Cytological Studies of *Luzula purpurea*, *L. multiflora*, and *Vicia faba*--Differential Staining and DNA Replication Patterns and Characterization of *L. purpurea* DNA

A Dissertation

Presented to

the Faculty of the Department of Biology

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In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

James Hubert Ray

May 1976

For Vicki and Geoff

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ABSTRACT

The cytological studies of Vicia faba, Luzula purpurea, and L. multiflora and the biophysical characterization of L. purpurea DNA revealed several interesting features to occur in both the organisms and DNA of organisms lacking localized centromeres.

Vicia faba, an organism having chromosomes with localized centromeres, exhibited a very restricted nuclear and chromosomal distribution for C-positive, Q-positive, and DNA late-replicating regions. These regions were concentrated in the vicinity of the centromeres. Multiple C-positive and DNA late-replicating regions were scattered throughout both the nuclei and chromosomes of L. purpurea and L. multiflora. The C-positive, DNA late-replicating chromosome regions of L. purpurea were also Q-positive and composed of highly repeated DNA sequences as revealed by ^{3}H-RNA in situ hybridization experiments. The different techniques permitted recognition of each of the three chromosome pairs of L. purpurea even though the chromosomes are equal-sized and lack primary constrictions.

L. purpurea DNA has a T_m value of 88.5° - 90.0° C, a main band buoyant density of 1.6819 ± 0.0003 g/cm^3, and two satellite DNA fractions having buoyant densities of 1.6928 g/cm^3 and 1.6972 g/cm^3. The discrepancy in the GC content of the DNA calculated from the T_m and buoyant density data was attributable to a high content of methylated bases being present (30.5 mole %). The heavier satellite DNA fraction

probably represents chloroplast DNA, but the lighter, low melting, satellite DNA fraction appears to be nuclear in origin being scattered throughout the DNA for the most part and comprising approximately 10% of the total DNA. DNA re-association revealed the highly repeated, moderately re-peated, and unique DNA sequences to make up 25%, 21%, and 54%, respectively, of the total DNA and the haploid DNA content to be 1.4×10^9 nucleotide pairs.

The cytological and biophysical data are discussed with regard to the nonlocalized nature of the centromeres of Luzula purpurea and L. multiflora chromosomes.

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LIST OF ABBREVIATIONS

A - adenine

C - cytosine

C-banding - banding patterns produced in chromosomes on staining with Giemsa following dissociation and reassociation.

Cot - moles per liter X seconds; can be determined by

$$\frac{\text{Denatured } OD_{260}}{2} \text{ X Hrs incubation}$$

$Cot_{1/2}$ = time required for one-half of the DNA to be reassociated

DNA - deoxyribonucleic acid

EDTA - ethylenedinitrilotetracetic acid

G - guanine

G-banding - banding patterns produced in chromosomes on staining with Giemsa following enzyme digestion.

HAP - hydroxyapatite; calcium phosphate

PB - phosphate buffer, pH 6.8; made by mixing equimolar concentrations of Na_2HPO_4 and NaH_2PO_4

Q-banding - banding patterns produced in chromosomes following staining with quinacrine mustard

RNA - ribonucleic acid

S stage of interphase - stage of interphase during which DNA is replicated.

SDS - sodium dodecyl sulfate

SSC - standard saline citrate; 1 X SSC contains 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0; 0.1 X, 2 X, 6 X, etc., are multiples of this concentration.

T - thymidine

T_m - melting temperature; temperature corresponding to point at which 50% of DNA is denatured.

INTRODUCTION

Chromatin is composed of two distinctive types of DNA-protein complexes. This was first recognized by Heitz (1928) from his study of stained intermitotic nuclei of moss. While stain was evenly distributed throughout most of the nucleus, a few regions appeared to have taken up more stain. The term heterochromatin was used to denote the more intensely stained regions while the more evenly stained nuclear regions were called euchromatin. Euchromatin is now known to be the portion of the chromatin which is alternately found in the condensed and extended state depending on the stage of the cell cycle being examined. During the intermitotic stage of the cell cycle it is in the extended state and, therefore, exhibits a low staining intensity. Heterochromatin is always condensed except for the short period during which it is being replicated. The stain is more heavily concentrated in the condensed regions causing these regions to appear to be stained darker in intermitotic nuclei.

Two types of heterochromatin are found in most cells-facultative heterochromatin and constitutive heterochromatin (Brown, 1966). Facultative heterochromatin may consist of either individual chromosomes or sets of chromosomes. Individual chromosomes are frequently found to be heterochromatic in species in which one of the sexes has a pair of sex chromosomes while the other does not thus permitting the heterogametic sex to have genetic potential equivalent to that of the homogametic one. In several groups of lower

animals whole sets of chromosomes may be heteropycnotic. Usually the condensed set is totally of either maternal or paternal origin, and the organisms possessing such chromosomes are functional haploids even though they contain a diploid chromosome complement. In contrast to these two cases of facultative heterochromatin, constitutive heterochromatin usually comprises only parts of chromosomes. It is found in the centromere regions, the nucleolus organizer regions, and the terminal regions of some homologous chromosomes.

The roles played by euchromatin and heterochromatin in the functioning of cells is not known with any degree of certainty. Euchromatin is thought to be the portion of the DNA containing structural genes which code for proteins necessary for the survival of the cell. The function of heterochromatin is much less clear, but it has been generally supposed that it plays some role in chromosome structure and recognition.

Until the last two decades most of the ideas on the structure and function of chromosomes or chromosome parts were inferred solely from cytological observations. Through a combination of new biochemical and cytological techniques analysis of chromosome structure on the molecular level has become possible. One such area of concentration has been the study of highly repeated DNA sequences, and this is the subject of the paper which follows.

REVIEW OF THE LITERATURE

Three DNA frequency classes are generally present in eukaryotic DNA (Walker, 1971). The first class, highly repetitive DNA, consists of families of sequences repeated on the order of 10^6 times; the second class, moderately repetitive DNA, consists of families of sequences containing 10^2 - 10^5 repetitions; the final class, unique DNA, consists of sequences present in one copy per haploid genome. DNA may be fractionated into the individual classes by dissociation and reassociation to different Cot values. Highly repetitive DNA reassociates between Cots 0 - 0.1; moderately repetitive DNA sequences reassociate between Cots 1 - 100; unique DNA sequences reassociate at Cots greater than 100 (Comings, 1972). Repetitive DNA has been found in all eukaryotic genomes that have been studied (Britten and Kohne, 1966; 1967; 1968; 1969; Bostock, 1972) with the proportion of the genome occupied by the repeated sequences being on the order of 10 - 30% (Rae, 1972). Variation occurs over a wide range, however. Salmon sperm DNA, for example, contains 80% of its genome as repetitive sequences (Britten and Kohne, 1967) as does Amphiuma (Britten and Kohne, 1968).

Highly Repeated DNA Sequences--Satellite DNA

Distribution. Highly repetitive DNA sequences sometimes exhibit buoyant densities different from that of the main

band causing them to appear as satellite bands. Satellite DNAs were first reported in mice and guinea pigs (Kit, 1961; 1962a; 1962b) and the actuality of their existence was quickly confirmed by reports of their presence in calf and salmon sperm DNAs (Schildkraut et al, 1962). Both calf and guinea pig satellite DNAs appeared as shoulders on the heavy side of main band DNA. Calf satellite DNA had a density of 1.713 g/cm^3 as compared to a main band density of 1.699 g/cm^3; guinea pig satellite DNA had a density of 1.703 g/cm^3 while that of the main band was 1.697 g/cm^3. Mouse and salmon sperm DNAs, on the other hand, appeared as distinct bands on the light side of main band DNA. The satellite buoyant densities were 1.690 g/cm^3 and 1.688 g/cm^3, respectively, while the densities of the main band DNAs were 1.701 g/cm^3 and 1.703 g/cm^3, respectively.

Although all organisms do not possess satellite DNA sequences, they have been found throughout both the plant and animal kingdoms (Coudray et al, 1970). A study of the buoyant density of DNA from 93 mammals showed 49 to contain satellite DNA bands. Of these 49 organisms, 11 had two satellite bands (Arrighi et al, 1970b). When the buoyant densities of DNA from 59 dicotyledons and 11 monocotyledons were determined, 27 of the dicots were found to contain satellite DNAs while none of the monocots exhibited satellites probably indicative of the small sample size studied (Ingle et al, 1973).

While most organisms that contain satellites contain only one, or at the most two, satellite DNA species, more occasionally occur. Three satellite DNAs were found in Drosophila virilis (Gall et al, 1971; Gall and Atherton, 1974), guinea pig (Corneo et al, 1970a), kangaroo rat (Hatch and Mazrimas, 1970; 1974), and Mus caroli (Sutton and McCallum, 1972). The satellite DNAs of D. virilis were all of lighter density than main band DNA (1.700 g/cm^3) having densities of 1.692 g/cm^3, 1.688 g/cm^3, and 1.671 g/cm^3. Guinea pig DNA contained one satellite DNA having a buoyant density of 1.706 g/cm^3 and two satellite DNA sequences having identical buoyant densities of 1.704 g/cm^3 in neutral CsCl but separable in alkaline CsCl gradients. This was similar to the situation in the kangaroo rat where two of the satellites, HS-α and HS-β, exhibited buoyant densities of 1.713 g/cm^3 in neutral CsCl but were separable in Ag^+-$CsSO_4$ gradients. The remaining satellite had a buoyant density of 1.707 g/cm^3 intermediate between the heavy satellites and the main band (1.698 g/cm^3). The satellite DNAs of M. caroli had densities of 1.695 g/cm^3, 1.697 g/cm^3, and 1.709 g/cm^3 being both lighter and heavier than main band DNA (1.701 g/cm^3).

Four satellites were found in calf DNA (Kurnit et al, 1973). They had densities of 1.706 g/cm^3, 1.709 g/cm^3, 1.716 g/cm^3, and 1.722 g/cm^3 all being heavier than main band DNA (1.699 g/cm^3). At least three and possibly four different human satellite DNAs have been isolated in Hg^+-

and Ag^+-$CsSO_4$ gradients. The first satellite DNA having a density of 1.687 g/cm^3 was detected in an Hg^+-$CsSO_4$ gradient (Corneo et al, 1967; 1968a). This was followed by reports of three additional satellite DNAs. One had a density of 1.693 g/cm^3 (Corneo et al, 1970b); another had a density of 1.696 g/cm^3 (Corneo et al, 1971). The final satellite had a density of 1.703 g/cm^3 (Saunders et al, 1972) although data from cytological localization experiments have suggested this satellite to be identical to a previously isolated one.

The proportion of the genome occupied by satellite DNA has ranged from 0.2% to over 50% (Jones, 1973a). Individual satellite DNA sequences in Cancer artennarius (Smith, 1964) and muskmelon (Bendich and Anderson, 1974) comprise 30% of the diploid DNA. The three satellite DNAs of the kangaroo rat account for 52% of the diploid genome (Hatch and Mazrimas, 1974) while the two satellite DNAs of cucumber comprise 44% of the DNA (Ingle et al, 1973). These examples represent extreme cases, however, and the satellite DNAs generally make up less than 10% of the cell's DNA.

Characteristics. Satellite DNA has several characteristics not shared by other repetitive and unique DNA sequences. The first and most obvious is their different buoyant density in CsCl and Ag^+- and Hg^+- $CsSO_4$ gradients indicating their differing G + C content from main band DNA. One of the more striking characteristics is the sharp melting profile of most satellite DNAs. The temperature at which dissociation of a DNA fragment occurs on heating is

characteristic of its G + C content. When main band DNA or total cellular DNA is heated, dissociation occurs over a rather broad temperature range since different regions of an organism's DNA contain differing quantities of G + C. Native mouse DNA dissociation was found to occur over the range of 81° - 91° C (Bolton et al, 1965; Flamm, 1972). When mouse satellite DNA was heated, however, complete dissociation occurred over a 3° C temperature range (Flamm et al, 1966; Corneo et al, 1968b; Flamm, 1972). Dissociation over narrow temperature ranges was also found in three guinea pig satellite DNAs (Corneo et al, 1968b; 1970a), two satellite DNAs of the hermit crab (Skinner and Beattie, 1974), all three human satellite DNAs (Corneo et al, 1968a; 1970b; 1971), chimpanzee satellite DNA (Prosser et al, 1973), Rhynchosciara hollaenderi satellite (Eckhardt and Gall, 1971), Heteropeza pygmaea satellite (Kunz and Eckhardt, 1974), and the three satellite DNAs of Drosophila virilis (Blumenfeld et al, 1973). Satellite DNA from muskmelon exhibited a somewhat different melting profile (Bendich and Anderson, 1974; Sinclair et al, 1975). The melting curve was biphasic with 2/3 of the satellite DNA having a T_m of 84° - 86° C and 1/3 having a T_m of 92° - 94° C.

Satellite DNAs frequently exhibit interstrand compositional differences. Ultracentrifugation of the satellite DNAs of guinea pig (Flamm et al, 1969a; Walker et al, 1969; Corneo et al, 1970a), human (Corneo et al, 1968a; 1970b; 1971), chimpanzee (Prosser et al, 1973), hermit crab (Skinner

and Beattie, 1974), kangaroo rat (Hatch and Mazrimas, 1970), and mouse (Walker et al, 1969) in alkaline CsCl gradients resulted in the separation of the individual satellite strands with each strand having a different buoyant density. Only two organisms, the European wood mouse (Walker et al, 1969) and muskmelon (Bendich and Anderson, 1974), had satellite DNAs with strands that failed to separate on alkaline CsCl ultracentrifugation. Since separation in alkaline gradients was known to be on the basis of G + T content of individual DNA strands (Wells and Blair, 1967), the results suggested that there was a great difference in the G + T content of the individual satellite strands.

Satellite DNA reassociates extremely rapidly following dissociation. This was first shown in mouse DNA where re-chromatography of DNA previously thermally eluted from HAP columns and elution by increasing the buffer concentration resulted in a portion of the DNA's being eluted as if it were native (Walker and McLaren, 1965). Dissociation of the sample by heating showed it to have a denaturation profile similar to that of native DNA. Incubation of sheared, denatured mouse DNA at 60° C for 4 hr resulted in 10% of the DNA's being present as duplex molecules (Bolton et al, 1965; Waring and Britten, 1966). Mouse satellite DNA was found to be reassociated by a Cot of 10^{-3} indicating it to consist of DNA sequences present on the order of 10^{6} times (Bolton et al, 1965). Similar, although slightly slower, reassociation rates were obtained with guinea pig satellites and calf

satellite I (Corneo et al, 1970a).

Dissociation of reassociated satellites result in very broad melting profiles quite unlike those of native DNA (Britten and Kohne, 1966; Corneo et al, 1968a; 1968b; 1970a; 1970b; 1971; Prosser et al, 1973; Chuang and Saunders, 1974; Flamm et al, 1966). Both the hyperchromicity and T_m of reassociated satellites have generally differed significantly from those of native satellites indicative of much mismatching in the reassociated satellite DNAs. Mismatching was also very apparent in electron micrographs of reassociated mouse satellite DNA (Salomon et al, 1969) and was suggested by differences in the buoyant densities of native and reassociated satellite DNA (Bond et al, 1967).

Sequence analysis. The mismatching which accompanied satellite DNA reassociation caused a large over-estimation of the length of the repeating units. Mouse satellite DNA was originally thought to be made up of a sequence 300 - 400 nucleotides long (Waring and Britten, 1966) as was guinea pig α-satellite (Southern, 1970). Analysis of the base composition of the individual strands of guinea pig α-satellite DNA by pyrimidine tract analysis of depurinated DNA showed each strand to be composed of a basic hexanucleotide repeating unit (Southern, 1970). The major hexanucleotide sequence was (5'-CCCTAA-3')·(3'-GGGATT-5'). Variations in this sequence which were found were explainable by transitions and transversions of the basic sequence or mutation by nucleotide insertion or deletion. A basic repeating sequence

of 8 - 13 nucleotides was suggested to occur in mouse satellite DNA with the sequence TTTTTC being present in the repeating unit. Later analyses of mouse satellite DNA using restriction endonucleases to break the satellite DNA into smaller fragments (Southern, 1975) and pyrimidine tract analysis of light and heavy satellite strands or chemical analysis of digests of ^{32}P-labeled RNA complementary to light and heavy strands (Biro et al, 1975) showed satellite DNA to be made up of repeated segments 220 - 240 nucleotide pairs long. Within each of the longer repeated segments were shorter repeated segments less than 20 nucleotide pairs in length with the nucleotide sequence of the majority of the light strand segments being d(GA_5TGA). Southern (1975) proposed that the present-day mouse satellite DNA resulted from four individual multiplications of the original 9 - 18 nucleotide long segment so that the 9 - 18 nucleotide pairs became 36 - 72, 110 - 130, and finally 220 - 260 nucleotide pairs. Such long range periodicities as those of mouse satellite DNA were also found in bovine satellite I DNA (Botchan, 1974) and the satellites of two species of Apodemus (Cooke, 1975). Bovine satellite I consisted of repeats 1400 base pairs in length while the satellites of Apodemus were made up of repeated segments 370 nucleotide pairs in length.

Several other satellite DNA sequences have been analyzed, and they, too, consist of short repeating units. The nucleotide sequence of kangaroo rat HS-β satellite was

determined by analysis of RNase T1 digests of RNA synthesized using HS-β DNA as the template and KOH digests of GTP-substituted HS-β DNA (Fry et al, 1973). The basic repeating unit was 10 nucleotides consisting of the sequence (5'-ACACAGCGGG-3')·(3'-TGTGTCGCCC-5'). Analysis of the base composition of RNA transcripts of satellite I of the hermit crab, Pagurus pollocaris, showed it to be composed of the repeating nucleotide sequence (5'-TAGG-3')·(3'-ATCC-5') (Skinner et al, 1974). The three satellite DNAs of Drosophila virilis were also found to consist of simple repeating units (Gall, 1973; Gall and Atherton, 1974; Gall et al, 1974). Analysis of RNA transcripts of individual strands of the different satellites showed each to be made up of repeating heptanucleotide sequences. Satellite I contained the sequence (5'-ACAAACT-3')·(3'-TGTTTGA-3'), satellite II had the sequence (5'-ATAAACT-3')·(3'-TATTTGA-5'), and satellite III's nucleotide sequence was (5'-ACAAATT-3')·(3'-TGTTTAA-5'). The three satellite sequences are clearly related to one another. Satellite I differs from both satellite II and III by a single nucleotide pair while two nucleotide pair differences exist between satellites II and III. The base sequences of three of the satellite DNAs of D. melanogaster were determined (Endow et al, 1975). The nucleotide sequence of satellite II was the only one that could definitely be established. It consisted of the ten nucleotides A-A-T-A-A-C-A-T-A-G. Satellite I was composed of A-A-T and A-T in the molar ratios of 2 : 3 while satellite

IV consisted of A-A-G, A-G, and G in the molar ratios 3 : 4 : 1. Although nucleotide sequencing data have shown satellite DNA relatedness to exist, this was not thought to be the case until recently.

Inter-relatedness of satellite sequences. Initial studies of the inter-relatedness of highly repeated DNA sequences were mainly carried out on rodents. Walker (1968) and Hennig and Walker (1970) observed much variation in the physical properties of DNA from sixteen rodent species. CsCl banding profiles showed some organisms to possess light satellites, some heavy satellites, and some no satellites. Even within a single genus, Apodemus, satellite DNAs from different species were found to differ both in their buoyant densities and the fraction of the genome occupied. DNA reassociation studies of the different rodent DNAs indicated the fast reassociating fraction of most species to comprise from 5 - 12% of the total DNA, but Microtus agrestis and Clethrionomys glareolus had no detectable fast reassociating fraction. These differences in physical properties led to the conclusion that satellite DNAs were species-specific.

Satellite DNA relatedness was also studied by testing the ability of Mus musculus satellite DNA to hybridize to the DNA of other rodents. No complementarity was found to exist between M. musuclus satellite DNA and DNA from Rattus rattus (Flamm et al, 1969b), R. norvegicus (Sutton and McCallum, 1972), Cavia porcellus (Flamm et al, 1969a), Apodemus

sylvaticus (Sutton and McCallum, 1972), or Peromyscus polionotus and P. maniculatus (Walker et al, 1969). Species specificity was also indicated from the study of the ability of RNA complementary to the heavy satellites of Drosophila neohydei and D. pseudoneohydei to hybridize to total DNA from each other or D. hydei. Although all three organisms were very closely related, each RNA exhibited complementarity only with DNA from which it was synthesized (Hennig et al, 1970). The reassociation conditions used in all these studies except that of Sutton and McCallum (1972) were very stringent. Rodent DNA reassociation experiments were carried out at 60° C while Drosophila RNA : DNA hybridization experiments were conducted at 75° C. The high temperatures could possibly explain the inability to detect hybrid molecules when heterologous DNA was used. The results of the experiments of Sutton and McCallum (1972) which were conducted at 50° C would seem to contradict this, at least in the species studied.

