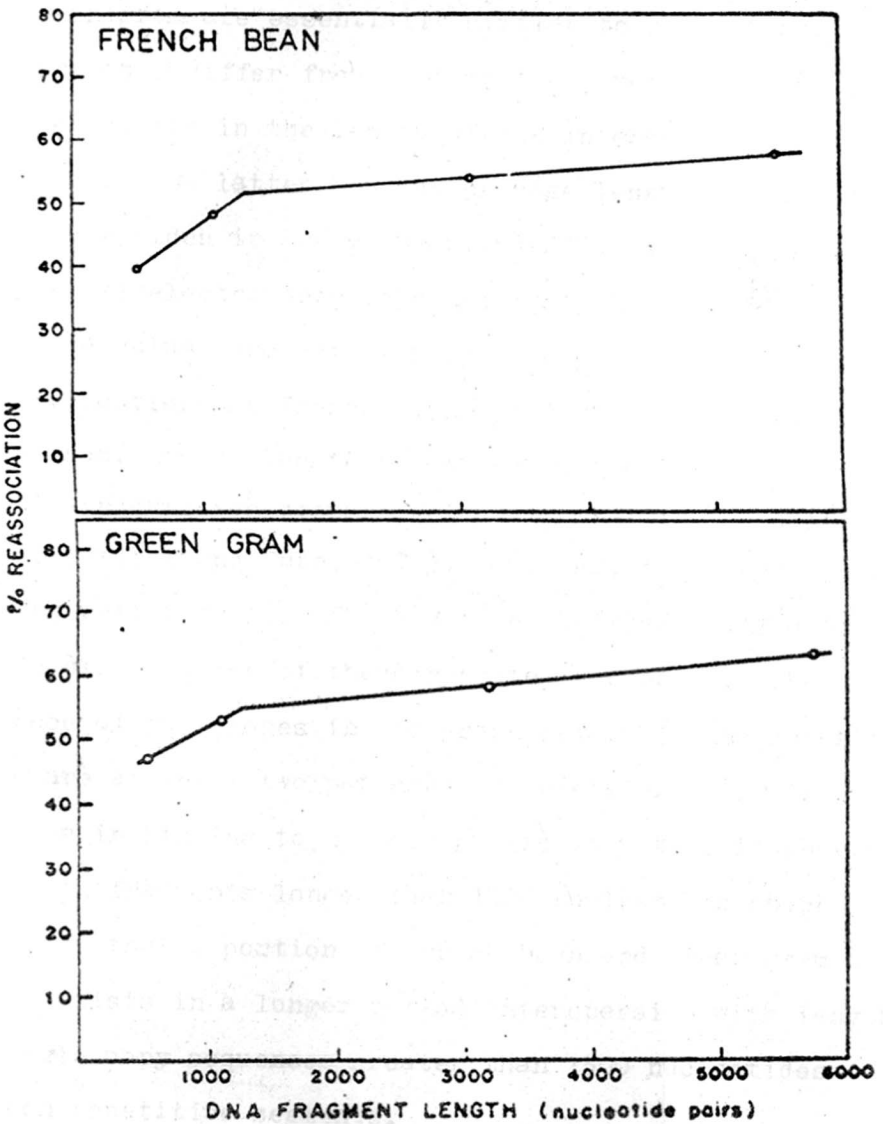

FIG. 31

The percentage binding of DNA to hydroxyapatite at Cot 50 in French bean and Cot 100 in green gram as a function of fragment length. All the experimental procedures are described in Materials and Methods.

DISCUSSION

The DNA sequence interspersion patterns in French bean and green gram are essentially similar to each other. However, they differ from most of the other plant species reported so far in the length of the interspersed repetitive stretches. The latter have an average length of the order of 1950 nucleotides in French bean and 2175 in green gram. The agarose gel electrophoresis experiments have revealed that the above values are actually lower and are of the order of 1545 nucleotides in French bean and 1900 nucleotides in green gram. These length estimates of the interspersed repetitive duplexes compare well with that in the cotton genome (Walbot and Dure, 1976). Further, the electrophoretic banding patterns (Fig. 30) indicate the absence of a broad length distribution of these repeated sequences. The presence of two slopes in the graph (Fig. 31) indicates that there are at least two patterns of interspersion. An increase in binding to hydroxyapatite at Cot 50 is observed even with fragments longer than 1300 nucleotides which indicates that a portion of French bean and green gram genome exists in a longer period interspersion with length of single copy sequences greater than 1300 nucleotides between repetitive segments.