Interspecies homology has been found to exist in the highly repeated DNA sequences of a number of species belonging to the genus Mus. Cross-reassociation was found to occur between M. musculus satellite DNA and DNA from several other Mus species (Sutton and McCallum, 1972; Rice and Straus, 1973). Hybridization of total DNA from M. caroli, M. cervicolor, and M. famalus with M. musculus satellite DNA resulted in heterologous duplexes with significantly lower thermal stabilities than homologous duplexes indicating that although complementarity existed between the DNAs, the

complementary sequences differed greatly from one another (Sutton and McCallum, 1972). Sequences identical to Mus musculus satellite DNA were found in M. castaneus and M. poshiovinus DNAs. The hybridization of purified M. caroli satellite DNAs to M. musculus satellite showed the heterologous hybrids produced in total DNA reassociation studies to be the result of complementarity existing between the satellite DNAs of the two species. Rice and Straus (1973) also found complementarity to exist between mouse satellite DNA and M. caroli and M. cervicolor DNAs. In addition, M. m. molossinus and M. m. castaneus, two subspecies of M. musculus, were found to have satellite sequences identical to that of M. musculus. The three density satellites of Drosophila virilis were also found to be inter-related (Blumenfeld et al, 1973; Blumenfeld, 1974). Cross-hybridization was found to occur between the strands of satellite I and those of satellites II and III, but no cross-reassociation was detectable between satellites II and III. Since sequence analysis has shown satellites II and III to differ from one another by only two base pairs, the inability to detect hybrid molecules between them suggests that minor sequence differences in satellite DNAs resulting in a lack of heteroduplex formation could lead to the conclusion that satellite DNA sequences are totally unrelated when they, in fact, belong to the same repeated sequence family.

In addition to interspecies satellite DNA relatedness, inter-genera satellite DNA relatedness also occurs. The

GC-rich satellite DNA of the land crab, Gecarcinus lateralis, exhibited complementarity with DNA from four other crustaceans (Graham and Skinner, 1973). Homology varied from 0.4 - 10% in different genera and even within the same genus, different species had different proportions of homologous DNA. Inter-relatedness was also found between chimpanzee satellite A and human satellite III (Prosser et al, 1973). Hybridization of ^{3}H-RNA complementary to the two satellites to CsCl-fractionated homologous and heterologous DNA showed 25 - 30% homology to exist between the satellites with both ^{3}H-RNAs hybridizing to DNA of identical buoyant densities in each species. Homology was also shown by the similar hybridization patterns of satellite III to in situ denatured DNA of chimpanzee, orangutan, and man.

Organization in DNA. Satellite DNA organization within total DNA has been studied in a number of ways. Buoyant density studies suggested a tandem arrangement for the repeated sequences. The molecular weight of mouse satellite DNA (10^{7} daltons) was appropriate for a sequence of 100 tandemly arranged units each consisting of 300 - 400 nucleotide pairs (Flamm et al, 1966). Such an arrangement was also found in Drosophila melanogaster satellite DNA where satellites were shown to comprise 20 μ lengths of DNA corresponding to molecular weights of 10^{7} daltons (Peacock et al, 1974).

More direct evidence on satellite DNA organization came from electron microscopic studies of sheared DNA that had been partially digested with exonuclease III, an enzyme which

exposes 5'-ended single nucleotide chains, and then incubated in SSC for 1 - 3 hr. Thomas et al (1970) first studied the DNAs of salmon sperm, trout, and Necturus by this method and found 20%, 20%, and 35%, respectively, of the DNA to be observed as circular molecules. These results indicated repeated sequences to be tandemly arranged within the DNA. Similar results were reported for Drosophila melanogaster, D. virilis, and D. hydei in which 15% of the DNA was found to form rings (Thomas et al, 1972; Lee and Thomas, 1973) and mouse DNA in which approximately 70% of the satellite band formed rings and 20% of the main band (Thomas et al, 1972; Pyeritz and Thomas, 1973). Some discrepancies appeared to exist in the organization of these repetitive regions. In all these organisms the percentage of cyclization depended on the fragment length. The percent of ring formation decreased as the fragment length increased above 2 μ, the optimum fragment length for ring formation, and were no longer formed if greater than 10 - 15 μ fragments were used. Conversely, as the fragment length decreased below 1 - 2 μ, a decrease in the cyclizability of the fragments was also found. These results suggested that the repetitive sequences were clustered in a relatively short region of 5 - 10 μ, but within the clustered region the repetitive sequences were separated by 600 - 6000 nucleotide long regions. A study of cyclized DNA of D. hydei and Necturus using increasing formamide concentrations in an attempt to find nonhomologous regions in the closure region that were suggested from the

above results showed nonhomologous regions to be absent from the regions where ring closure occurred indicating that interspersed nonrepetitive regions possibly did not exist (Bick et al, 1973).

One of the problems in obtaining clear-cut results concerning the arrangement of the repeated sequences capable of ring formation probably resulted from the fact that total cellular DNA was used. Two other studies involving Drosophila melanogaster DNA allowed more concrete conclusions to be made as to the cyclizable DNA fraction. In one study Thomas circles formed by D. melanogaster DNA were covalently sealed with DNA polymerase, ligase, and exonuclease III and isolated by propidium-CsCl ultracentrifugation (Schachat and Hogness, 1974). Dissociation-reassociation experiments on the isolated, sonicated DNA showed 66% to renature with a $Cot_{1/2}$ value characteristic for highly repetitive DNA sequences and 34% to renature with a $Cot_{1/2}$ value characteristic for moderately repeated sequences. The majority of the isolated Thomas circles were also found to have a buoyant density similar to D. melanogaster satellite DNAs. That satellite DNA was indeed responsible for the major fraction of Thomas circles formed was shown by experiments conducted with isolated satellites (Peacock et al, 1974). Total nuclear DNA formed circles with a 23% frequency. However, when total DNA was separated into main band and satellites, only 10 - 11% of the main band DNA containing moderately repeated and unique sequences formed circles while 60 - 70% of the four satellites exhibited circle

formation. While these experiments showed the majority of the cyclizable sequences to be confined to satellite DNA and suggested satellite DNA to consist of tandemly arranged repeated sequences, they offered no suggestion as to the localization of satellite DNA within the nucleus.

Organization in chromatin. Several studies have been conducted on the nuclear location of satellite DNA. When DNA that had been isolated from mouse chromosomes fractionated into different size classes by centrifugation through sucrose gradients was analyzed by CsCl ultracentrifugation, all chromosome classes were found to contain satellite DNA sequences (Maio and Schildkraut, 1969). Treatment of the fractions with 2 M NaCl resulted in differential extraction of main band and satellite DNA with the main band DNA's being removed from the chromosomes. Satellite DNA remained associated with the chromosomes and nucleoli indicating that its association with protein was perhaps different from that of main band DNA.

Studies to determine the heterochromatic or euchromatic association of satellite DNA were also conducted. Calf thymus chromatin had earlier been separated into active and inactive portions (Frenster et al, 1963). Modification of this technique involving nuclei swelling in sucrose and a short sonication permitted chromatin to be fractionated into a heterochromatic portion by centrifugation at 3500g, a portion containing mostly euchromatin but with a small amount of heterochromatin pelleted at 78,000g, and a euchromatic

portion (Yasmineh and Yunis, 1969). Analysis of mouse DNA by this method followed by CsCl ultracentrifugation of the separated fractions showed mouse satellite DNA to be present in heterochromatin (Yasmineh and Yunis, 1969; 1970; Duerksen and McCarthy, 1971). Similar satellite DNA enrichment in heterochromatin has been found in kangaroo rat (Mazrimas and Hatch, 1970), guinea pig (Yunis and Yasmineh, 1970), man (Corneo et al, 1971), crab (Duerksen and McCarthy, 1971), and calf (Yasmineh and Yunis, 1971).

Organization in chromosomes. Direct visualization of the chromosomal localization of specific DNA sequences became possible with the development of the in situ hybridization technique. The cytological localization of DNA sequences complementary to rRNA was simultaneously reported in the toad Xenopus laevis (Gall and Pardue, 1969), HeLa cells (John et al, 1969), and Chinese hamster cells (Buongiorno-Nardelli and Amaldi, 1970). All three groups of workers studied the hybridization of ^{3}H-labeled 28 S and 18 S rRNA to in situ denatured DNA of interphase nuclei and found only certain restricted regions of the interphase nuclei to be hybridized. These reports were quickly followed by the chromosomal localization of mouse satellite DNA (Jones, 1970; Jones and Robertson, 1970; Pardue and Gall, 1970). ^{3}H-RNA complementary to satellite DNA was found to hybridize to the centromere regions of all mouse chromosomes with the exception of one chromosome which Pardue and Gall (1970) suggested to be the Y-chromosome. Innumerable reports have since

appeared concerning the chromosomal localization of different DNA sequences. The experiments have generally involved the hybridization of ^{3}H-labeled DNA or *in vitro* synthesized ^{3}H-RNA complementary to a particular DNA fraction to *in situ* alkaline or heat denatured DNA and visualization of the hybridized regions by autoradiography.

Several experiments have utilized rapidly reassociating DNA fractions as the template for ^{3}H-RNA synthesis. Hybridization of *Microtus agrestis* ^{3}H-RNA complementary to the rapidly reassociating DNA fraction (Cot $\leq$ 8) to chromosome preparations resulted in label concentration over the centromeric regions of autosomes, all of the X-chromosome except the distal 3/4 of the short arm, and the entire Y-chromosome showing the centromeric regions of chromosomes to consist of highly repetitious DNA even in the absence of satellite DNA (Arrighi *et al*, 1970a). *In situ* hybridization of ^{3}H-RNA complementary to *Cavia porcellus* rapidly reassociating DNA (Cot 1) (Natarajan and Raposa, 1974) and *Mus musculus* DNA (Cot 1) (Natarajan *et al*, 1973; Ahnstrom and Natarajan, 1974) resulted in label concentration over the centromere region of the chromosomes. A similar centromeric localization of highly repeated DNA (Cot 0.1) of *Schistocerca gregaria* was found, but label was also located over the telomeres of the short chromosomes (Brown and Wilmore, 1974).

In situ hybridization of ^{3}H-RNA complementary to *Drosophila melanogaster* light density DNA (Rae, 1970) and rapidly reassociating DNA (Rae, 1970; Botchan *et al*, 1971)

or one of the satellite strands (Gall et al, 1971) resulted in label being mainly concentrated over the α-heterochromatin, the region consisting of the fused centromeres of chromosomes, again suggesting satellite DNA sequences to make up centromeric heterochromatin. Study of the satellite DNA sequences of Drosophila neohydei and D. pseudoneohydei yielded somewhat different results (Hennig et al, 1970). ^{3}H-RNA complementary to the heavy satellite of D. neohydei was found to be complementary to sites distributed throughout the polytene chromosomes with the RNA preferentially binding to the dark staining bands. With both the heavy and light satellites of D. pseudoneohydei, however, label occurred mostly in the α-heterochromatin. Rhynchosciara hollaenderi satellite DNA was found to be located in the centromeric region of all four chromosomes and the telomere regions of two of the chromosomes (Eckhardt and Gall, 1971). Centromeric localization of satellite DNA has also been reported in the gall midge Heteropeza pygmaea (Kunz and Eckhardt, 1974) and the salamander Plethodon cinereus cinereus (Macgregor and Kezer, 1971; Macgregor et al, 1973). In situ hybridization of ^{3}H-RNA complementary to GC-rich satellite DNA of the Japanese quail Coturnix coturnix japonica showed it to be confined largely to the microchromosomes (Brown and Jones, 1972).

In cases where multiple satellites occur, individual satellite DNAs have usually been restricted to certain chromosomes or chromosome regions. Study of the two satellite

peaks present in Dipodomys ordii Ag^{+}-$CsSO_4$ centrifuged DNA showed the low density satellite, HS-β , to be present in the centromere regions of all but three chromosome pairs while the high density satellite, HD, was present in the short arms of the chromosomes containing HS-β satellite regions and in the centromeric regions of the three chromosome pairs from which HS-β was absent (Prescott et al, 1973).

The four satellite DNAs of calf also exhibited different chromosome distributions (Kurnit et al, 1973) although this was not nearly so distinct as that of the kangaroo rat. Satellite I (1.715 g/cm^3) was localized in the centromeric regions of all chromosomes except the X- and Y-chromosomes. Satellite II (1.722 g/cm^3) was distributed mostly over centromeric regions but approximately 1/3 was found over interstitial and telomeric regions. Satellite III (1.706 g/cm^3) was present in the X- and Y-chromosomes and at the centromeres of all but four autosomal pairs. Satellite IV (1.709 g/cm^3) was localized at the centromeres of all but two autosomal pairs with occasional grains randomly located over the sex chromosomes.

Human satellite DNA sequences also show somewhat restricted chromosome distribution patterns. Satellite DNA I (1.688 g/cm^3) has been found in the centromeres of chromosomes 1, 3, 4, 5, 9, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22, and the distal part of the Y-chromosome (Jones et al, 1974; 1975; Gosden et al, 1975). Satellite DNA II (1.693 g/cm^3) was found in major amounts on chromosomes 1 and 16 and in lesser

amounts on chromosomes 9, 15, 16, 17, 21, and 22 (Jones and Corneo, 1971; Gosden et al, 1975). Satellite III (1.696 g/cm^3) was present at the centromere region of chromosomes 9, 13, 14, 15, 20, 21, and 22 (Jones et al, 1973; Gosden et al, 1975). A similar distribution was found for the satellite (1.703 g/cm^3) isolated by Saunders et al (1972).

A single report has appeared concerning the chromosomal localization of satellite DNA sequences in plants (Timmis et al, 1975). ^{3}H-RNA complementary to a satellite band of Scilla siberica DNA having a density of 1.705 g/cm^3 exhibited restricted chromosome distribution. Three pairs of chromosomes had grains localized over only the end of one arm, another pair showed terminal and interstitial localization, one pair contained only interstitial grains, and a final pair had no grains over the chromosomes. A satellite of Vicia faba isolated by Ag^{+}-$CsSO_4$ ultracentrifugation had a more widely distributed arrangement in the nucleus and chromosomes.

C-banding patterns. In the initial report concerning the chromosomal localization of mouse satellite DNA, Pardue and Gall (1970) noted that the chromosome regions containing satellite DNA were more darkly stained with Giemsa than the remainder of the chromosomes. A similar correlation between darker stained chromosome regions and highly repetitious DNA localization was also found in Microtus agrestis (Arrighi et al, 1970a) where the centromeric regions of the autosomes, all but 3/4 of the short arm of the X-chromosomes, and the entire Y-chromosome exhibited both darker staining and label

concentration. Treatment of human chromosomes by the method of Pardue and Gall (1970) resulted in autosomes and X-chromosomes with distinctly stained centromeric regions and Y-chromosomes with the distal 2/3 of the long arm darkly stained (Arrighi and Hsu, 1971). Since female chromosome complements exhibited no totally stained X-chromosomes, the method was suggested to be specific for the localization of constitutive heterochromatin. The procedure has come to be known as the C-banding method and has been applied to innumerable organisms. The method involves the *in situ* dissociation of DNA in air-dried or squash preparations followed by reassociation for varying periods of time in some concentration of SSC at elevated temperatures and staining in buffered Giemsa.

Probably the most exhaustively studied group of organisms has been mammals. Denaturing agents that have been used in the studies include NaOH (Arrighi *et al*, 1970a; Arrighi and Hsu, 1971; Bianchi and Ayres, 1971a; 1971b; Chen and Ruddle, 1971; Gagne *et al*, 1971; Hsu *et al*, 1971; Hsu and Arrighi, 1971; Natarajan *et al*, 1971; Sinha *et al*, 1972; Voiculescu *et al*, 1972; Evans *et al*, 1973; Hansen, 1973a; Natarajan and Raposa, 1974), $Ba(OH)_2$ (Sumner *et al*, 1971; Sumner, 1972; Chandley and Fletcher, 1973; Evans *et al*, 1973; Prescott *et al*, 1973; Sharma and Dhaliwal, 1974), hot water (Takagi, 1971), hot phosphate buffer (Yunis *et al*, 1971; Polani, 1972), alkaline Giemsa (Bobrow *et al*, 1972; Gagne and Laberge, 1972), alkaline SSC (Cooper and Hsu, 1972; Forejt, 1973; Pathak *et al*, 1973a; 1973b; Stock and Hsu, 1973; Jalal

et al, 1974; Markvong et al, 1975; Stock, 1975), hot SSC (Pera, 1972), formamide in SSC (Dev et al, 1972; 1975), and hot Earle's BSS medium (Eiberg, 1974). In all cases examined except one constitutive heterochromatin was found in the autosomal and X-chromosome centromeres. Heterochromatin was also occasionally located in the arms of autosomes (Bianchi and Ayres, 1971a; 1971b; Hsu and Arrighi, 1971; Polani, 1972; Pathak et al, 1973a; 1973b; Prescott et al, 1973; Stock and Hsu, 1973; Jalal et al, 1974; Bostock and Christie, 1975; Markvong et al, 1975; Stock, 1975) and X-chromosomes (Arrighi et al, 1970a; Hsu and Arrighi, 1971; Cooper and Hsu, 1972; Pera, 1972; Polani, 1972; Voiculescu et al, 1972; Pathak et al, 1973a; 1973b; Jalal et al, 1974; Markvong et al, 1975; Stock, 1975). Didelphis virginiani exhibited no autosomal heterochromatin (Sinha et al, 1972). Heterochromatin was located in the centromere and one distal band in each arm of the X-chromosome. Greatest variation occurred in the constitutive heterochromatin content of the Y-chromosome. Mus musculus (Hsu et al, 1971; Chen and Ruddle, 1971; Dev et al, 1972; Polani, 1972; Forejt, 1973), Carollia perspicillata (Hsu and Arrighi, 1971; Pathak et al, 1973b), and Capra hircus, Ovis aries, and Bos taurus (Evans et al, 1973) exhibited none; Muntiacus muntjak (Hsu and Arrighi, 1971; Sharma and Dhaliwal, 1974; Pardue and Hsu, 1975), Saimiri sciureus (Jalal et al, 1974), Macaca mulatta (Stock and Hsu, 1973), and Cercopithecus aethiops (Bianchi and Ayres, 1971a; Stock and Hsu, 1973) showed centromeric

localization. About 2/3 of the long arm of the Y-chromosome of Homo sapiens (Arrighi and Hsu, 1971; Chen and Ruddle, 1971; Gagne et al, 1971; Takagi, 1971; Yunis et al, 1971; Sumner, 1972; Chandley and Fletcher, 1973) and Mesocricetus newtoni (Voiculescu et al, 1972) and most or all of the Y-chromosome of Microtus agrestis (Arrighi et al, 1970a; Natarajan et al, 1971; Cooper and Hsu, 1972; Pera, 1972), M. dunni (Markvong et al, 1975), Cricetulus griseus, M. auratus, Peromyscus maniculatus, Reithrodontomys fulvescens (Hsu and Arrighi, 1971), Didelphis virginiani (Sinha et al, 1972), and Cavia porcellus (Bianchi and Ayres, 1971b) contained constitutive heterochromatin.

The constitutive heterochromatin distribution of the amphibians Plethodon cinereus cinereus (Macgregor et al, 1973), Triturus vulgaris meridionalis and T. italicus (Nardi et al, 1973), and Notophthalmus viridescens (Hutchinson and Pardue, 1975) has been studied. The chromosomes of P. c. cinereus exhibited constitutive heterochromatin at the centromere regions. Most of the chromosomes of T. v. meridionalis showed two bands on each arm close to the centromeres while most chromosomes of T. italicus exhibited single bands on each arm near the centromeres. The chromosomes of N. viridescens exhibited centromeric and interstitial heterochromatin on all chromosomes. Study of constitutive heterochromatin distribution in birds using 2 X SSC, pH 12 (Stefos and Arrighi, 1971; 1974; Stock et al, 1974) or NaOH (Comings and Mattoccia, 1972) as the denaturing agent showed a centromeric localization in

the microchromosomes and macrochromosomes of Columba livia domestica, Streptopelia risoria, Phasianus colchicus, Anas platyrhynchos, Colinus virginianus, and Coturnix coturnix and the microchromosomes of Gallus domesticus while neither the micro- nor macro- chromosomes of Melopsittacus undulatus contained constitutive heterochromatin. The W chromosomes of all the organisms showed total staining while constitutive heterochromatin was present only on the Z chromosomes of G. domesticus.

Insect autosomal chromosomes have exhibited centromeric constitutive heterochromatin (Bianchi et al, 1971; Hsu, 1971; Takagi, 1971; Bregman, 1973; Gallagher et al, 1973; Brown and Wilmore, 1974; Dolfini, 1974; Drets and Stoll, 1974; Ennis, 1974; Fiskesjo, 1974a; Klasterska et al, 1974; Bedo, 1975) together with occasional interstitial and telomeric locations (Bianchi et al, 1971; Takagi, 1971; Bregman, 1973; 1975; Gallagher et al, 1973; Brown and Wilmore, 1974; Drets and Stoll, 1974; Bedo, 1975). Constitutive heterochromatin was restricted to the centromeres of X-chromosomes of Bryodema tuberculata (Klasterska et al, 1974), Schistocerca gregaria (Brown and Wilmore, 1974), and Drosophila melanogaster (Hsu, 1971; Dolfini, 1974). Acheta domesticus (Fiskesjo, 1974a) and Myrmeleotettix maculatus (Gallagher et al, 1973) X-chromosomes also showed interstitial regions containing constitutive heterochromatin while Gryllus argentinus X-chromosomes had terminal heterochromatin (Drets and Stoll, 1974). The Y-chromosome of D. melanogaster was almost totally

composed of constitutive heterochromatin (Hsu, 1971; Dolfini, 1974).

Plant chromosomes have also been studied in some detail. Denaturing agents that have been used include $Ba(OH)_2$ (Vosa and Marchi, 1972; Sarma and Natarajan, 1973; Stack and Clarke, 1973a; 1973b; Vosa, 1973a; Filion, 1974; Gill and Kimber, 1974a; 1974b; Marks and Schweizer, 1974; Marks, 1974; Natarajan and Sarma, 1974; Schweizer, 1974; Stack, 1974; Stack et al, 1974; Verma and Rees, 1974; Vosa, 1974; El-Gadi and Elkington, 1975; Klasterska and Natarajan, 1975; Weimarck, 1975), HCl (Fiskesjo, 1974b; 1975), NaOH (Natarajan and Natarajan, 1972; Takehisa and Utsumi, 1973a; 1973b; Yamasaki, 1973; Natarajan and Sarma, 1974), hot potassium phosphate buffer (Stack and Clarke, 1973a; Stack, 1974; Stack et al, 1974; Stack, 1975), urea (Dobel et al, 1973), and hot 2 X SSC or McIlvaine's buffer (Merker, 1973; Schweizer, 1973; Stack, 1974; 1975). Centromeric constitutive heterochromatin is absent in many plants that have been studied. Only one organism, Rhoeo discolor, has been found to have heterochromatin restricted to the centromeric region (Natarajan and Natarajan, 1972). Two of the first organisms studied, Allium carinatum and Tulbaghia leucantha, contained only interstitial and telomeric heterochromatic bands (Vosa and Marchi, 1972). A similar situation was found in Tulipa sp. (Filion, 1974), Anemone blanda, A. coronaria, A. pavonina, A. riparia, and A. virginiana (Marks and Schweizer, 1974), Fritillaria meleagris, F. recurva, and F. lanceolata (Schweizer, 1973), and A. cepa,

Allium fistulosum, and A. galanthum (El-Gadi and Elkington, 1975).

In Secale cereale some differences have been reported in constitutive heterochromatin. Two groups found heterochromatin to be restricted to telomeric and interstitial chromosome regions (Sarma and Natarajan, 1973; Verma and Rees, 1974) while two others also found it in the centromeres (Gill and Kimber, 1974a; Vosa, 1974). Since all groups used identical denaturation procedures, the differences appear to be real and probably result from the fact that different varieties of rye were used in the studies. Differences also occurred in the C-banding patterns of Triticum monococcum. Natarajan and Sarma (1974) found only centromeric staining while Gill and Kimber (1974b) found additional staining in the telomeres of some of the chromosomes. Differences in banding patterns were found in Vicia faba chromosomes depending on the pretreatment used (Schweizer, 1973). Differences also occurred in A. cepa, A. proliferum, and Ornithogalum virens depending on the C-banding procedure used. Treatment of A. cepa chromosomes with saturated $Ba(OH)_2$ resulted in telomeric and nucleolar organizer staining by Giemsa while chromosomes subjected to hot potassium phosphate buffer (90° C) showed centromeric and nucleolar organizer staining (Stack, 1974; Stack et al, 1974; Stack, 1975). O. virens chromosomes treated with hot potassium phosphate showed nucleolar organizer and centromeric staining while $Ba(OH)_2$-treated chromosome preparations had additional interstitial bands. The hot potassium

phosphate treatment actually produced two darkly stained structures at the centromere region leading to the later conclusion (Stack, 1974; 1975) that the method was specific for revealing the position of kinetochores in the chromosomes.

Slide storage also affected banding patterns (Fiskesjo, 1974b). Immediate C-banding of Allium cepa and A. proliferum chromosomes using HCl as the denaturing agent led to no bands; after three days telomeric regions were differentially stained in both organisms; after two to three weeks telomeric, centromeric, and interstitial bands appeared. These results suggested heterochromatin in the telomere and centromeric and interstitial regions of plant chromosomes to possibly be different and could perhaps explain some of the discrepancies which occur in C-banded plant chromosomes. The other plants that have been studied have contained centromeric heterochromatin, but telomeric heterochromatin has also been present in each case and sometimes interstitial bands.

Mechanism of C-banding. Several studies have been undertaken to determine the basis for Giemsa staining of highly repeated DNA sequence-containing chromosome regions. During the C-banding procedure much of the DNA is lost from the chromosomes (Comings et al, 1973; Crossen, 1973) with the DNA in the chromosome arms being disproportionately lost (Pathak and Arrighi, 1973). Double-strandedness of the repeated DNA sequences remaining does not seem to be the cause of Giemsa staining because 70% of the in situ denatured DNA was found to be single-stranded after reassociation (Kurnit, 1974).

Since strong bases, salts at alkaline pH, and protein denaturants such as urea and guanidine-HCl induce C-banding (McKenzie and Lubs, 1973; Kato and Moriwaka, 1972; Comings et al, 1973), repetitive DNA-protein interactions seem to be responsible for C-band induction. Histone removal has no effect on banding (Comings et al, 1973; Comings and Avelino, 1974) indicating that acidic nuclear protein-repetitive DNA interactions are probably the cause of C-band production.

DNA late-replicating patterns. Attempts have been made to study the relationship, if any, between C-banding patterns and those of DNA late-replicating regions. The C-positive short arms of Peromyscus eremicus chromosomes were found to be late-replicating (Pathak et al, 1973a) as were the C-banded regions of Carollia perspicillata (Pathak et al, 1973b). However, in both organisms facultative heterochromatin replication occurred even later than that of the C-bands. The DNA replication order was euchromatin, G-bands, C-bands, and facultative heterochromatin. Late-replication was also characteristic for the C-banded chromosome regions of Bos taurus, Ovis aries, and Capra hircus (Schnedl, 1972; Schnedl and Czaker, 1974), HeLa cells (Czaker, 1973), Cercopithecus aethiops (Parker et al, 1973), and Mesocricetus newtoni (Voiculescu et al, 1972). A somewhat different late-replicating pattern occurred in the chromosomes of Dipodomys merriami (Bostock and Christie, 1974), D. panamintinus (Bostock and Christie, 1975), and Muntiacus muntjak (Sharma and Dhaliwal, 1974). Noncentromeric C-bands were generally

late-replicating while centromeric C-banded regions were replicated earlier in the S phase of the cell cycle. A study of three species of Mus showed variation in late-replicating chromosome regions to occur even within the same genus. In M. fulvidiventris the C-banded regions were the latest to finish replication while in M. musculus and M. dunni C-positive heterochromatin finished replication earlier than many euchromatic regions.

Q-banding patterns. Another cytological technique that has been used in conjunction with C-banding is Q-banding. Differential fluorescence of chromosome regions was first reported in Vicia faba, Cricetulus griseus, Trillium erectum, and Scilla siberica when squash preparations were stained with quinacrine mustard and observed (Caspersson et al, 1968; 1969a; 1969b). Each chromosome exhibited characteristic Q-banding patterns permitting homologous chromosome identification. The Q-banding patterns observed in plants and animal chromosomes in the initial studies have been characteristic for the most part of the Q-banding patterns observed in other organisms. C. griseus chromosomes exhibited bright Q-bands alternating with dull ones with the alternating pattern extending from one end of the chromosome to the other. In V. faba, T. erectum, and S. siberica the chromosomes exhibited either an over-all dull fluorescence with a few bright Q-bands or an over-all bright fluorescence with a few dull Q-bands.

Q-banding patterns similar to those of C. griseus have been observed in Bos taurus (Schnedl, 1972; Hansen, 1972),

Capra hircus (Hansen, 1973b), Cercopithecus aethiops (Parker et al, 1973), Rattus norvegicus (Mori and Sasaki, 1973), Mus poschiavinus (Zech et al, 1973), M. musculus (Schnedl, 1971; Natarajan et al, 1973), Microtus agrestis (Natarajan et al, 1971; de la Chapelle et al, 1971; Gropp et al, 1973), Muntiacus muntjak (Fredga, 1971; Brown and Cohen, 1973; Pardue and Hsu, 1975), Cavia porcellus (Natarajan and Raposa, 1974), Apodemus agrarius, Aethechinus algirus, and Sorex gemellus (Gropp et al, 1973), Homo sapiens (Caspersson et al, 1970a; 1970b; Evans et al, 1971; Manolov et al, 1971; Gagne et al, 1971; Ganner and Evans, 1971; Aula and Saksela, 1972; Zech, 1973; Chen, 1974), Potorous tridactylis (Grewal et al, 1971; Brown and Cohen, 1973), pig (Gustavsson et al, 1972), Gallus domesticus (Stahl and Vagner-Capodano, 1972), Saimiri sciureus, Balaenoptera physalus, and Peromyscus eremicus (Jalal et al, 1974), and Samoaia leonensis (Ellison and Barr, 1972). Centromeric C-positive bands are Q-negative for the most part although Q-positive regions close to the centromeres of chromosomes A_3 and two D-group chromosomes of man have been observed (Gagne et al, 1971). Noncentromeric C-bands occasionally appear to be Q-positive (Jalal et al, 1974) as do entire chromosome arms (Natarajan and Raposa, 1974). The Y-chromosome is generally totally Q-negative or Q-positive. Only in M. cervicolor (Dev et al, 1973), Drosophila melanogaster (Vosa, 1970; Adkisson et al, 1971), D. virilis (Adkisson et al, 1971), and Miastor sp. (Bregman, 1975) have Q-positive regions corresponded to C-banded chromosome regions. The use

of the fluorescent stain 33258 Hoechst resulted in the C-banded chromosome regions of Mus musculus and M. poschiavinus (Hilwig and Gropp, 1972; Gropp et al, 1973), Drosophila melanogaster, D. virilis, and D. eohydei (Holmquist, 1975), and Chilocorus sp. (Ennis, 1974; 1975) being brightly fluorescent.

The Q-banding patterns of plant chromosomes rarely are of the alternating Q-positive, Q-negative type. Only in Zea mays has such a Q-banding arrangement been observed (Horn and Walden, 1971). The Q-positive chromosome regions found in Secale cereale (Sarma and Natarajan, 1973; Vosa, 1974), Tricale sp. (Sarma and Natarajan, 1973), Allium carinatum (Vosa, 1970; 1971; Vosa and Marchi, 1972), A. flavum (Vosa, 1973b), Rhoeo discolor (Natarajan and Natarajan, 1972), and Vicia faba (Vosa, 1970; Zech, 1973) and the reduced fluorescence chromosome regions of Tulbaghia sp. (Vosa, 1970; Vosa and Marchi, 1972) and Scilla siberica (Vosa, 1973a) correspond to C-banded chromosome regions.

Mechanism of Q-banding. The mechanism of Q-banding has been studied by a number of workers. Quinacrine mustard was initially thought to act by alkylating the N-7 atom of guanine and was, therefore, thought to bind to GC-rich chromosome regions (Caspersson et al, 1968). Recent evidence indicates the fluorescent stain to be specific for AT-rich DNA regions, however (Pachmann and Rigler, 1972; Weisblum and de Haseth, 1972; Michelson et al, 1972). This was also suggested by Comings et al (1975), but they found the decrease

in quinacrine fluorescence to be mainly due to non-histone proteins.

Function of highly repetitive DNA sequences. There appears to be little or no possibility that highly repeated DNA sequences are transcribed and translated into protein. Isolation of RNA from different mouse tissues and hybridization with the light and heavy strands of satellite DNA indicated no complementarity to exist between RNA sequences and satellite DNA (Flamm et al, 1969b; Flamm, 1972). Sequence analysis data indicate that proteins produced from satellite sequences would consist of only 2 - 3 amino acids. Since neither a protein nor a function for such a protein is known, an absence of genetic activity is suggested for satellite DNA.

Many possible functions have been ascribed to satellite DNA. Britten and Kohne (1968; 1969) suggested that highly repeated sequences represented new genetic potential for organisms. They envisioned satellite sequences arising as sudden processes. With time these sequences would be distributed throughout the genome and either alone or by combining with other gene sequences would give rise to new genetic material.

Walker et al (1969) suggested certain "housekeeping" roles for the repeated DNA sequences. They might serve as regions for the initiation of replication or transcription or as specific sites recognized by enzymes involved in recombination. They could be important in centromere recognition or as sites for the pairing of homologous chromosomes

in the prophase stage of meiosis. Finally, they suggested that repetitive sequences might be important in the folding of DNA into the chromosome structure.

Yunis and Yasmineh (1971) suggested that repeated sequences might serve as "fertility barriers" preventing interspecies breeding and encouraging speciation. They further suggested (Yunis and Yasmineh, 1972) that these sequences might serve to protect vital areas of the genome such as centromeres and nucleolus organizers from evolutionary change.

Hsu (1975) proposed heterochromatin to serve as the cell's "bodyguard" to protect euchromatin. This would be accomplished by heterochromatin's forming a layer around the periphery of the nucleus where it would come in contact with damaging chemicals and radiation and would be altered rather than euchromatin.

Finally, Smith (1976) suggested that the presence of highly repeated DNA sequences in eukaryotes represents the natural state of DNA and that only when selection pressure is applied to a given set of nucleotides are unique DNA sequences produced and maintained in the genome.

Support for and against all of these possible functions can be found in the literature (see review of Bostock, 1972). One or more or none of these roles may actually be carried out by highly repeated DNA sequences, but its ubiquity within eukaryotic DNA suggests that it does serve some important function in the genome.

STATEMENT OF THE PROBLEM

In spite of the voluminous quantity of literature that has been generated from the study of chromosome banding patterns and DNA reassociation, only five reports have appeared in which organisms with chromosomes possessing nonlocalized centromeres were studied. The chromosomes of Triatoma infestans exhibited the typical G-banding pattern of alternating dark and light bands when treated with trypsin (Maudlin, 1974) as did those of Tetranychus urticae (Pijnacker and Ferwerda, 1976). Pijnacker and Ferwerda (1976) stated that they also unsuccessfully attempted to detect C-bands in the chromosomes of T. urticae and suggested that the reason for their lack of success might have resulted from the fact that the chromosomes were holokinetic and thus lacked distinct centromeres. Characterization of DNA from Oncopeltus fasciatus (Lagowski et al, 1973), Bombyx mori (Gage, 1974), and Ascaris lumbricoides (Tobler et al, 1972) indicated them not to markedly differ from those of organisms with localized centromeres.

Chromosomes with nonlocalized centromeres have been found in organisms from both the plant and animal kingdoms. They look and behave differently from those with localized centromeres. There are no constricted regions on such chromosomes. The chromosomes are generally more-or-less equal-sized resulting in an inability to distinguish homologous pairs. During the anaphase stage of mitosis either the chromatids remain parallel to each other as they

move toward opposite poles of the cell or the chromosome ends lead slightly. In meiosis, the first division is equational with the chromatids being separated while the second division is reductional resulting in a halving of the chromosome number (Darlington, 1965).

Probably the best studied group of organisms possessing nonlocalized centromeres are members of the genus Luzula, a group of monocotyledonous plants belonging to the rush family Juncaceae. The diploid chromosome number in the group ranges from 6 - 66 (Nordenskiold, 1949; de Noronha Wagner, 1949; Nordenskiold, 1951). Three different centromeric organization patterns have been suggested for the chromosomes of Luzula. The nature of the centromeres of L. purpurea was studied by following the fate of chromosome fragments produced by X-ray irradiation (de Castro et al, 1949; La Cour, 1953). The chromosome fragments were maintained in both experiments moving as though they contained centromeres. This led de Castro et al (1949) to conclude that diffuse centromeres extended along the length of the chromosomes while La Cour (1953) concluded that the chromosomes were polycentric with discrete kinetochores along their length. A study of meiosis of L. purpurea showed that the chromosome ends led in the movement toward the poles in the reductional stage of meiosis (Kusanagi and Tanaka, 1959) and that distinct structures were present on the ends of the chromosomes in the pachytene stage (Kusanagi and Tanaka, 1960). These results indicated the chromosomes

to be dicentric with the centromeres being located on either end of the chromosomes. It now appears that the polycentric organization is the actual one for ultrastructural studies of Luzula purpurea (Braselton, 1971) and L. albida (Lambert, 1971) chromosomes have shown the kinetochores to exist as discrete entities in recesses along the poleward surfaces of the chromosomes. Serial sections of at least some of the L. purpurea chromosomes (Braselton, 1971) showed the distances between adjacent kinetochores to be 0.2 - 0.3 μ.

At the time this project was initiated, Braselton's (1971) and Lambert's (1971) ultrastructural work on the polycentric nature of Luzula chromosomes had not appeared, and the only C-banding patterns that had been reported were those conducted on animal chromosomes. Since C-positive regions were typically found at the centromeres of animal chromosomes, it was assumed, incorrectly as it later turned out, that the same would apply to plant materials. In view of the controversy surrounding the nature of the centromere arrangement in Luzula chromosomes, a study of the C-banding patterns appeared worthwhile. If centromeres were distributed along the entire chromosome surface, then it was reasoned that the entire chromosome would be C-positive; if the chromosomes were dicentric, then two C-positive regions on either end of the chromosomes were expected; if the chromosomes were polycentric, then discrete bands were expected along the length of the chromosomes.

Two species of Luzula, L. purpurea Link. and L. multiflora Lej., were chosen for the study as was Vicia faba, an organism with chromosomes possessing localized centromeres. In this way a comparison would be possible between organisms with different centromeric organizations. Several chromosome techniques were used in the study. All organisms were studied in terms of their normal chromosome constitutions, C-banding patterns, and DNA late-replicating patterns. An additional study of the Q-banding patterns of V. faba and L. purpurea was undertaken. L. purpurea chromosomes were further studied by in situ hybridization of ^{3}H-RNA complementary to rapidly reassociating DNA.

The DNA of L. purpurea was also analyzed. It was reasoned that if the chromosomes were truly polycentric and that if the centromeres were composed of constitutive heterochromatin, an unusual quantity of repeated DNA sequences might be present in the L. purpurea genome and that they might exist as a satellite DNA fraction.

MATERIALS AND METHODS

Culturing of Organisms

Source of organisms. Luzula purpurea Link seeds were the gift of Dr. Miguel Mota, Department of Genetics, Estao Agronomica Nacional, Oeiras, Portugal. L. multiflora Lej. seeds were supplied by the Jardin Botanique de l'Universite Louis Pasteur de Strasbourg, Strasbourg, France. Seeds of Vicia faba were purchased from Carolina Biological Supply, Burlington, North Carolina.

Surface sterilization. Seeds of L. purpurea, L. multiflora, and V. faba were sterilized by soaking in a sterile 10% solution of commercial Clorox for 10 min. Following Clorox removal by rinsing in 4 changes of autoclaved distilled water, the seeds were germinated either in sterile water or on 1% agar.

Tissue culture of L. purpurea. Seedlings that had germinated on 1% agar were gently chopped to injure their roots and transferred aseptically onto medium basically like that previously used in rye embryo callus production (Carew and Schwarting, 1958). The components used in the medium together with their final concentrations are listed in Table 1. Yeast extract was not used in the initiation medium but was added after the callus ceased to grow. The pH of the medium was adjusted to 5.8 prior to the addition of agar, and the medium was sterilized by autoclaving (15 psi,

Table 1. Components of the modified Heller's medium used for the culture of Luzula purpurea. Concentrations given are final concentrations obtained by the addition of small quantities of more concentrated stock solutions. Medium was brought to volume and pHed prior to agar addition.

Constituents	mg/l
I. Salts	
$NaNO_3$	120.00
$CaCl_2 \cdot H_2O$	11.30
KCl	150.00
$MgSO_4 \cdot 7\ H_2O$	50.00
$NaH_2PO_4 \cdot H_2O$	25.00
$MnSO_4 \cdot 4\ H_2O$	0.10
$ZnSO_4 \cdot 7\ H_2O$	1.00
H_3BO_3	1.00
KI	0.01
$AlCl_3$	0.03
$CuSO_4 \cdot 5\ H_2O$	0.03
$FeCl_3$	1.00
$NiCl_2 \cdot 6\ H_2O$	0.06
II. Vitamins	
Nicotinic acid	5.00
Thiamine hydrochloride	1.00
Pyridoxine hydrochloride	1.00
Riboflavin	0.50
Calcium pantothenate	0.50
Folic acid	0.50
Biotin	0.01
Choline chloride	1.00
p-aminobenzoic acid	0.50
Inositol	10.00
III. Other compounds	
2,4-D	2.00

Na_2EDTA	1.00
Yeast extract	1000.00
Sucrose	20000.00
Agar	10000.00

120° C, 20 min). Callus appeared along the roots of the seedlings after approximately 2 weeks in a constant light (3000 lux), constant temperature (28° C) chamber. Subcultures were made, thereafter, at monthly intervals.

Cytological Preparations

Stains. Feulgen stain was made by dissolving 1 gm basic fuchsin in 200 ml boiling distilled water, adding 30 ml 1 N HCl followed by 3 gm potassium metabisulfite to the filtered, cooled solution (50° C), and decolorizing the solution with activated charcoal after overnight dark storage. The stain was refrigerated until it was used (Purvis et al, 1966). Stock Giemsa stain was made by dissolving 0.8 gm Giemsa in 100 ml of a mixture of equal volumes of glycerol and absolute methanol. After shaking for 2 - 3 days on a mechanical shaker, the stain was ready for use (Conn et al, 1960). Alternately, Giemsa's Blood Stain was purchased from Curtin-Matheson Scientific.

Normal squash preparations. Once the secondary roots of Vicia faba and the primary roots of Luzula purpurea and L. multiflora had reached 2 - 3 mm in length, the excised root tips of V. faba and the entire seedlings of L. purpurea and L. multiflora were placed in colcemid (10 µg/ml; GIBCO) at a final concentration of 2 µg/ml in the dark at room temperature for 4 - 6 hr. The root tips were then fixed at least overnight in fixative consisting of 3 parts 95% ethanol : 1 part glacial acetic acid. Immediately before squash

preparations were made, the roots were washed in several changes of distilled water and hydrolyzed in 1 N HCl at 60° C for 10 min. The roots were again rinsed several times in distilled water and stained for 1 hr in feulgen stain. After excess stain was washed off the root tips, they were placed onto 0.1 N HCl-washed microscope slides in a drop of 45% acetic acid, and a coverslip was applied. The slide was gently heated in the flame of a methanol burner, and cell dispersal was facilitated by tapping on the coverslip with the blunt eraser end of a pencil. After another passage through the flame of the methanol burner, excess liquid was removed and the mitotic figures spread by placing the slide between two pieces of absorbent paper and applying pressure with the thumb. The slides were frozen on a block of dry ice, and the coverslips were flipped off with a razor blade. Dehydration was accomplished by placing the slides in 2 changes of 95% ethanol for 10 min. After air-drying, Permount and a coverslip were added (Conger and Fairchild, 1953).