Although our work on DNA sequence organization has thrown some light on the probable nucleotide sequence arrangement of repeated and single copy sequences in French bean and green gram, we do not know what fraction of the

total repetitive DNA are fold back sequences and how are they distributed in the total genomes. Since we have not bean able to get the radioactive DNAs of sufficient specific activity, the reassociation experiments at Cot values lower than 0.1 have not been carried out. The hydroxyapatite binding at Cot 0.1 is found to increase with increase in the DNA fragment length (Figs. 24,25). Such results are assumed to indicate that fold back and highly repetitive sequences are interspersed throughout the genomes (Davidson et al., 1973; Cech and Hearst, 1975).

Single copy DNA in French bean and green gram represents 60 to 65% of the total genome. Reassociation kinetics experiments of long DNA fragments indicate that 35 to 40% of the single copy sequences have an average length of 1300 nucleotides in French bean and 1250 nucleotides in green gram. Since most of the single copy sequences are actually the structural genes, the average length of the single copy DNA stretch is expected to be comparable to that of mRNA. We have not carried out the length determination of mRNA of either French bean or green gram. However, from the literature the average length of mRNA containing Poly(A) is shown to be 1500 nucleotides (Walbot and Dure, 1976; Gurley et al., 1979). If we assume that the size of mRNA in French bean and green gram is of the order of 1500 nucleotides, then there would appear to be a close correspondence between the size of the interspersed single copy sequences and that of mRNA.

From the limited information that is available in plants, it is of interest to note that a considerable variation in the interspersion pattern exists. For example, wheat (Flavell and Smith, 1976), rye (Smith and Flavell, 1977), soybean (Goldberg, 1978 ; Gurley et al., 1979) and tobacco (Zimmerman and Goldberg, 1977) exhibit mainly a short period interspersion pattern, typical of animal genomes with the lengths of alternating repetitive and nonrepetitive DNA sequences as 300-800 and 800-1500 nucleotides respectively. In pea genome (Murray et al., 1978), short period interspersion exists but the length of interspersed single copy sequences is unusually short (300-400 nucleotides). In the cotton genome (Walbot and Dure, 1976), two types of interspersion patterns are apparent. About 60% of this genome consists of single copy sequences averaging 1800 nucleotides interspersed with repeated sequences of 1250 nucleotides and nearly 20% of the genome contains nonrepetitive segments of 4000 nucleotides interspersed with repeated sequences of 1250 nucleotides. The pearl millet genome (Wimpee and Rawson, 1979) is conspicous by the absence of short period interspersion pattern . In this genome 50% of the DNA is composed of alternating single copy and repetitive sequences of 750-1400 and 5000 nucleotides respectively. In the remaining 50% of the genome, single copy sequences vary in length from 1400 to 8600 nucleotides and the length of repeated sequences is longer than 5000 nucleotides. It is tempting

to speculate about the basis of these variations in the DNA sequence organisation in plant genomes. The latter, as against those of animals exhibit a wide variation in their DNA content, proportion of repetitive DNA and nuclear organization. The DNA content, for example, exhibits a hundred fold variation. Likewise the proportion of the repetitive DNA varies in the range of 40 to 90% of the total genome (Flavell et al., 1974). It is, therefore, possible that the differences in the lengths of the interspersed repetitive DNAs in plant genomes are due to the significant variations observed in their content in nuclear DNA and total repetitive DNA.

CHAPTER VI

GENERAL DISCUSSION

GENERAL DISCUSSION

Although some data were available on the satellite DNA in Phaseolus (Beridze, 1972; Lima De Faria et al., 1975; Beridze and Bragvadze, 1976), the present study constitutes the first attempt to carry out a detailed comparative investigation of the DNAs of four Phaseolus plant species. Two main approaches have been used to analyze the Phaseolus genomes. In the first approach, the extent of changes in the contents and the properties of repetitive DNA sequences within four Phaseolus species are assessed. For this purpose, the reassociation kinetics of the sonicated DNAs of the four Phaseolus species are studied in detail. To enable better characterization of the rapidly reassociating sequences (Cot 1), the latter were isolated by hydroxyapatite column chromatography and their denaturation-reassociation properties were studied. In the second approach, the DNA sequence arrangement of the two Phaseolus species, namely French bean and green gram are compared.