C-banding method. The procedure of Marks and Schweizer (1974) was found to give consistent C-banding results for plant chromosomes after many initial unsuccessful attempts to obtain C-bands using the methods of Arrighi and Hsu (1971), Hsu (1971), Yunis et al (1971), Sumner (1972), Vosa and Marchi (1972), and Dobel et al (1973).

Colcemid-treated (2 μg/ml) excised secondary root tips of Vicia faba or seedlings of Luzula purpurea and L. multiflora

were fixed in ethanol-acetic acid (3 : 1) for 7 and 5 hr, respectively. The fixed material was then transferred into 90% ethanol and stored in the refrigerator (4° C) for 12 - 18 hr until squash preparations were made. Slides used for the squash preparations were washed in 0.1 N HCl and coated with a solution containing 0.1% gelatin-0.01% chromium potassium phosphate that had been heated to near boiling for gelatin dissolution and cooled to 50° C (Arrighi and Hsu, 1971). The meristematic regions of the seedlings and root tips were placed in 45% acetic acid on thoroughly dried gelatin-coated slides and teased apart. A coverslip was added, and the slide was gently heated in the flame of a methanol burner. The cells were dispersed by tapping on the coverslip, and the slide was again gently heated. Excess liquid was removed by placing the slide between two pieces of absorbent paper and applying pressure to the coverslip as mentioned before. Coverslips were removed from dry ice-frozen slides, and the squash preparations were dehydrated by quickly passing them through two changes of 90% ethanol and two changes of absolute ethanol. Following overnight storage at room temperature, DNA of the squash preparations was denatured by incubating the slides in $Ba(OH)_2 \cdot 8\ H_2O$ at 50° C. The optimum $Ba(OH)_2 \cdot 8\ H_2O$ concentrations and times were 6% and 15 - 20 min for Vicia faba, 5% and 15 min for Luzula purpurea, and 4.5 or 5% and 15 min for L. multiflora. To prevent a film from forming on the slides, $Ba(OH)_2 \cdot 8\ H_2O$ (50° C) was poured over slides already in staining jars

in a 50° C water bath. At the end of the denaturation period $Ba(OH)_2 \cdot 8\ H_2O$ was removed by diluting it out under running distilled water. After a thorough rinsing the slides were incubated in 2 X SSC (65° C) for 1.5 - 2.0 hr. Then the slides were again rinsed in running distilled water and stained in Giemsa diluted 1 : 50 with M/15 Sorensen's phosphate buffer (equimolar concentrations of KH_2PO_4 and $Na_2HPO_4 \cdot 2\ H_2O$, pH 6.9). The time required for adequate staining varied but was never greater than 1.5 hr. Staining progress was monitored by removing slides from the staining jar and observing them under the microscope. When staining was adequate, the slides were washed in several changes of distilled water and air-dried prior to the addition of Permount and a coverslip.

Q-banding method. Q-banding experiments were conducted according to the method of Caspersson et al (1969a). Excised secondary root tips of Vicia faba or entire seedlings of Luzula purpurea were treated with colcemid (2 µg/ml) for 6 hr in the dark on a gyrorotary shaker (80 rpm). The root tips were then fixed in absolute ethanol-acetic acid (3 : 1) for 15 min after which they were washed once with absolute ethanol and stored in a final change of absolute ethanol in the refrigerator (4° C) until slides were made approximately 6 hr later. Squash preparations were then made on 1 N HCl - washed microscope slides according to the procedure used in the C-banding method. Following coverslip removal, the preparations were dehydrated for 10 min in absolute ethanol

and stored overnight in the refrigerator in a final change of absolute ethanol.

Prior to staining, the squash preparations were hydrated by transferring stepwise through 80%, 50%, 20%, and 5% ethanol and MacIlvaine's buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, mixed to an appropriate pH). The slides of Vicia faba were then stained in a solution containing 50 µg/ml quinacrine mustard dihydrochloride (Sigma) in MacIlvaine's buffer (pH 4.1) for 20 min (22° C). The optimum buffer pH for Luzula purpurea staining was tested using buffers ranging from pH 4.0 - 7.0 and was determined to be 5.0. Staining time was 20 min as before. Excess stain was removed by washing the slides in 4 changes of MacIlvaine's buffer at the same pH as that of the staining buffer for a total of 2 min. The slides were then drained and mounted in the same buffer. The edges of the coverslips were sealed with clear fingernail polish to prevent buffer evaporation. Slides were examined with a Wild M-20 microscope adapted for use as a fluorescent microscope. The light source was a HBO 200 W mercury vapor lamp, and a BG12 exciter filter and a 530 nm barrier filter were used.

DNA replication patterns. DNA replication patterns were studied by continuous labeling experiments utilizing tritiated thymidine (specific activity--71.5 Ci/mM; ICN Pharmaceuticals, Inc.). Excised secondary root tips of V. faba or entire seedlings of L. purpurea and L. multiflora were placed in 4 ml of ^{3}H-thymidine solution (20 - 25 µCi/ml)

and incubated in the dark on a gyrorotary shaker. Colcemid was added to a final concentration of 2 µg/ml at different times. Colcemid was added to Vicia faba root tips 0, 2, 4, and 6 hr after the start of incubation, and fixation was 10 hr later in each case. Colcemid was added at 0, 0, 2, 4, and 6 hr after the start of incubation of Luzula purpurea seedlings and fixation was at 2, 4, 6, 8, and 10 hr, respectively, after incubation initiation. L. multiflora seedlings were exposed to colcemid 0, 2, 4, 6, and 8 hr after incubation initiation and were fixed 4 hr later in each case. Following overnight fixation in ethanol-acetic acid (3 : 1) squashes were made on 95% ethanol-cleaned slides as described before for normal squash preparations. After a thorough air-drying the slides were covered with autoradiographic stripping film (Kodak AR-10) prepared in the following way (Schmid, 1965).

A thin strip was cut along the outer edge of the glass plate (4 3/4" X 6 1/2") containing the stripping film and discarded to remove film possibly contaminated by handling. The remainder of the film was cut into approximately 12 equal-sized, rectangular sections. The plate was placed in a tray containing 70% ethanol for 3 min after which it was transferred into a tray containing absolute ethanol. Sections were then lifted from the plate with forceps and placed emulsion-side down onto the surface of a tray of distilled water. After the film had spread out, a slide containing the radioactive squash preparation was maneuvered under the

film and lifted out of the water. The stripping film was smoothed and wrapped around the edges of the slide with a camel hair brush, and the slides were allowed to air-dry for several hours in the darkroom. Slides were exposed in the refrigerator in aluminum foil-wrapped slide boxes containing packets of silica gel. After several days (always less than a week) the slides were developed. The appropriate time was determined by developing test slides. Prior to development the stripping film was affixed to the slide by painting a strip along the edge of the film on the backside of the slide with a mixture of Permount and xylene (1 : 5). Slides were developed in Kodak D-19 for 2 min, dipped twice in water, and fixed in Kodak Rapid Fixer for 2 min. The slides were washed in running water for 10 min, air-dried, and stained for 7 min in a Giemsa staining solution containing 100 ml distilled water, 3 ml absolute methanol, 3 ml 0.1 M citric acid, 3 ml 0.2 M sodium dibasic phosphate, and 5 ml Giemsa stain. Finally, the slides were rinsed by dipping twice in distilled water, air-dried, and mounted in Permount.

^{3}H-RNA in situ hybridization. Sonicated Luzula purpurea DNA (2.037 denatured OD_{260}/ml) that had been dialyzed against 0.12 M PB containing 2 mM EDTA was dispensed into 4 test tubes (4 ml/tube) and covered with teflon tape. Denaturation was accomplished by holding the tubes in a boiling water bath for 15 min. The DNA was then reassociated to Cot 1 in a 60° C water bath and fractionated on a HAP (1.6 cm

X 2.5 cm) pre-equilibrated with 0.12 M PB. The temperature of the column was maintained at 60° C by wrapping it with heating tape which was connected to a calibrated rheostat. The entire set-up was placed in a chamber the temperature of which was above room temperature in order to prevent temperature fluctuation of the column. The column was washed with 10 column volumes of 0.12 M PB (60° C) to remove single-stranded DNA. Cot 1-reassociated DNA was eluted from the column with 0.4 M PB (60° C). Fractions containing OD_{260} absorbing material were pooled, concentrated by lyophilization, and dialyzed against two 4 l changes of 0.12 M PB. Following an additional dissociation, reassociation to Cot 1, and fractionation according to the above procedure, the pooled, reassociated fractions were dialyzed against two 4 l changes of distilled water. The dialysate was frozen and lyophilized to dryness.

The lyophilized DNA was dissolved in 0.1 M Tris HCl, pH 7.9, 0.5 mM EDTA (Rae, 1970) and used as a template for RNA synthesis. The reaction mixture for RNA synthesis was a combination of that used by Arrighi et al (1970a) and Rae (1970). The reaction mixture contained 40 mM Tris HCl, pH 7.9, 100 mM KCl, 12 mM $MgCl_2 \cdot 6\ H_2O$, 70 μM EDTA, 2 mM $MnCl_2 \cdot 4\ H_2O$, 4.8 mM β-mercaptoethanol, 120 nmoles GTP, 100 μCi each of ^{3}H-ATP (7.93 Ci/mM), ^{3}H-UTP (21 Ci/mM), and ^{3}H-CTP (22.5 Ci/mM) (New England Nuclear), 5 units of Escherichia coli RNA polymerase (Sigma), and 10 μg of Cot 1-reassociated Luzula purpurea DNA. The total reaction volume

was adjusted to 0.25 ml by the addition of sterile, distilled water. The three radioactive nucleoside triphosphates were added to the reaction tube and dried under a stream of nitrogen prior to the addition of the other reaction components. Incubation was for 3 hr at 37° C. The template was digested by adding 20 ug of pancreatic DNase (RNase free, Worthington Biochemicals) in 0.05 M Tris HCl, pH 7.5 (500 µg/ml) and incubation of the mixture at room temperature for 20 min. The reaction was stopped by the addition of 2 ml of 2 X SSC, 0.5% SDS. The mixture was shaken with an equal volume of water-saturated phenol, and the phases were separated by centrifugation for 10 min at 10,000 rpm (SS-34 rotor) in a refrigerated Sorvall RC2-B centrifuge. The upper aqueous phase was removed, and the lower phenolic phase was re-extracted with an additional 1 ml of 2 X SSC, 0.5% SDS. Following centrifugation as before the aqueous phase was added to the previous one. The combined aqueous phases were placed in dialysis tubing, prepared by heating in a 1 mM EDTA solution and rinsing in several changes of deionized, distilled water, and dialyzed overnight against 2 l of 2 X SSC.

Further purification of the ^{3}H-RNA was accomplished by chromatography on a Sephadex G-50 (Sigma) column (Rae, 1970). The column (1 cm X 40 cm) was prepared by adding Sephadex that had been swollen overnight in distilled water to a glass wool-plugged column. ^{3}H-RNA along with 100 µg of yeast RNA (Sigma) was added to the column, and the column was

washed with distilled water. The radioactivity in the 1 ml fractions was determined by adding 0.01 ml aliquots to toluene-based scintillation cocktail (5 gm PPO, 0.3 gm POPOP/liter of toluene (Cowles and Key, 1972)) to which a drop of Biosolv (Beckman) had been added to facilitate dispersal of the aqueous sample. Counting was done using a Packard scintillation counter. The elution profile of the yeast RNA was determined by combining two 1 ml fractions and determining the OD_{230} using a Beckman Dual-beam Spectrophotometer. Fractions containing high radioactivity were pooled, lyophilized to dryness, and redissolved in 1 ml of 6 X SSC. The TCA-precipitable radioactivity in the ^{3}H-RNA sample was determined by placing a 0.01 ml aliquot onto a glass fiber disc (Type GFC). After having been air-dried, the disc was placed in three successive changes of 0.5% TCA and three changes of 70% ethanol. All the solutions were in an ice bath, and the disc was allowed to remain in each solution for 2 min. The disc was then again air-dried, and the radioactivity on the disc was determined by liquid scintillation counting as before.

Slides of Luzula purpurea used for ^{3}H-RNA in situ hybridization were prepared like those used in the C-banding procedure. The slides were pretreated with pancreatic RNase (Sigma). Any DNase present in the RNase was destroyed by holding the RNase solution (1 mg/ml in 0.02 M sodium acetate, pH 5.0) in a boiling water bath for 3 min. The hot RNase solution was quickly cooled in an ice bath before dilution

with 2 X SSC to a concentration of 100 µg/ml (Jones, 1973b). 50 µl of diluted RNase was added to each slide. Coverslips, previously washed in 0.1 N HCl, were added, and the slides were placed in pre-warmed moist chambers (37° C) made by placing a 2 X SSC-soaked piece of filter paper in plastic petri plates (Gall and Pardue, 1971). The slides were suspended over the filter paper by a rubber washer. After 1 hr at 37° C, the coverslips were removed by placing the slides in a jar containing 2 X SSC. The slides were rinsed successively in 2 more changes of 2 X SSC, 50%, 70%, and 95% ethanol, and air-dried. In situ hybridization was performed according to the method of Timmis et al (1975). DNA was denatured by holding the slides in 0.1 X SSC at 100° C for 30 sec. The slides were then quickly submerged in 0.1 X SSC at 0° C and dehydrated through 50%, 70%, and 95% ethanol. After the slides had been thoroughly air-dried, 100 µl of ^{3}H-RNA in 6 X SSC was added along with 0.1 N HCl-washed coverslips, and the slides were placed in pre-warmed moist chambers (60° C) containing 6 X SSC-soaked filter paper. After 6 hr or 18 hr at 60° C, the coverslips and excess ^{3}H-RNA were removed by dipping the slides in 3 - 4 changes of 2 X SSC. 50 µl of an RNase solution (20 µg/ml in 2 X SSC) and a pre-cleaned coverslip were added to each slide. Following a 30 min incubation at 37° C in a moist chamber with 2 X SSC-soaked filter paper, the slides were rinsed in 3 changes of 2 X SSC and dehydrated by passage through 70% and 95% ethanol. The slides were allowed to thoroughly air-dry,

and autoradiographic stripping film was added by the procedure used in the DNA replication studies. At weekly intervals slides were processed as before to determine the correct exposure time. Slides were stained for approximately 30 min in diluted Giemsa stain made by adding 5 ml stock Giemsa to 95 ml Sorensen's PB, pH 6.9 (Gall and Pardue, 1971). Stain was removed by dilution under running distilled water.

Photographic procedures. Photographs of normal squash preparations (except Luzula multiflora), DNA replication patterns, and ^{3}H-RNA in situ hybridization patterns were taken on Kodak High Contrast Copy Film. The film was developed in D-19 according to the manufacturer's directions. Photographs of C-banding patterns and L. multiflora normal squash preparations were taken on Kodalith Ortho film which was developed in Kodalith developer according to the directions included with the film. A green filter was generally used when taking the pictures of C-bands to create a greater contrast between the banded regions and the remainder of the chromosome. Q-banding photographs were taken with Kodak Panatomic-X film and development was in Microdol-X according to the directions included with the film. Prints were made on Kodabromide paper F2, F3, or F4.

DNA Studies

HAP preparation. HAP was made according to the method

of Miyazawa and Thomas (1965). 2 l of 0.5 M $CaCl_2 \cdot 2 H_2O$ and 2 l of 0.5 M $Na_2HPO_4 \cdot 7 H_2O$ were added to two separatory funnels and allowed to drip at the rate of 5 ml/min into a 4 l beaker containing 400 ml of double-distilled, deionized water which was being stirred at slow speed. The precipitate was washed 4 times by decantation with 4 l quantities of distilled water and allowed to remain overnight in a final change of water. The water was again decanted and another 4 l was added. To this, 100 ml of 40% NaOH (w/w) was added, and the suspension was boiled for 1 hr with simultaneous stirring. After again being washed 4 times with 4 l quantities of distilled water during which many of the fines were poured off, the precipitate was resuspended in 4 l of 0.01 M PB and brought just to boiling. The hot buffer was removed by decantation, and the precipitate was boiled 2 more times in 0.01 M PB, once for 5 min and once for 15 min. Each time the boiling was accompanied by slow stirring, and the buffer was removed by decantation. Finally, the HAP was stored in a refrigerator in 0.001 M PB with a drop of chloroform.

DNA isolation. Two different procedures were used for DNA isolation. The HAP-urea method of Britten et al (1968) was used to isolate DNA for use in dissociation-reassociation experiments while the method of Bendich and Bolton (1967) was used to isolate DNA that was used in the buoyant density studies. Each procedure offers advantages over the other for the particular use to which the DNA was put. The

HAP-urea method permits large quantities of DNA to be isolated, but the homogenization step could possibly permit extensive shearing of the DNA. Since high molecular weight DNA is needed for buoyant density studies, the method of Bendich and Bolton (1967) is more useful since a minimal amount of shearing of the DNA occurs during isolation.

For DNA isolation by the HAP-urea method (Britten et al, 1968), Luzula purpurea callus was suspended in a volume of lysis buffer approximately equivalent to the weight of the callus (20 gm/extraction). The lysis buffer consisted of 8 M urea, 1% SDS, 0.01 M Na_2EDTA, and 1 M sodium perchlorate in 0.24 M PB. The tissue was sheared in an Omni mixer (Lourdes) at a maximum setting of 100 for two 1 min intervals separated by a 2 min cooling period. The cup of the Omni mixer was in an ice bath during the entire procedure. The extract was shaken with an equal volume of chloroform-1% octanol for 1 min. Following a 10 min centrifugation at 10,000 rpm (SS-34 rotor) in a Sorvall RC2-B refrigerated centrifuge, the upper aqueous phase was pipetted off and added to a HAP column (1.4 cm X 16 cm) equilibrated with 0.12 M PB. A glass wool plug was used as the column bed support. The column was washed with 4 column volumes (1 column volume equals approximately 30 ml) of 8 M urea in 0.24 M PB and 4 column volumes of 0.01 M PB. The column was then stirred, and the DNA was eluted with 0.4 M PB. The OD_{230}, OD_{260}, and OD_{280} of each 3 ml fraction were determined, and those fractions in which the 260/280 and 260/230 ratios

approached 2.0 were combined. If an excessive amount of pigment was present in the DNA preparation as is frequently the case when fresh plant tissue is used for DNA isolation, it was removed by using a combination of the following two procedures.

The DNA preparation was repeatedly extracted with water-saturated phenol (Bendich and Bolton, 1967) followed each time by centrifugation for 10 min at 10,000 rpm as described before. When precipitate was no longer observed at the interface of the aqueous and phenolic phases, the aqueous phase was overlayered with 2 volumes of ice-cold 95% ethanol. The layers were mixed, and the precipitated DNA was pelleted by centrifugation at 10,000 rpm for 5 min. DNA was redissolved in 0.1 X SSC, and the SSC concentration was adjusted to 1 X SSC by the addition of the appropriate quantity of 6 X SSC. The DNA was dialyzed against 2 changes of 2 X SSC (4 l/change). If colored contaminants were still present in the DNA sample, they were removed by chromatography on a Sephadex G-50 column (Stern, 1968). The Sephadex was swollen by soaking overnight in 1 X SSC. A column (1 cm X 30 cm) was then packed under slight pressure from a syringe. Glass wool was used as the bed support. After the column had been washed with several column volumes of 1 X SSC, Luzula purpurea DNA was added, and the column was again washed with 1 X SSC. L. purpurea DNA was eluted with the solvent front. The fractions containing 260/280 and 260/230 ratios approaching 2.0 were pooled and dialyzed

against two 4 l changes of 2 X SSC and stored frozen for later use.

For DNA isolation by the method of Bendich and Bolton (1967), callus tissue (approximately 10 gm) was added to an ice-cold mortar and ground for 3 min with a pestle. A small quantity (3 ml) of grinding solution consisting of 1% SDS, 0.1 M Na_2EDTA, and 3 X SSC, pH 7.0, was added, and the grinding was continued for 1 min. Additional grinding solution (8 ml) was added, and the homogenate was transferred to a flask containing an equal volume of chloroform-1% octanol. The flask was rapidly shaken for 30 sec after which the phases were separated by centrifugation at 10,000 rpm for 10 min. The upper aqueous phase was added to a pre-heated flask (60° C) and allowed to remain for 5 min. The extract was rapidly cooled in an ice bath, adjusted to 1 M sodium perchlorate by the addition of the appropriate quantity of 5 M sodium perchlorate, and re-extracted with an equal volume of chloroform-1% octanol. Following centrifugation as before, the upper aqueous phase was added to an ice-cold beaker. Two volumes of cold 95% ethanol were added onto the aqueous phase, and the nucleic acids were precipitated by mixing the two layers by a gently swirling of the beaker. The precipitate was collected on the end of a glass rod and redissolved in 0.1 X SSC. The salt concentration was adjusted to 1 X SSC, and the solution was subjected to pancreatic RNase (Sigma) (final concentration of 25 µg/ml; preheated as before to remove DNase activity) for 30 min at 37° C. This

was followed by extraction with chloroform-1% octanol as before. DNA of the aqueous phase was again precipitated with 95% ethanol and redissolved in 0.1 X SSC. The SSC concentration was adjusted to 1 X SSC, and pigment and RNase were removed by extraction with water-saturated phenol and Sephadex G-50 column chromatography as previously described. The DNA was dialyzed against 1 X SSC and stored frozen for later use.

DNA purity. The purity of the isolated DNA was determined in a number of ways. The OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios were determined for each DNA sample. Samples were also scanned between the wavelengths 220 and 320 nm in a Beckman Dual-beam Spectrophotometer. The hyperchromicity of each DNA sample was determined by heating 3 ml portions in teflon tape-covered test tubes in a boiling water bath for 15 min. The OD_{260} of the denatured sample was then determined, and the percentage hyperchromicity was calculated using the equation:

$$\% \text{ hyperchromicity} = \frac{A - B}{B} \times 100$$

where

A = denatured OD_{260}

B = native OD_{260}

T_m determination. The HAP melting temperature (Flamm, 1972) was determined for both sonicated and native Luzula purpurea DNA isolated by the HAP-urea method (Britten et al, 1968) using sonicated Escherichia coli B DNA (Sigma) as a

control. Approximately 300 μg (6 OD_{260} units) of DNA were added to a HAP column (1.7 cm X 4.0 cm) at 40° C. The column was then exhaustively washed with 0.12 M PB at the same temperature to remove any unadsorbed material. Once the OD_{260} of the eluants approached zero, the column temperature was raised in approximately 5° C increments. This was accomplished by adjusting a rheostat connected to heating tape wrapped around the column. Once the desired temperature was reached, the column was allowed to stabilize for 10 min after which any single-stranded DNA was eluted by washing the column with seven 3 ml aliquots of 0.12 M PB at the same temperature. The OD_{260} of each aliquot corrected for absorbance at OD_{320} was determined, and the total corrected OD_{260} of the 7 fractions was used as the single-stranded DNA eluting at a given temperature.

The melting profiles of Luzula purpurea DNA isolated by both the HAP-urea (Britten et al, 1968) and Bendich and Bolton (1967) methods and Escherichia coli B DNA in 0.12 M PB were also spectrophotometrically determined according to the method of Mandel and Marmur (1968). A Beckman Acta CIII Spectrophotometer equipped with a multi-sample holder and jacketed cuvettes was used for the melting profile determinations. The initial OD_{260} of each sample was approximately 0.5. Sample evaporation was prevented by overlayering the DNA sample in each cuvette with mineral oil and closing the tops of the cuvettes with teflon stoppers. The temperature was raised at the rate of 0.5° C/min using a Lauda temperature

programmer attached to a Lauda circulating water bath filled with 50% glycerol. The OD_{260} values at the different temperatures were corrected for the thermal expansion of water (Table, Mandel and Marmur, 1968). %GC was calculated using the equation:

$$\%GC = 2.44\ (T_m - 81.5 - 16.6 \log M)$$

where

%GC = percent guanine + cytosine

T_m = temperature by which half of the increase in OD_{260} had occurred

M = monovalent cation concentration (for 0.12 M PB = 0.18 M Na^+

Buoyant density determination. The buoyant density of Luzula purpurea DNA was determined in a Spinco Model E Ultracentrifuge. Two samples of L. purpurea DNA that had been isolated at different times by the method of Bendich and Bolton (1967) and one sample of L. purpurea DNA isolated by the HAP-urea method (Britten et al, 1968) were used. Each cell contained approximately 0.4 µg of DNA in 0.01 M Tris HCl (pH 8.1), 0.001 M EDTA. 0.5 µg of Micrococcus luteus DNA was used as a buoyant density marker (1.7245 gm/cm^3). The initial density of the solution was made to 1.691 gm/cm^3 by the addition of saturated CsCl solution. Centrifugation was carried out at 44,000 rpm in a 4-hole An-F rotor for 24 hr (25° C). The cells were scanned with a photoelectric scanning-multiplex system with the monochromator set at 265.4 nm. The buoyant density of L. purpurea DNA was then determined by the method of Vinograd and Hearst (1962) and

the %GC was calculated by the method of Schildkraut et al (1962).

The two Luzula purpurea DNA samples isolated by the Bendich and Bolton method (1967) were then digested with 0.5 mg/ml nuclease-free pronase (Calbiochem) for 2 hr at 37° C. This was followed by extraction with 1 X SSC-saturated phenol as described before to precipitate the protein and repeated ether extractions to rid the deproteinized DNA samples of phenol. Ether extractions were conducted by repeatedly adding ether to the deproteinized DNA sample, mixing it with the DNA sample, and drawing off the ether layer with a Pasteur pipette until the DNA sample was no longer milky in appearance. The DNA samples were then dialyzed against 4 l of 1 X SSC and recentrifuged in the analytical ultracentrifuge as described before.

DNA reassociation. DNA at an approximate concentration of 2 OD_{260}/ml in 2 X SSC was fragmented in 10 ml quantities with a Branson Sonified Model W-125 fitted with a microtip. The DNA solution was placed in a rosette sonication vessel which was in turn placed in an ice bucket during the entire sonication period. Sonication was for a total of 50 min in 5 min intervals followed by 2 min cooling periods. The size of the DNA fragments was determined by boundary sedimentation in a Spinco Model E Ultracentrifuge using a 4-hole An-F rotor. The DNA was dissolved in a solution containing 0.2 M NaCl, 0.1 mM EDTA, and 0.1 M PB (pH 7.0). Centrifugation was at 50,000 rpm (25° C). Scans of the sedimenting DNA were made

at 6 min intervals for a total of 54 min. The molecular weights were then determined by the method of Studier (1965). The hyperchromicity of the sonicated DNA samples was determined by heating them in a boiling water bath as was done for native DNA.

If concentrated samples were needed for DNA reassociation experiments in order to obtain large Cot values in relatively short periods of time, the samples were concentrated by lyophilization and then dialyzed against 0.24 or 0.48 M PB. Other DNA samples were dialyzed against 0.06 or 0.12 M PB. All PB contained 2 mM EDTA for metal chelation (Britten et al, 1974).

The DNA reassociation profile of Luzula purpurea was determined using Escherichia coli B DNA as a control. DNA samples were denatured in 1 or 2 ml portions as in the hyperchromicity determination except when long incubation times were needed. Then the DNA samples were placed either in small screw cap vials covered with teflon tape onto which the caps were tightly screwed during reassociation or in sealed microliter pipettes when high concentrations were used. For reassociation, the tubes or vials were placed in a 60° C Temp block, and the sealed microliter pipettes were placed in a 60° C incubator.

DNA reassociation experiments were conducted over Cots ranging from 0.1 - 100 for E. coli and 0.01 - 6000 for L. purpurea DNA. The incubation times required for the different Cot values were determined using the equation:

$$\text{Cot} = \frac{\text{Denatured } OD_{260}}{2} \text{ X Time of Incubation (Hr)}$$

(Church, 1973).

After incubation to the desired Cot value, samples were fractionated according to the HAP batch method of Flamm (1972). A 2 ml packed volume of HAP in a tapered centrifuge tube was used for the fractionation of each sample. The HAP was pre-equilibrated with 0.12 M PB by repeatedly suspending it in 0.12 M PB and pelleting it by centrifugation in a table-top centrifuge. The centrifuge was in a chamber at 60° C which was also the temperature to which the packed HAP was heated prior to sample fractionation. The DNA samples were added to the HAP along with enough 0.12 M PB to make a total volume of 3 ml. In cases where the molarity of the PB in the sample was above 0.12 M the molarity was adjusted to 0.12 M by addition of the appropriate quantity of water at 60° C. The HAP was resuspended by vortexing and pelleted by centrifugation for approximately 1 min in the 60° C chamber. The liquid was drawn off with a Pasteur pipette, and the HAP was washed 5 more times with 3 ml volumes of 0.12 M PB (60° C). This was followed by six 3 ml washes with 0.4 M PB (60° C) for elution of reassociated DNA. The OD_{260} and OD_{320} of each wash were determined and the OD_{320} was subtracted from the OD_{260} to correct for absorbance due to the presence of HAP. The total corrected OD_{260} eluted with 0.12 M PB and 0.4 M PB was determined, and the percent reassociation was determined by the equation:

$$\% \text{ Reassociated DNA} = \frac{A + B}{A + B + C}$$

where

A = total corrected OD_{260} eluted with 0.4 M PB

B = A X % hyperchromicity

C = total corrected OD_{260} eluted with 0.12 M PB

Since DNA reassociation experiments were conducted at different PB concentrations, the Cots were corrected for the increase or decrease in Na^+ concentration from the normal 0.18 M Na^+ in 0.12 M PB using the table in Britten (1970), and the data were plotted as equivalent Cots.

RESULTS

Normal Squash Preparations

Different mitotic stages from feulgen-stained squash preparations of the root tips of *Vicia faba*, *Luzula purpurea*, and *L. multiflora* are shown in Figures 1 - 3. The diploid chromosome complement of *V. faba* consists of 12 chromosomes (Fig. 1c). There are two large metacentric chromosomes (M chromosomes) each of which contains a secondary constriction or nucleolus-organizer region. In the remaining 5 pairs of chromosomes, referred to as S chromosomes, the centromeres are located near the end of the chromosomes. The centromeres are most evident on the M chromosomes, but they can also be seen on some of the S chromosomes (Fig. 1c).

The diploid chromosome number for *L. purpurea* is 6 (Fig. 2) while that for *L. multiflora* is 36 (Fig. 3). The chromosomes of *L. purpurea* are much larger than those of *L. multiflora*, but in each organism all of the chromosomes are approximately the same size. Those of *L. purpurea* are approximately 6 μ in length (Fig. 2c) while those of *L. multiflora* measure 1 μ in length (Fig. 3d). In both organisms primary and secondary constrictions are lacking in all chromosomes. An unusual feature of the chromosomes of the two organisms is their apparent stickiness. Numerous inter- and intra- chromosomal fibers are routinely observed in squash preparations of both organisms (Figs. 2d; 2e; 3d).

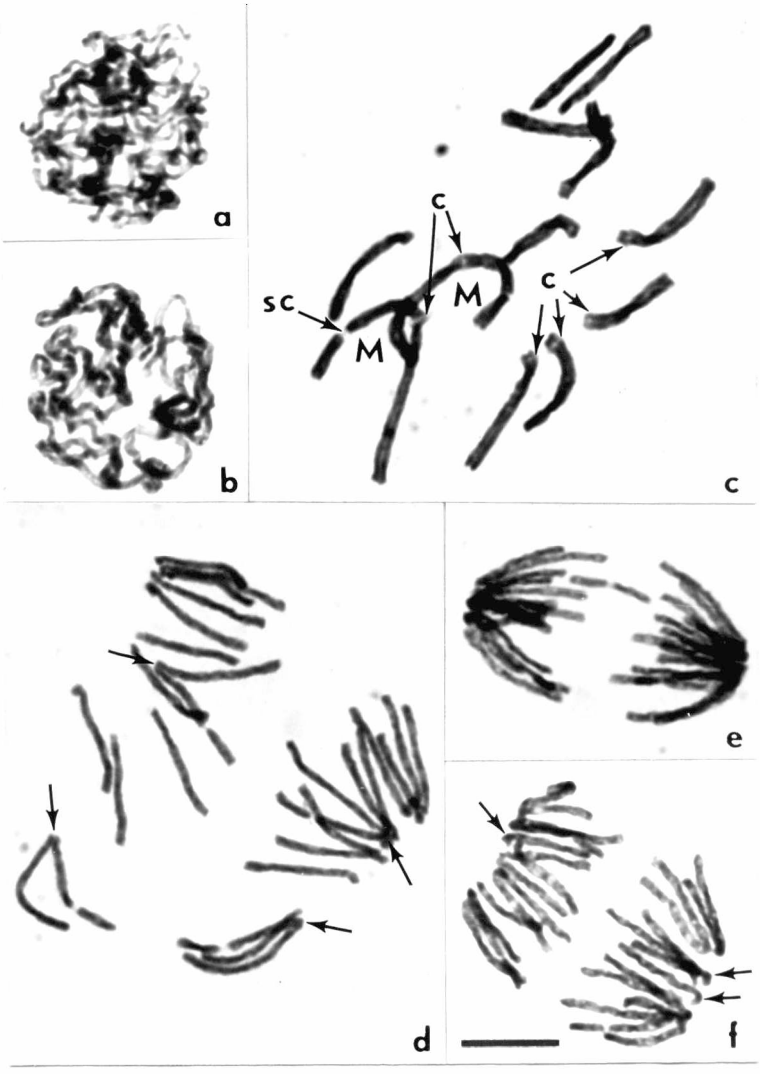
Figure 1. Vicia faba mitotic figures (2N = 12) from feulgen-stained squash preparations. Slides were prepared as described in Materials and Methods. Bar represents 10 μ.

a - b. Two stages of prophase.

c. Metaphase showing the primary and secondary constrictions of the M chromosomes and the primary constrictions of other chromosomes.

d - f. Anaphase stages depicting the mode of chromosome movement toward poles of cells.

M = M chromosomes; c = primary constriction; sc = secondary constriction.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M
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A comparison of the mitotic stages of Vicia faba (Fig. 1) with those of Luzula purpurea (Fig. 2) and L. multiflora (Fig. 3) reveals many of the differences which characteristically exist between chromosomes with localized and nonlocalized centromeres. The only mitotic stage in which the chromosomes have similar appearances is prophase (Figs. 1a; 1b; 2a; 3a). In all cases the chromosomes exist as long, thin fibers with no indication of primary or secondary constrictions. By metaphase, constrictions are very apparent in chromosomes with localized centromeres (Fig. 1c) while none are observed in either late prophase (Figs. 2b; 3b; 3c) or metaphase (Figs. 2c; 2d; 2e; 3d) chromosomes containing nonlocalized centromeres. The two chromatids of each metaphase chromosome are generally distinct along at least a part of their length if localized centromeres are present (Fig. 1c). The chromatids making up chromosomes with nonlocalized centromeres usually lie so close together that individual chromatids cannot be identified (Figs. 2c; 2e; 3d). If, as is rarely the case, a metaphase spread is found in which the chromatids are distinct (Fig. 2d), the chromatids will be seen to lie parallel to one another along their entire length. A final difference between chromosomes with nonlocalized and localized centromeres is the difference in their movement during the anaphase stage of mitosis. Chromosomes with localized centromeres characteristically exhibit V- (Fig. 1d) or J- (Fig. 1f) shapes as they move toward the poles of the cell. The V-shaped configurations

Figure 2. Luzula purpurea mitotic figures (2N = 6) from feulgen-stained squash preparations. Slides were prepared as described in Materials and Methods. Bar represents 10 μ.

a. Prophase.

b. Late prophase.

c. Metaphase showing lack of primary and secondary constrictions in chromosome complement.

d. Metaphase showing chromatids comprising each chromosome. Arrows point to interconnecting fibers between chromosomes and chromatids.

e. Metaphase figure with interconnecting fibers between chromosomes.

f. Early anaphase showing parallel movement of chromatids toward poles of cells.

g. Late anaphase depicting chromatid movement as in 2f above but with chromatid ends leading slightly.

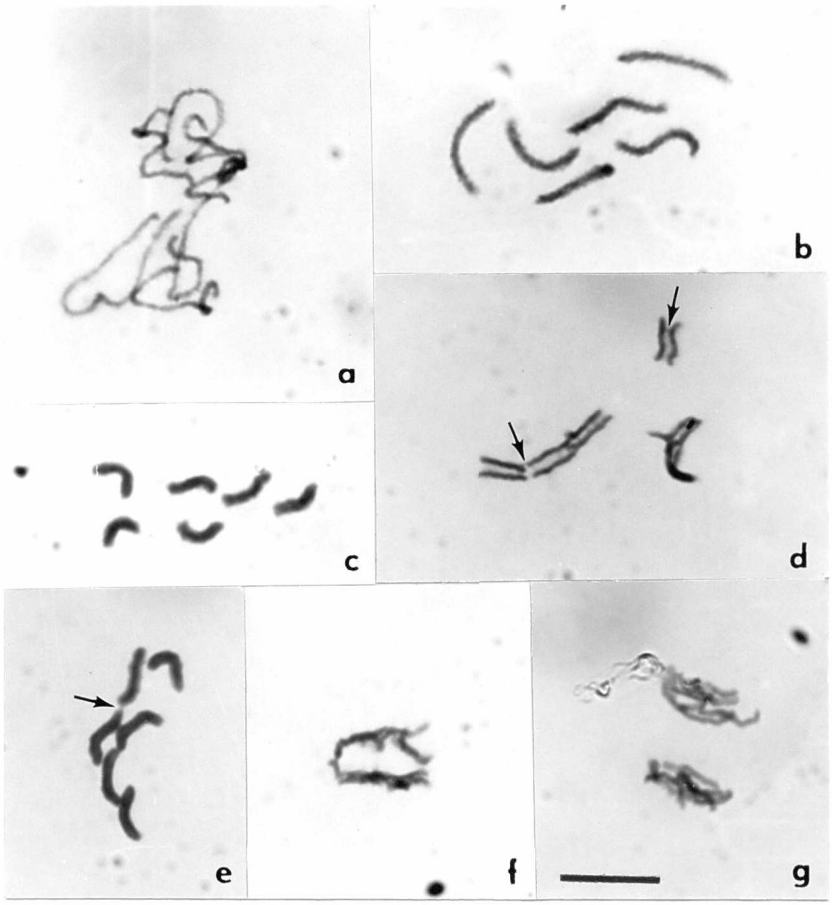
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Figure 3. Luzula multiflora mitotic figures (2N = 36) from feulgen-stained squash preparations. Slides were prepared as described in Materials and Methods. Bar represents 5 μ.

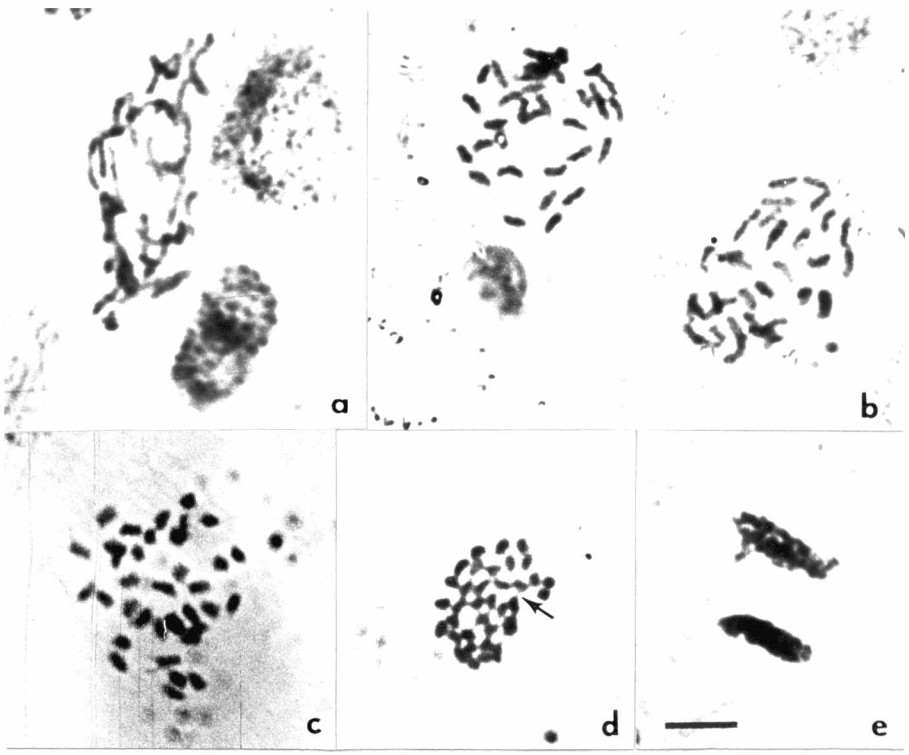
a. Early prophase stage with many overlapping chromosomes.

b. Two later prophase stages with distinct chromosomes.

c. Prometaphase.

d. Metaphase spread showing interchromosomal fibers.

e. Anaphase showing parallel movement of chromatids to poles of cell.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result during metacentric chromosome movement while the J-shaped configurations result during submetacentric or subtelocentric chromosome movement. The major portion of the chromosome which lacks a centromere moves perpendicular to the equatorial plate lagging behind the centromere-containing portion of the chromosome (Figs. 1d; 1e; 1f). The chromosomes of organisms with nonlocalized centromeres move parallel to the equatorial plate during anaphase (Figs. 2f; 3e) with the chromosome ends sometimes leading slightly (Fig. 2g).

DNA Replication Patterns

Vicia faba. The late-replicating regions of V. faba DNA are localized within restricted regions of interphase nuclei (Fig. 4a), prophase chromosomes (Fig. 4b), and metaphase chromosomes (Figs. 4e; 4f). The late-replicating regions do not appear to be centromeric (see arrows in Figs. 4b; 4d; 4e; 4f) but are concentrated in regions of the chromosomes close to the centromeres. As DNA replication patterns from chromosomes exposed to ^{3}H-thymidine at successively earlier stages of S are studied (Figs. 4c; 4d), the label patterns are discontinuously distributed throughout the chromosomes until eventually label is located all along their lengths (Fig. 4g).

Luzula purpurea. The DNA replication patterns of L. purpurea chromosomes can be seen in Figures 5 - 7. The

Figure 4. DNA replication patterns in Vicia faba nuclei and chromosomes. ^{3}H-thymidine continuous labeling experiments were carried out as described in Materials and Methods. Bar represents 10 μ.

a. Distribution of late-replicating DNA regions in interphase nuclei.