Analysis of Tm and buoyant density data:

Prior to studying the DNA reassociation kinetics of the sonicated DNAs of the four Phaseolus species, determinations of Tm and buoyant density are carried out in each case. Remarkable similarity is noticed in these two characteristics among black gram, dew gram and green gram. French bean DNA, however, has a biphasic melting

profile with a high melting DNA component having a Tm of 93.5°C and representing 15% of the total DNA. A question arises whether this high melting DNA component corresponds to the G + C rich satellite DNA component observed in neutral CsCl gradients. Our present data cannot throw any light on such correlation. However, the work carried out by Beridze and Bragvadze (1976) on isolation and characterization of the satellite DNA in French bean shows that this DNA reveals a major high melting DNA component. It, therefore, appears that the high melting component of French bean DNA is the major part of the satellite DNA. If we assume such relationship between the satellite DNA and the high melting component, the latter may also be present in at least black gram and dew gram. The differential data of the melting curves of these DNAs (Figs 2,3) actually show the presence of minor peaks in the temperature range of 90 - 95°C. Even the green gram DNA shows the presence of a minor high melting component in its differential melting pattern. It, therefore, appears that the high melting DNA component may be present in black gram, dew gram and green gram except for the fact that its presence is not evident as in the case of French bean DNA. This may reflect about the differences in the distribution patterns of the high melting DNA components within French bean DNA and the DNAs of black gram, dew gram and green gram.

Repetitive DNA content in Phaseolus:

Reassociation kinetics studies of the sonicated DNAs of the four Phaseolus plant species have revealed the content of repetitive DNA. The fast reassociating DNA observed in the Cot curves is assumed to consist of mainly repeated DNA sequences whereas the slow reassociating DNA is shown to consist mainly of single copy DNA sequences. The estimate of repeated DNA sequences in the four Phaseolus species is in the range of 34.5 - 47% of the total DNA. The actual proportion of the repetitive DNA in these Phaseolus species may be lower since reassociation kinetics measurement by hydroxyapatite column chromatography tend to overestimate the actual fraction of the repetitive DNA. In green gram and French bean for example, the proportion of repetitive DNA is 47% and 40% respectively by hydroxyapatite column chromatography. From hyperchromicity and S_1 nuclease experiments, the proportion of repeated DNA sequences is found to be 41% in green gram and 36% in French bean.

At this point a possible relationship between nuclear DNA content and repetitive DNA can be discussed. Among angiosperms, DNA content per nucleus varies over a hundred fold range (Rees and Jones, 1972; Sparrow et al., 1972). Likewise, repetitive DNA content also exhibits a large variation in the range of 40 - 90% of the total DNA. Part of the variation in DNA content apparently results from

the differences in repetitive DNA content in plant genomes. Flavell et al.(1974), for example, in their extensive survey of 23 angiosperms ranging in DNA content from 1.7 - 98 pg have shown a mean value of repetitive DNA of 80% for species with nuclear DNA content above 5 pg and 62% for species with DNA content of less than 4 pg. They further suggest that most of the changes in DNA content during evolution are either due to addition or loss of repetitive sequences. Studies on amphibians (Baldari and Amaldi, 1976), Cichorieae (Bachmann and Price, 1977), conifers (Miksche and Hotta, 1973) and Lathyrus (Narayan and Rees, 1977) tend to support the conclusions of Flavell et al. (1974) while contradictory results are obtained in the case of the genomes of Anemone (Cullis and Schweizer, 1974), Orthopteran (Wilmore and Brown, 1975) and Allium (Ranjekar et al., 1978b). Cytophotometric DNA content determinations have shown that the DNA content in French bean is 1.8 pg (Ayonoadu, 1974; Bennet and Smith, 1976) and that in green gram is 0.5 pg (Wimpee and Rawson, 1979). No data are available on the DNA content determinations in dew gram and black gram. If we assume that the haploid nuclear DNA content in all the four Phaseolus species is approximately in the range of 0.5 - 2.0 pg then the trend described by Flavell et al. (1974) appears to be true in the four Phaseolus species under present consideration.

Properties of repetitive DNA:

The thermal stability of the repetitive and the highly repetitive DNA fractions in each Phaseolus species is broadly similar except in black gram thus indicating that the highly as well as intermediately repetitive DNA sequences resemble each other in the extent of base mismatching. Such similarity in base mismatching of reassociated DNA sequences belonging to families with large differences in their rate of reassociation may appear unexpected. However, to our knowledge corresponding studies in both animal as well as plant species have led to some what conflicting results in this respect. From our data, it is clear that although the average Tms of the two repetitive DNA fractions are similar, these consist of nucleotide sequences with remarkable differences in their degree of repetition and complexity (Table 4).