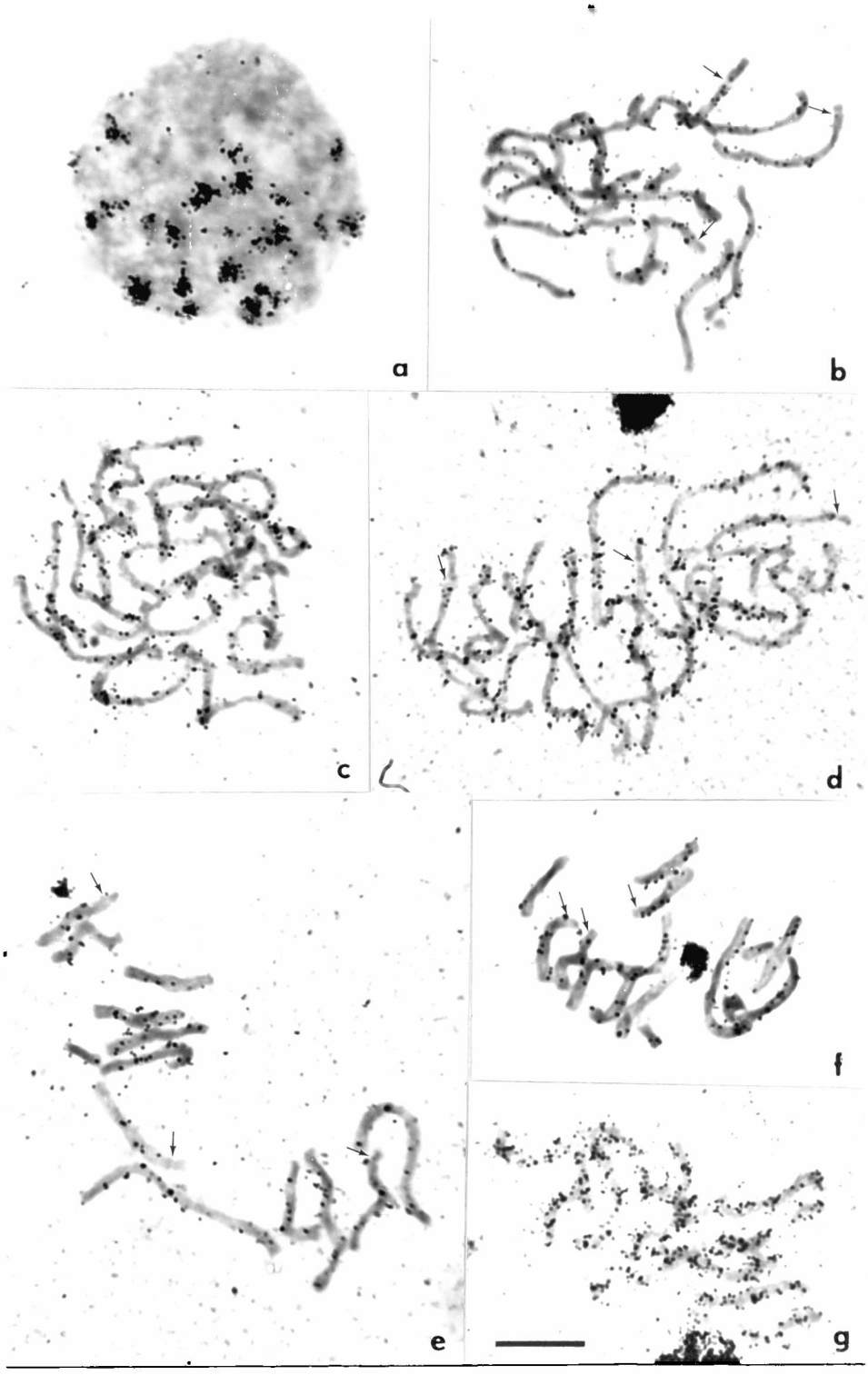
b. DNA late-replicating region distribution in prophase chromosomes.

c - d. Discontinuous labeling patterns in prophase chromosomes replicated successively earlier in the S stage of interphase.

e - f. Distribution of DNA late-replicating regions in metaphase chromosomes.

g. Metaphase figure showing labeling patterns in chromosomes exposed to ^{3}H-thymidine during the early stage of S.

Arrows point to centromere regions of some of the chromosomes.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photographs in Figure 5 are of cells exposed to ^{3}H-thymidine during the late stages of DNA replication. DNA late-replicating regions exhibit a random though restricted distribution in interphase nuclei (Fig. 5a) quite unlike the situation in Vicia faba where late replicating regions are localized in certain regions of the nucleus (Fig. 4a). The distribution of DNA late-replicating regions is more evident in nuclei that have progressed into early prophase (Fig. 5b; 5c) and late prophase (Fig. 5d; 5e). Labeled regions are interspersed with unlabeled ones throughout the length of the chromosomes. Three different late-replicating patterns can be discerned in the late prophase chromosomes. One pair of chromosomes contains three DNA late-replicating regions. One region is located on either end of the chromosome, and another is located near the middle of the chromosome. In another pair of chromosomes, late-replicating regions are restricted to one end and comprise approximately one-half of the total chromosome length. The final chromosome pair contains DNA late-replicating regions throughout the length of the chromosome. The chromosome pairs have been arbitrarily numbered 1, 2, and 3, respectively (Figs. 5d; 5e).

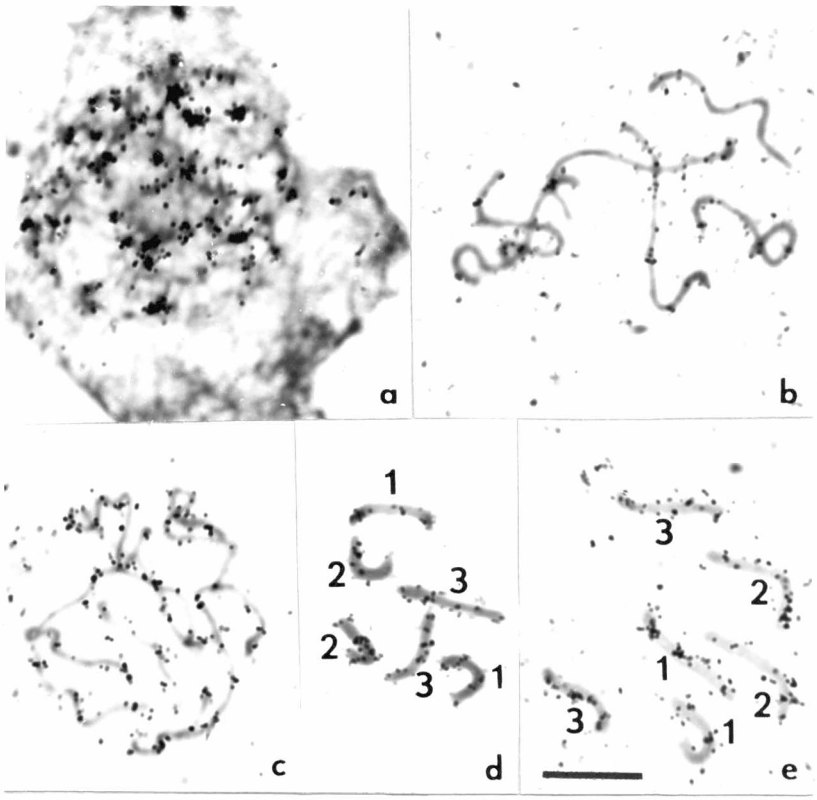
Examination of late prophase stages prepared from nuclei that were in progressively earlier stages of DNA replication when first exposed to ^{3}H-thymidine shows the grain number to progress from practically none (Fig. 6a) where nuclei were in the final stages of DNA replication to

Figure 5. DNA late-replication patterns in Luzula purpurea nuclei and chromosomes. Continuous labeling experiments were conducted according to the procedure described in Materials and Methods. Bar represents 10 μ.

a. Distribution of DNA late-replicating regions in interphase nuclei.

b - c. Discontinuous labeling patterns in early prophase chromosomes exposed to ^{3}H-thymidine late in the S stage of interphase.

d - e. Discontinuous labeling patterns in very late prophase chromosomes exposed to ^{3}H-thymidine late in the S stage of interphase.


a
b
c
1
2
3
2
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1
d
3
2
1
2
3
1
e

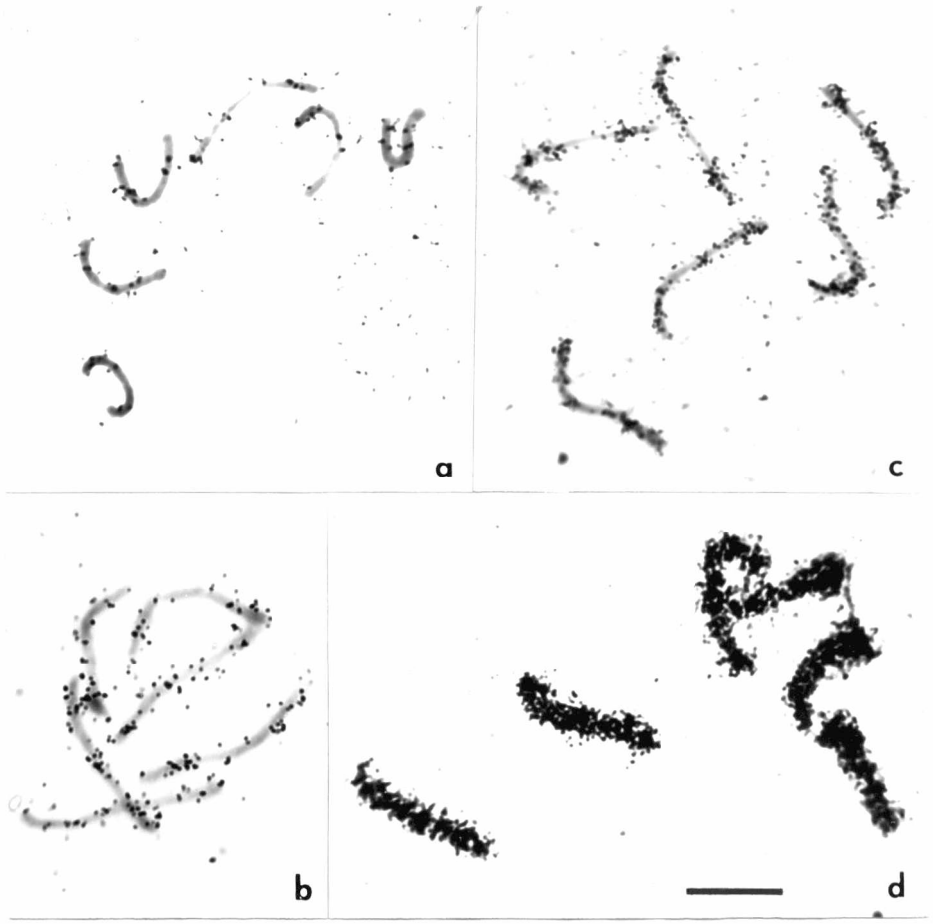
Figure 6. DNA replication patterns in Luzula purpurea chromosomes. ^{3}H-thymidine experiments were conducted according to the procedure described in Materials and Methods. Bar represents 10 μ.

a. Labeling pattern in chromosomes exposed to ^{3}H-thymidine very late in the S stage of interphase.

b. Label pattern in chromosomes exposed to ^{3}H-thymidine earlier in the interphase S stage.

c. Label pattern in chromosomes exposed to ^{3}H-thymidine near the beginning of the S stage of interphase.

d. Total label pattern in chromosomes exposed to ^{3}H-thymidine at the beginning of the S stage of interphase.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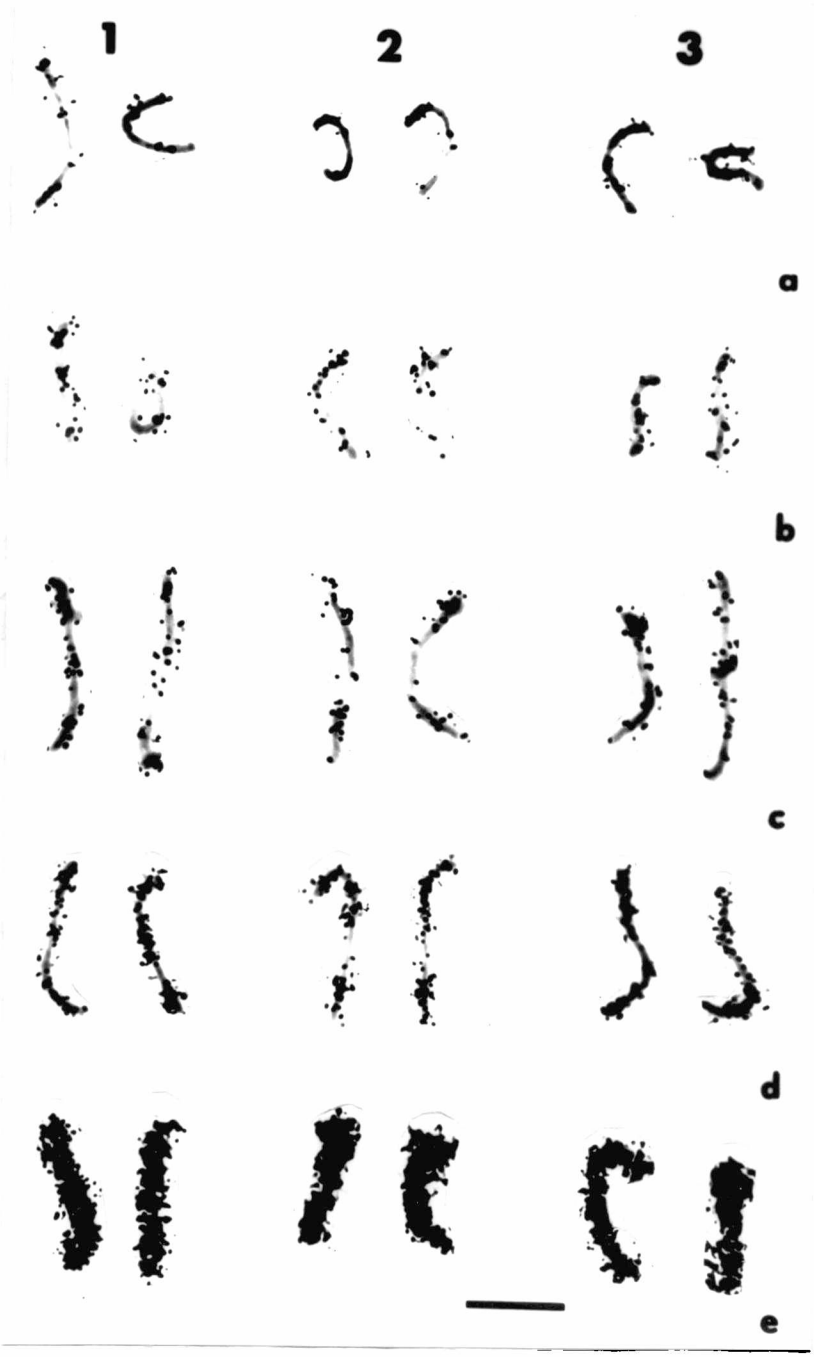
total chromosome labeling (Fig. 6d) where nuclei began DNA replication after ^{3}H-thymidine addition. The chromosomes from nuclei already undergoing DNA replication when radioisotope was added exhibit an intermediate degree of labeling (Fig. 6b; 6c) with the grain number being dependent upon the amount of DNA replication that occurred prior to the time of ^{3}H-thymidine addition.

Chromosomes from nuclei undergoing successively longer periods of DNA replication in the presence of ^{3}H-thymidine exhibit characteristic labeling patterns enabling homologous chromosomes to be identified and their replication patterns to be determined (Fig. 7). As one progresses to earlier and earlier stages of S, the DNA late replication pattern (Fig. 7a) for chromosome pair 1 is maintained (Fig. 7b; 7c). DNA replication occurs at the ends and middle of the chromosomes. The next earlier portion to be replicated is the region between the middle and the ends of the chromosomes with replication in one end being completed before the other (see arrow, Fig. 7d). Chromosome pair 2 maintains the same replicating pattern as that of the late replicating one (Fig. 7a) for only a short time (Fig. 7b). Then the DNA in the terminal half of the unlabeled chromosome region replicates (Fig. 7c). Replication continues in the already labeled chromosome regions (Fig. 7d) until DNA in the remaining unlabeled chromosome region is replicated. Chromosome pair 3 exhibits the same general over-all labeling pattern throughout the entire period of DNA replication (Figs. 7a;

Figure 7. Pairing of Luzula purpurea homologous chromosomes according to their DNA replication patterns. Bar represents 10 μ.

a - d. Arrangement of chromosomes replicated at successively earlier stages of S in pairs on the basis of their labeling patterns.

e. Totally labeled chromosomes showing inability to detect homologues.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3
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7b; 7c; 7d). Finally, all chromosome pairs are totally labeled and can no longer be distinguished from one another (Fig. 7e).

Luzula multiflora. DNA late-replicating regions are distributed throughout the interphase nuclei of L. multiflora (Fig. 8a). The late-replicating patterns in L. multiflora chromosomes fall into one of three categories. This is particularly evident in prophase chromosomes (Fig. 8b; 8d). Some chromosomes exhibit a concentration of label at one end; other chromosomes show label concentration at both ends; the remaining chromosomes exhibit label at both ends and near the center. These general replication patterns are also obvious in contracted metaphase chromosomes exposed to ^{3}H-thymidine late in the S stage of interphase (Fig. 8c). Some of the chromosomes exposed to ^{3}H-thymidine at earlier stages of S (Fig. 8e) still exhibit the general replication pattern, but chromosomes exposed to ^{3}H-thymidine at the beginning of S are totally labeled and are indistinguishable from one another (Fig. 8f).

C-banding Patterns

Vicia faba. Treatment of squash preparations of V. faba by the C-banding technique resulted in the differential staining of nuclei and chromosomes (Fig. 9). The C-positive regions of interphase nuclei were restricted in their distribution (Fig. 9a) in a similar way that DNA late-replicating

Figure 8. DNA replication patterns in Luzula multiflora nuclei and chromosomes. ^{3}H-thymidine experiments were conducted according to the procedures described in Materials and Methods. Bar represents 5 μ.

a. Distribution of DNA late-replicating patterns in interphase nuclei.

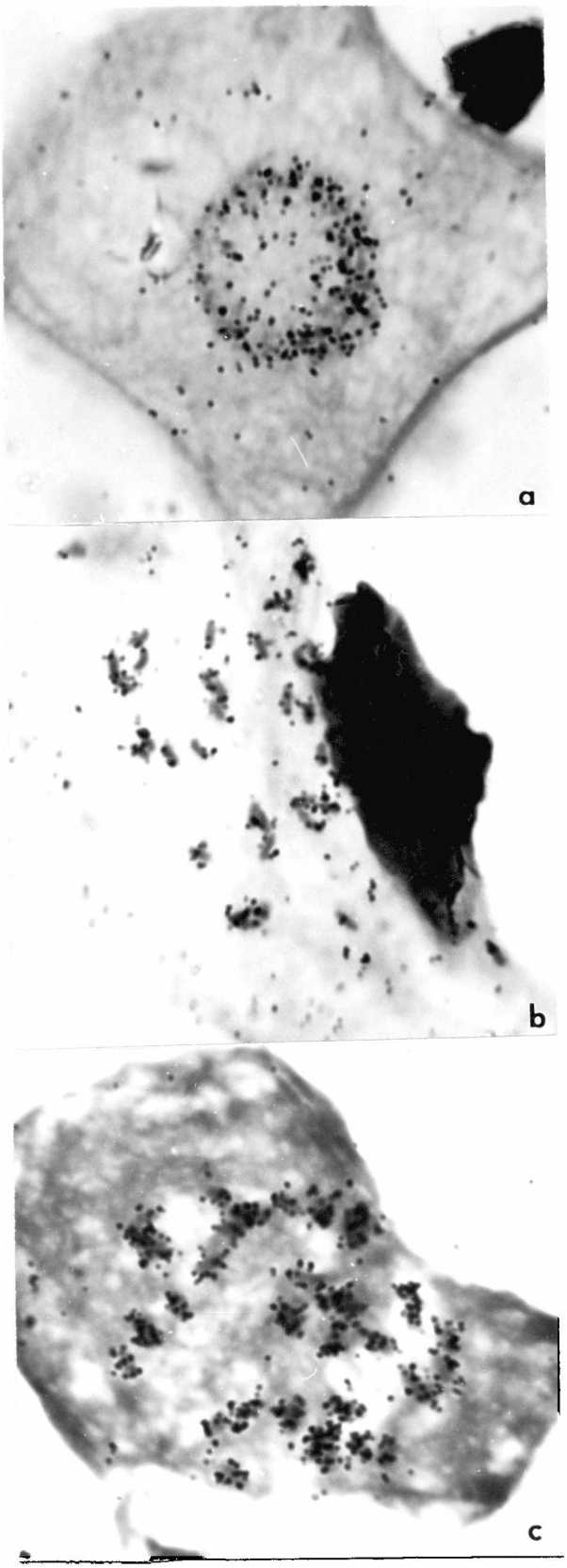
b. Distribution of extremely late DNA replication patterns in prophase chromosomes.

c. Distribution of DNA late-replicating patterns in metaphase chromosomes.

d. Distribution of DNA late-replication patterns in prophase chromosomes that were at a slightly earlier stage of S than those in 8b above when ^{3}H-thymidine was added.

e. Distribution of label over prophase chromosomes exposed to ^{3}H-thymidine early in the S stage of interphase.

f. Totally labeled metaphase chromosome complement exposed to ^{3}H-thymidine at the beginning of the S stage of interphase.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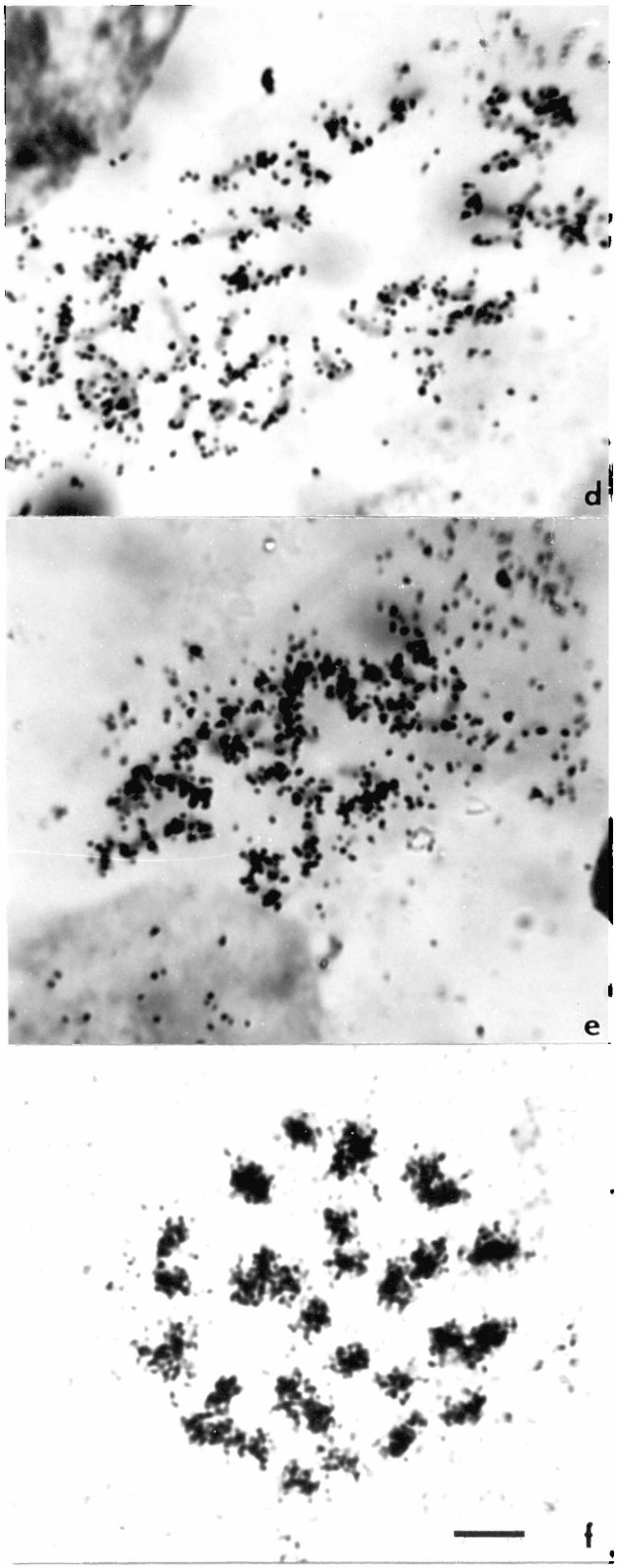
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regions were (Fig. 4a). Prophase chromosomes also exhibited a localized distribution of C-positive regions (Fig. 9b) with the C-positive regions being concentrated toward one end of the chromosomes. This uneven distribution is much more evident in metaphase (Figs. 9c; 9d) and anaphase (Fig. 9e) figures. The C-positive regions are located in the pericentromeric regions of the long arms of the S chromosomes and on either side of the centromeric region of the M chromosomes (Fig. 9d). C-positive material is absent from the secondary constriction of the M chromosomes. The C-positive material has a distinct appearance in each chromosome enabling homologous chromosomes to be identified as described later. The number of C-positive regions varies from one region in one chromosome pair to as many as three in two chromosome pairs. The pericentromeric localization of C-positive regions is also indicated from their leading in the movement of chromosomes toward the poles of the cell in anaphase (Fig. 9e).

Luzula purpurea. The distribution of C-positive regions in L. purpurea nuclei and chromosomes (Figs. 10; 11) differs markedly from those of Vicia faba (Fig. 9). C-positive regions are randomly distributed throughout interphase nuclei (Fig. 10a). This random distribution is also evident in early prophase chromosomes (Figs. 10b; 10c) where C-positive regions can be seen all along the length of the chromosomes. At later prophase stages (Figs. 10d; 10e; 10f) the C-banded arrangement in each chromosome has a distinct

Figure 9. C-banding patterns of Vicia faba nuclei and chromosomes. Experiments were conducted as described in Materials and Methods. Bar represents 10 μ.

a. Interphase nucleus showing localized C-positive regions.

b. Prophase spread showing that C-positive regions are restricted to certain chromosome regions.

c - d. Metaphase chromosomes showing distribution of C-positive regions.

e. Anaphase mitotic stage showing a leading of C-positive chromosome regions toward poles of the cell.

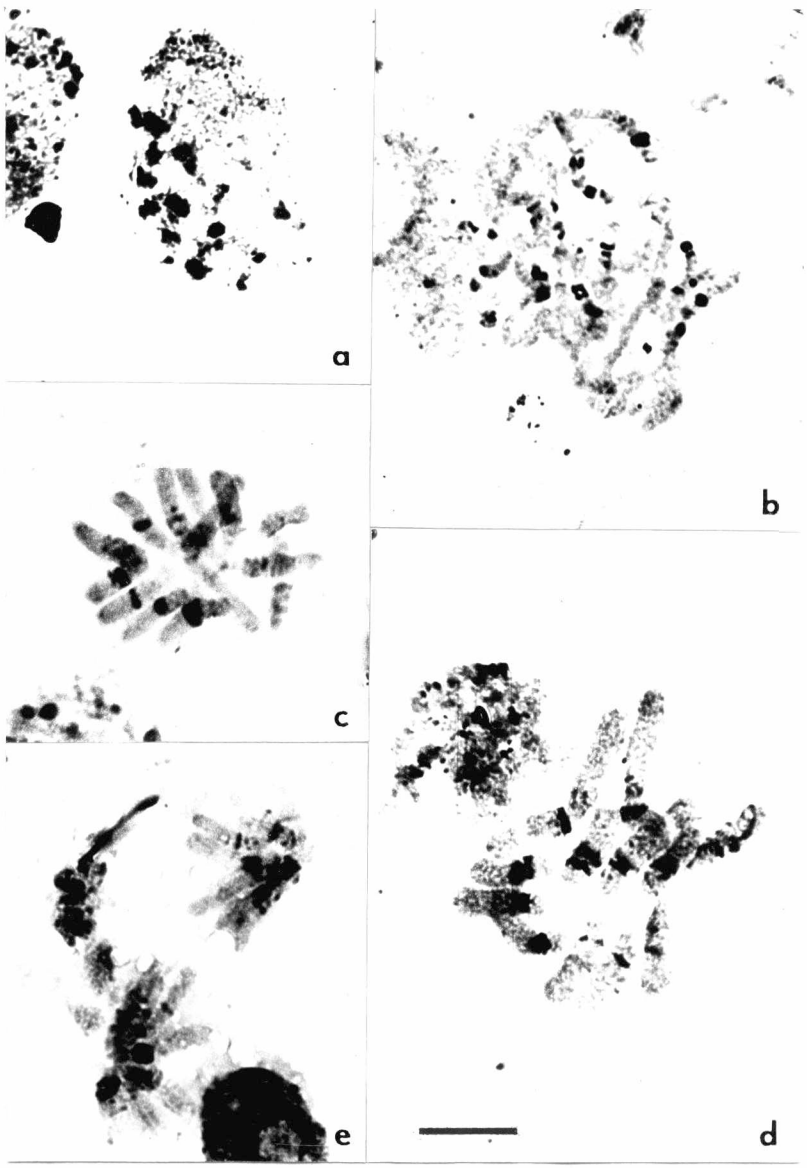
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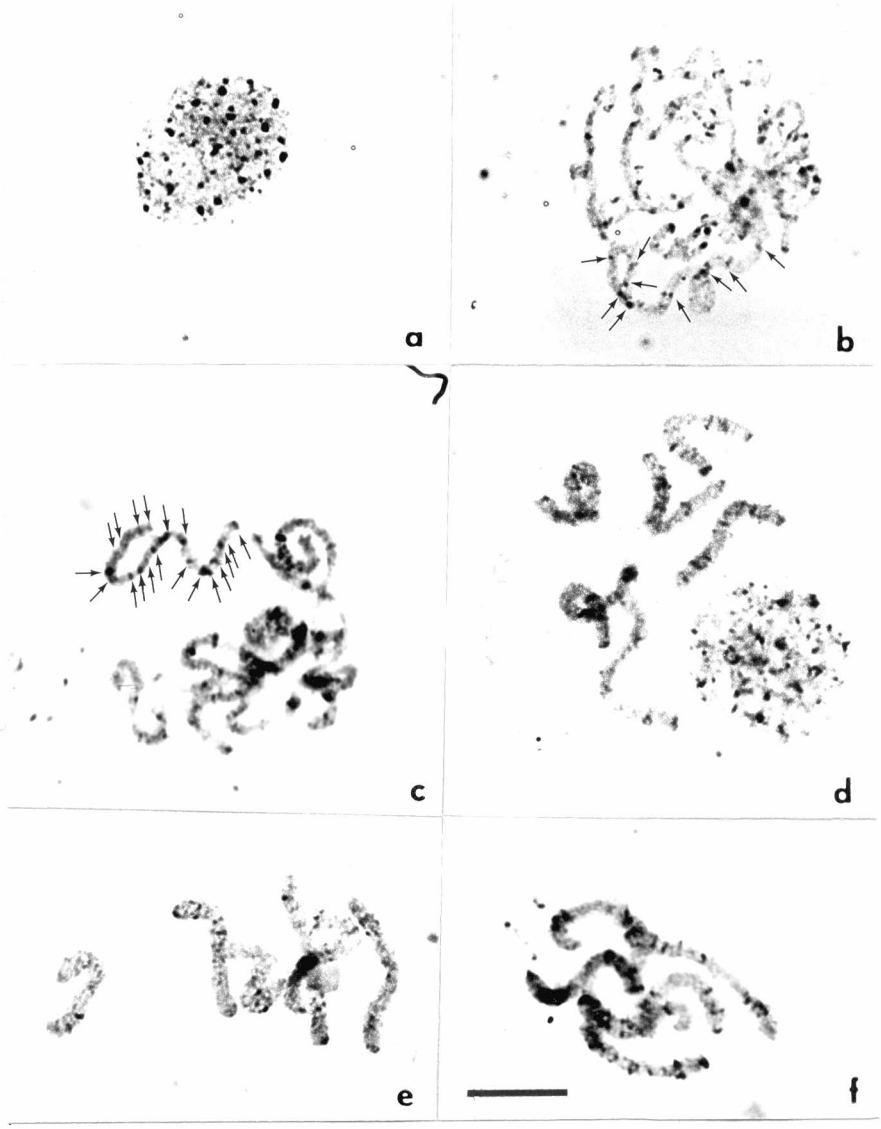
Figure 10. C-banding patterns of Luzula purpurea nuclei and and chromosomes. Details of experimental procedures are given in Materials and Methods. Bar represents 10 μ.

a. Interphase nucleus showing random distribution of C-positive regions.

b - c. Early prophase figures showing multiple C-positive regions to be present in all chromosomes.

d - f. Late prophase figures showing distribution of multiple C-positive regions in chromosomes.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pattern. This is more evident in the interpretative drawings of the C-band arrangements that have been made of chromosomes in different mitotic stages (Fig. 11). At early prophase (Figs. 11a; 11b) there is much overlapping of the chromosomes resulting in an inability to follow most of them from one end to the other and causing many of the C-banded regions to be obscured. In the chromosomes that have little or no overlapping, however, C-banded regions are distinctly distributed from one end of the chromosome to the other with no given pattern for their arrangement being evident. By the time the chromosomes have progressed to later prophase (Figs. 11c; 11d; 11e; 11f), distinct arrangements for the C-banded regions are present. One pair of chromosomes contains C-bands which extend from one end of the chromosome to the other at more-or-less regular intervals. Another pair of chromosomes contains C-bands on either end of the chromosomes while the final pair contains C-bands mostly on one end of the chromosomes. This arrangement is also found in metaphase chromosomes (Figs. 11g; 11h). Few bands are seen in metaphase chromosomes in comparison to the number present in prophase chromosomes indicating that each band is probably composed of more than one C-positive region.

Luzula multiflora. The distribution of C-positive regions in L. multiflora interphase nuclei (Fig. 12a) closely resembles their distribution in L. purpurea nuclei (Fig. 10a). The C-positive regions are randomly distributed throughout

Figure 11. Comparison of C-banded chromosome regions in different mitotic stages of Luzula purpurea. Experimental methods are described in Materials and Methods. Bar represents 10 μ.

a. C-band distribution in prophase chromosomes.

b. Interpretative drawing of 11a.

c. C-band distribution in late prophase chromosomes.

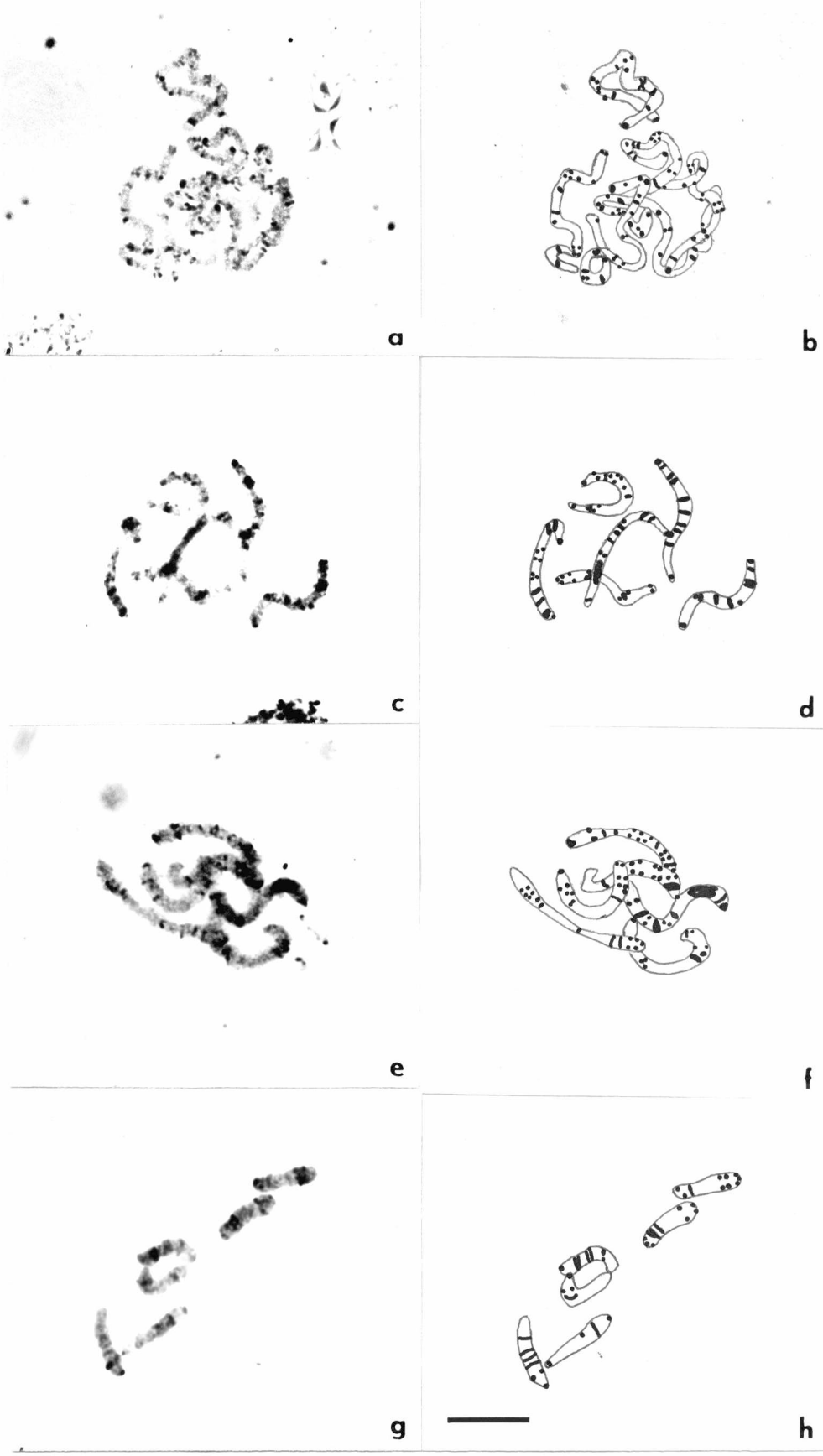
d. Interpretative drawing of 11c.

e. C-band distribution in late prophase chromosomes.

f. Interpretative drawing of 11e.

g. C-band distribution in metaphase chromosomes.

h. Interpretative drawing of 11g.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the entire nucleus. Multiple C-positive regions are present in at least some of the chromosomes in the prophase stage of mitosis (Fig. 12b) although chromosome overlapping precludes the observation of many of the chromosomes. The nature of the distribution of C-positive chromosome regions is much more apparent in late prophase (Fig. 12c) and metaphase (Fig. 12d) preparations. At least 3 C-band distributions are easily discerned. Many chromosomes have 3 C-positive regions--one on each end and one near the middle; other chromosomes have 2 C-positive regions--one on either chromosome end; several chromosomes have a single C-band located at one end of the chromosome.

Q-banding Patterns

Vicia faba. Distinct banding patterns were produced after staining with quinacrine mustard (Fig. 13). Interphase nuclei have localized brightly fluorescent regions (Fig. 13b) as do the chromosomes (Figs. 13a; 13b; 13c). The most distinct Q-banding patterns are observed on the M chromosomes (see large arrows, Figs. 13a; 13b; 13c). Two brightly fluorescent regions are present on one side of the centromere while one is present on the other side. In addition, another less brightly fluorescent region is present between the centromere and the secondary constriction. The region of the chromosome around the secondary constriction exhibits dull fluorescence. The Q-positive regions of the remaining chromosomes are much less clear, but close study reveals a

Figure 12. C-banding patterns of Luzula multiflora nuclei and chromosomes. Experimental procedures are described in Materials and Methods. Bar represents 5 μ.

a. Interphase nucleus demonstrating multiple, randomly distributed C-positive regions.

b. Early prophase preparation showing multiple C-positive regions on many of the chromosomes.

c. Late prophase chromosomes exhibiting three different C-banding patterns.

d. Metaphase chromosomes demonstrating multiple C-positive sites on many of the chromosomes.