Since the satellite DNA has been shown to be present in at least three Phaseolus plant species under present consideration, the question naturally arises whether this DNA component is present in the highly repetitive (Cot 1) DNA fraction. We do not have any information about the properties of satellite DNA in dew gram and green gram and hence it is rather difficult to assess its presence in the respective Cot 1 DNA fraction. In the case of French bean however, Beridze and Bragvadze (1976) have characterized the satellite DNA by studying its melting properties. The melting pattern of the French bean satellite DNA exhibits

two components: one minor low melting (Tm = 69.5°C) and one major high melting (Tm = 77.5°C). Since these denaturation experiments were carried out in 0.1 x SSC, the melting temperature of the two components would be higher by 15.4°C in 1 x SSC (Marmur and Doty, 1962). We have carried out all the denaturation experiments in 0.12 M PB and the Tm values determined in 0.12 M PB or in 1 x SSC have been found to be the same as verified in the case of E.coli DNA. Between Cot 50 and Cot 1 DNA fractions of French bean, the latter fraction exhibits a biphasic melting behaviour similar to that in French bean satellite DNA and the Tm (92.5°C) of its high melting component compares well with the salt corrected Tm value (93.0°C) of the high melting component of the satellite DNA. The Tm value (78.7°C) of the major low melting component of the Cot 1 DNA is however lower than that of low melting fraction (85°C) of the satellite DNA. From these comparative data it seems very likely that the French bean satellite DNA is mainly present in the Cot 1 DNA fraction. Furthermore, the high melting fraction of the Cot 1 DNA consists of nucleotide sequences with a very high degree of base pairing while the low melting fraction includes sequences with a nucleotide base mismatching of the order of 6% (Ullman and McCarthy, 1973; Bonner et al., 1973). It is therefore probable that the DNA sequences in the high melting fraction of the Cot 1 fraction are very similar.

The optical reassociation data of the Cot 1 DNA

fraction have revealed the presence of at least two kinetic components, a minor fraction reassociating by Cot 10^{-2} and a major fraction reannealing in the Cot range of 10^{-2} - 1.0. By determination of the extent of collapse hypochromicity in each Cot 1 DNA fraction, it is definite that the minor fraction is not completely due to single strand collapse. The available data on plant satellites indicate that most of these DNAs actually consist of two fractions differing from each other in their Tm values and rate of reassociation. The Cot 1 DNA fractions in the four Phaseolus plant species are no exception to this behaviour except that the melting curves in the case of black gram, dew gram and green gram are monophasic. The two kinetic components of the Cot 1 DNA in each Phaseolus specis significantly differ from each other in their kinetic complexity. For example, the minor fraction appears to consist of 840 np to 1440 np whereas the major fraction is more complex and is made up of 1.2×10^5 to 2.1×10^5 np. From the forgoing discussion, it seems likely that the Cot 1 DNA in each Phaseolus species may include the respective satellite DNA components.

DNA sequence interspersion in Phaseolus:

The study of DNA sequence organization in French bean and green gram has revealed the interspersion of repetitive and nonrepetitive DNA sequences and has provided a precise information about the lengths of these interspersed sequence elements. In plants, only a few species have been studied

with respect to their genome organization. From the limited data that are available, the size range of interspersed repetitive sequences appears to be broader in plants than in animals. For example in rye (Smith and Flavell, 1977), wheat (Smith and Flavell, 1975), soybean (Goldberg, 1978; Gurley et al., 1979) Umbelliferae (Kiper and Herzfeld, 1978) and tobacco (Zimmerman and Goldberg, 1977) the size of interspersed repetitive elements varies in the range of 200 - 800 nucleotide pairs. The repetitive length estimates in the case of French bean and green gram are somewhat close to that in cotton genome (0.8 pg, Walbot and Dure, 1976). Since the DNA sequence arrangement in cotton (Walbot and Dure, 1976) is considered as short period interspersion, we can also assume that the DNA sequence organization in French bean and green gram is also of similar type.

The precise biological role of DNA sequence interspersion patterns in eukaryotes is not yet known. The short period interspersion pattern in view of its wide occurrence in both animals and plants must have a high degree of selective value in eukaryotic evolution. Although the fossil record is incomplete, eukaryotic plant like organisms may have existed approximately one billion years ago in the protozoic year (Schopf, 1968). Since plants and animals must have diverged prior to this time, it is possible that the evolution of the short period interspersion pattern and the appearance of eukaryotes coincided. The correlation

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between the interspersed single copy length and the average size of m^{RNA} is compatible with the hypothesis proposed by Britten and Davidson (1969) and Davidson et al. (1977) that the interspersed short repetitive sequences may regulate gene transcription during eukaryotic differentiation. In fact in sea-urchin' (Davidson et al., 1975) most structural genes have been found to be contiguous with interspersed repetitive sequences.

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