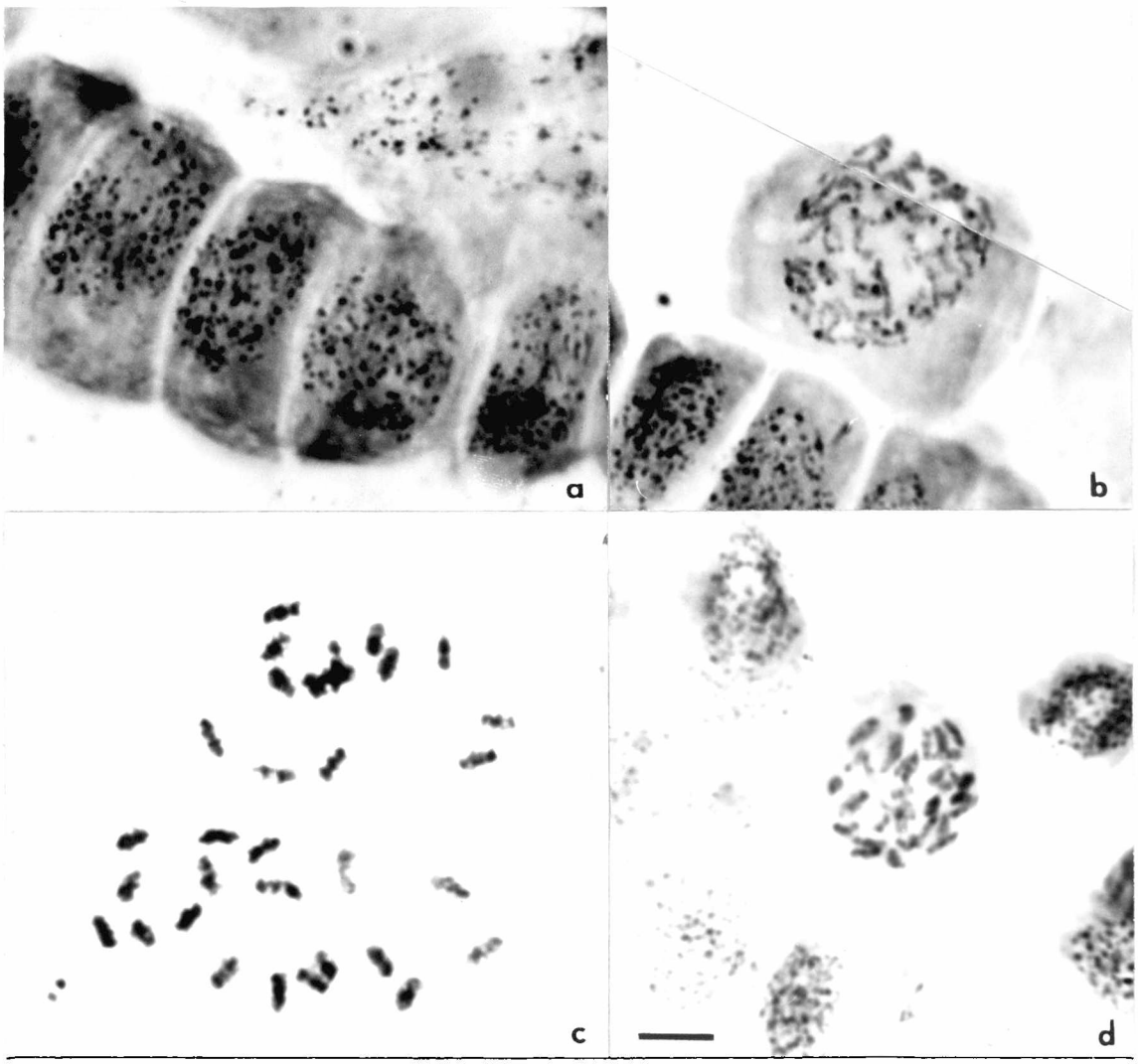
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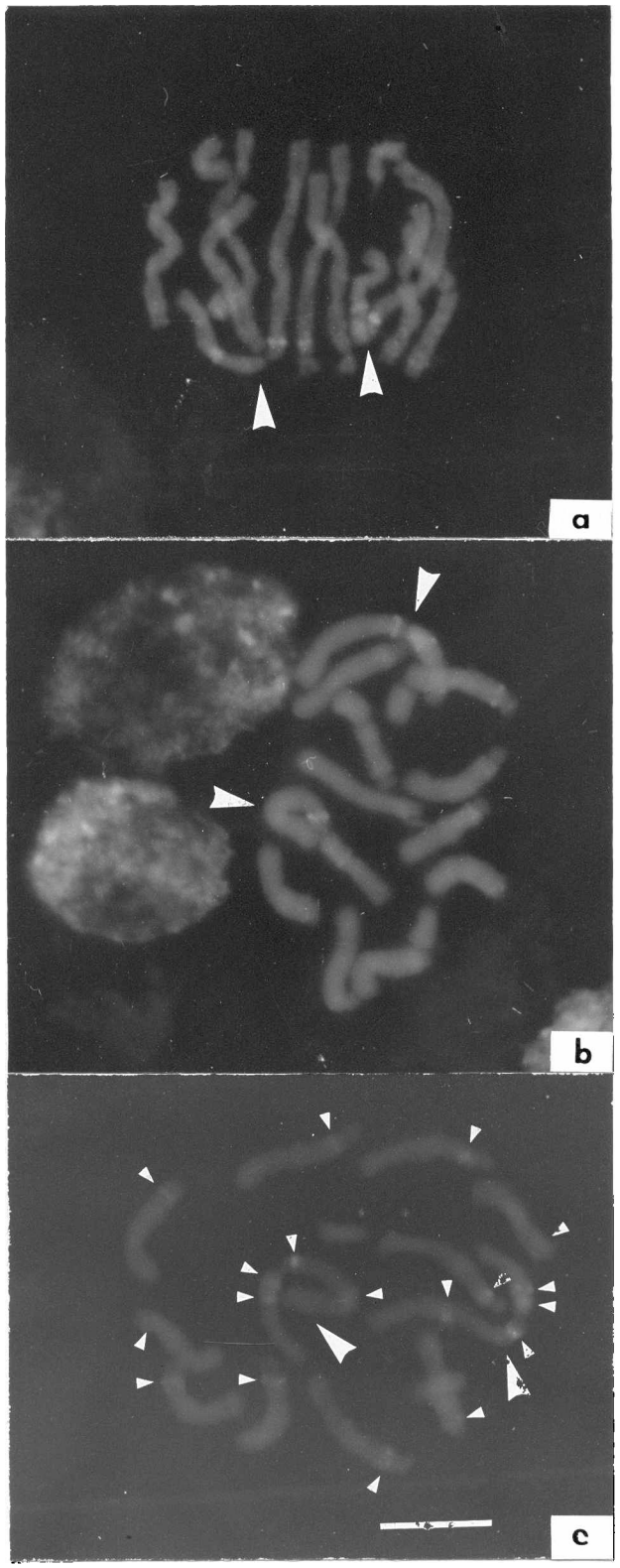
Figure 13. Q-banding patterns in Vicia faba nuclei and chromosomes. Squash preparations were stained with quinacrine mustard as described in Materials and Methods. Bar represents 10μ.

a. Mitotic figure showing prominent Q-bands in M chromosomes.

b. Metaphase and interphase preparation showing localized brightly fluorescent regions in interphase nuclei and Q-bands on the M- as well as other chromosomes.

c. Squash preparation showing Q-banded regions in each chromosome of the diploid complement.

Large arrows point to M chromosomes. Small arrows point to Q-positive chromosome regions.


a
b
c

Q-positive region to be present near the centromere of each chromosome pair (Fig. 13c).

Luzula purpurea. The Q-positive regions of L. purpurea nuclei and chromosomes (Fig. 14) are quite different from those of Vicia faba (Fig. 13). Bright quinacrine fluorescent regions are scattered throughout L. purpurea nuclei (Fig. 14a). The chromosomes exhibit an over-all bright fluorescence. Distinct Q-positive regions like those in V. faba are absent from L. purpurea chromosomes. Instead, Q-positive and Q-negative regions extend from one end of the chromosomes to the other (Figs. 14a; 14b). In the more contracted chromosomes of late prophase, the alternating bright and dull fluorescent regions cause some general chromosome regions to be more brightly fluorescent than others giving rise to patterns of bright and dull fluorescence (Figs. 14c; 14d).

^{3}H-RNA in situ Hybridization of Luzula purpurea

In an attempt to ensure that as little nucleotide contamination as possible was present in the in vitro synthesized ^{3}H-RNA sample, exhaustive dialysis was followed by chromatography on a Sephadex G-50 column using yeast RNA as a carrier (Fig. 15). Elution of yeast RNA occurred at two different times with a small portion being eluted in fractions 10 - 20 while the major portion was eluted in fractions 25 - 40. This was as expected since Crestfield et al (1955) reported that yeast RNA isolated by the method used consisted

Figure 14. Q-positive regions of Luzula purpurea nuclei and chromosomes. Procedures used are described in Materials and Methods. Two different photograph magnifications are included. Large bar represents 10 μ; smaller bar represents 5 μ.

a. Q-positive regions of early prophase chromosomes. The Q-positive regions of nuclei are also shown.

b. Later prophase stage showing the alternating arrangement of Q-positive and Q-negative chromosome regions.

c - d. Late prophase stages showing generalized regions of bright and dull fluorescence on chromosomes.

Arrows point to Q-positive chromosome regions.

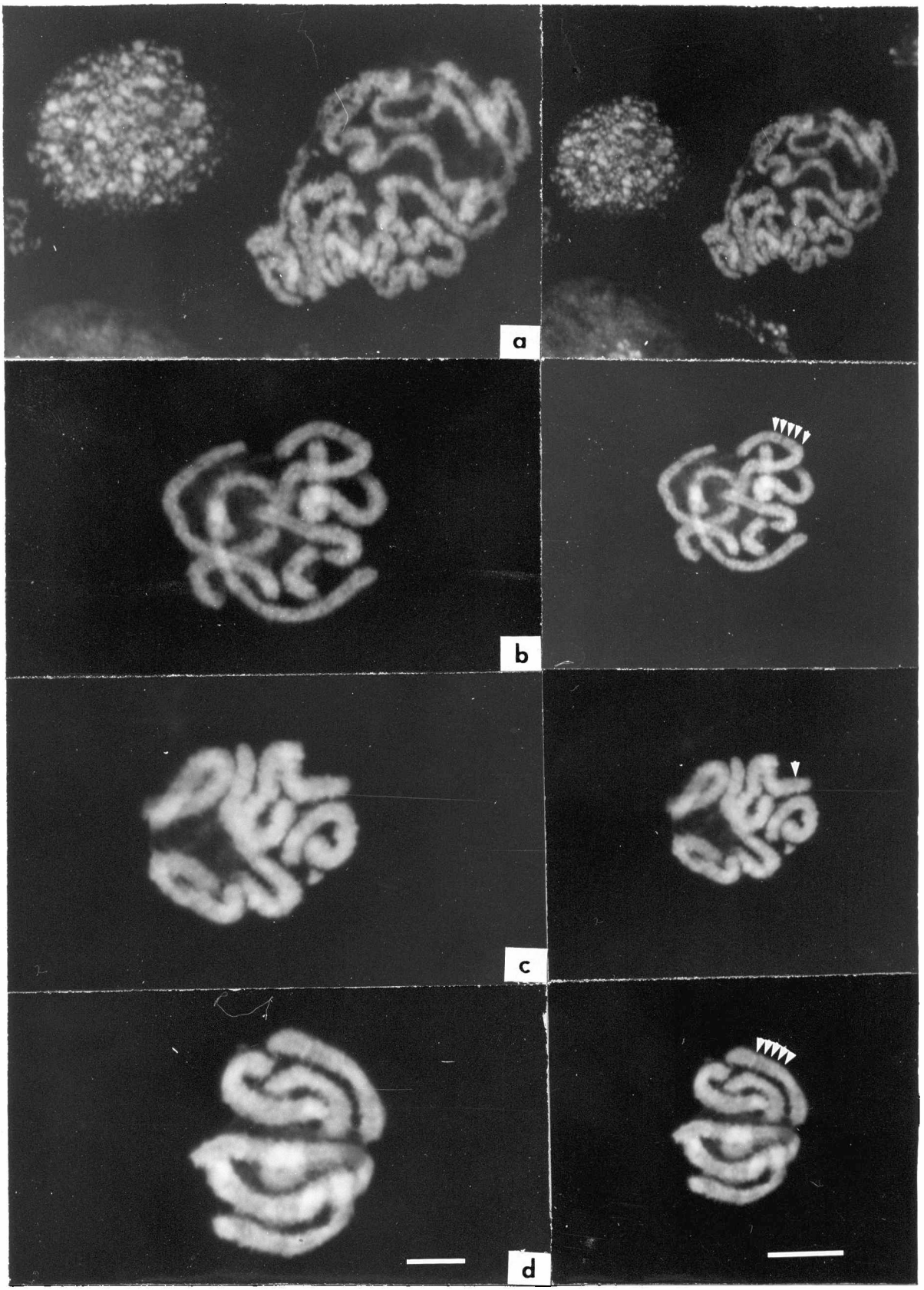
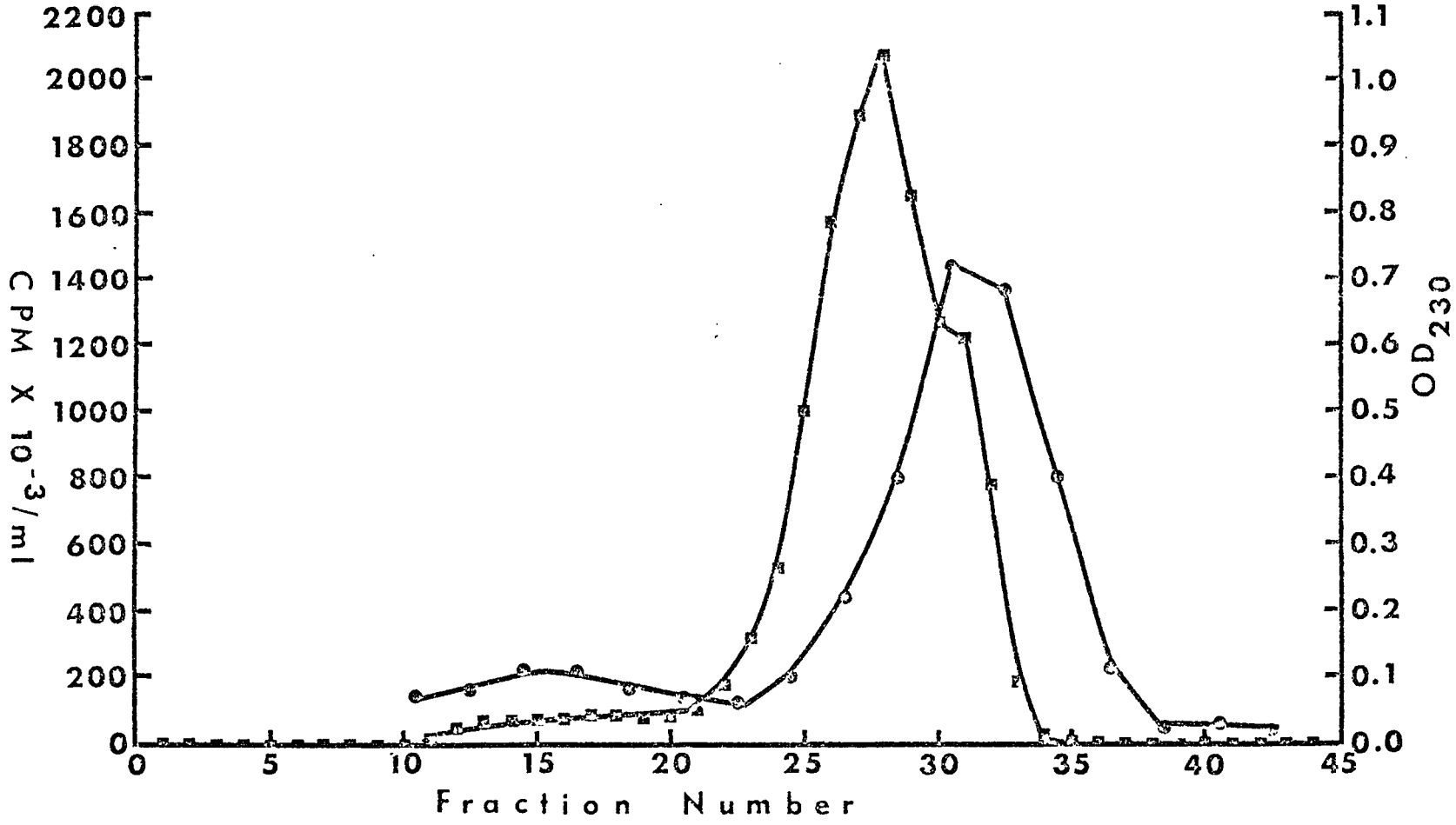

a
b
c
d

Figure 15. Elution profile of ^{3}H-RNA complementary to Cot-1 reassociated Luzula purpurea DNA from a Sephadex G-50 column. Details of ^{3}H-RNA preparation are described in Materials and Methods. ⟵●⟶, OD_{230}; ⟵■⟶, CPM X 10^{-3}/ml.


2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CPM X 10-3/ml
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD230
0
5
10
15
20
25
30
35
40
45
Fraction Number

of two different-sized fractions with the major portion having an S value of 2.8 - 4.0 and the minor portion having an S value of 7.0 - 9.5. ^{3}H-RNA was eluted from the column in fractions 21 - 33. Pooling, lyophilization, and redissolution of these fractions resulted in a ^{3}H-RNA solution used for *in situ* hybridization that had 2.3 X 10^{6} TCA-precipitable CPM/ml. No differences in grain distribution or density were found between 6 hr- and 18 hr- incubated slides.

The results of the ^{3}H-RNA *in situ* hybridization experiments are shown in Figure 16. Interphase nuclei exhibit a random grain distribution (Fig. 16d). Early prophase chromosomes also show a wide grain distribution, but some clustering of the grains is apparent (Figs. 16a; 16b). The nonrandomness of the ^{3}H-RNA *in situ*-hybridized chromosome regions is much more obvious in middle prophase (Figs. 16c; 16d) and late prophase (Figs. 16e; 16f) chromosome preparations. Although clear-cut grain distributions are absent, careful study of the chromosomes shows certain general labeling patterns to be present. One pair of chromosomes exhibits label concentration both at the ends and near the center of the chromosome (Fig. 16c). Another pair has grains concentrated mostly on one end of the chromosome ends with a few grains being located on the other end of the chromosomes (Fig. 16c). In the third chromosome pair, label is located all along their lengths (Figs. 16c; 16d).

Figure 16. ^{3}H-RNA *in situ* hybridized regions of *Luzula purpurea* nuclei and chromosomes. Procedures used are described in Materials and Methods. Bar represents 10 μ.

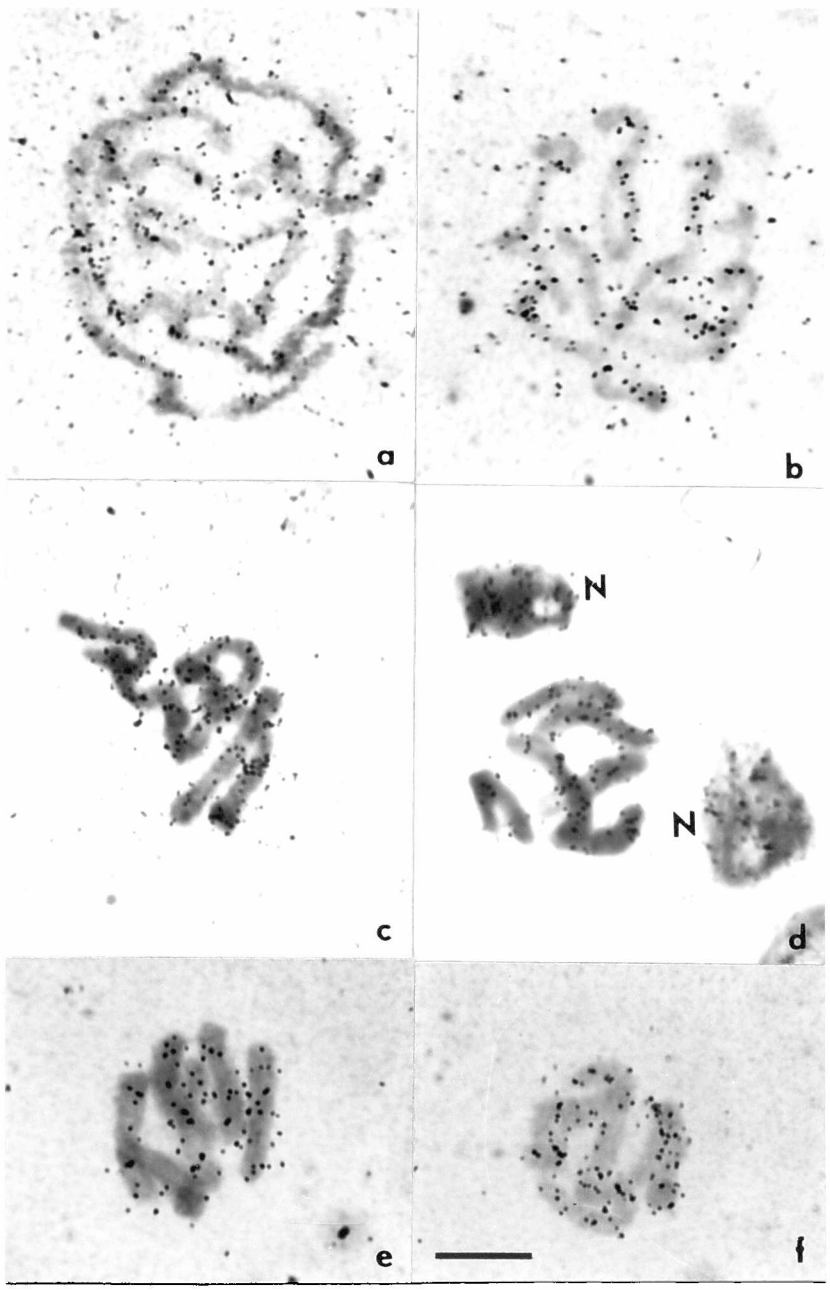
a - b. Early prophase chromosomes showing ^{3}H-RNA label distribution.

c. Middle prophase chromosomes showing label concentration in different chromosome regions.

d. Middle prophase chromosomes showing discontinuous label distribution as in 16c above. Two interphase nuclei exhibit random label distribution.

e - f. Late prophase figures showing ^{3}H-RNA concentrations in different chromosome regions.

N = nucleus.


a
b
c
N
N
d
e
f

Physical Properties of Luzula purpurea DNA

DNA purity. A typical elution profile for HAP-urea-purified DNA is shown in Figure 17. The 260/280 and 260/230 ratios of the major fractions of HAP-urea-purified DNA (Fractions 6 - 9) approached or exceeded 2.0 indicating the DNA to be relatively pure. A high degree of purity was also indicated from ultraviolet scans (220 - 320 nm) of the DNA preparations (Fig. 18). Maximum absorbance was found at 260 nm and rapidly decreased at both higher and lower wavelengths. The percent hyperchromicity of native DNA samples always exceeded 30% while that of sonicated DNA always exceeded 20% (Tables 2; 3).

DNA melting profiles. HAP melting profiles were determined for both native and sonicated L. purpurea DNA isolated by the HAP-urea method and sonicated Escherichia coli B DNA (Fig. 19). The T_m of native L. purpurea DNA was 90° C while that for sonicated L. purpurea DNA was 87.5° C. Such T_m differences between sonicated and native DNA were expected (Arrighi et al, 1970a; Stefos and Arrighi, 1974), and the T_m of native HAP-urea-purified DNA corresponded to 50.9% GC (Table 2). The T_m of E. coli B DNA was 92.5° C corresponding to a GC content of 57%.

Spectrophotometric melting profiles of L. purpurea DNA isolated by both the HAP-urea and Bendich and Bolton methods were determined (Fig. 20). E. coli B native DNA had a T_m of 93.5° C corresponding to a GC content of 58.2%. The

Figure 17. Elution of 260 nm-, 280 nm-, and 230 nm- absorbing material from HAP columns. Details of the HAP-urea method for Luzula purpurea DNA isolation and purification are described in Materials and Methods. ○——○ , OD_{260}; △——△ , OD_{230}; □——□ , OD_{280}.

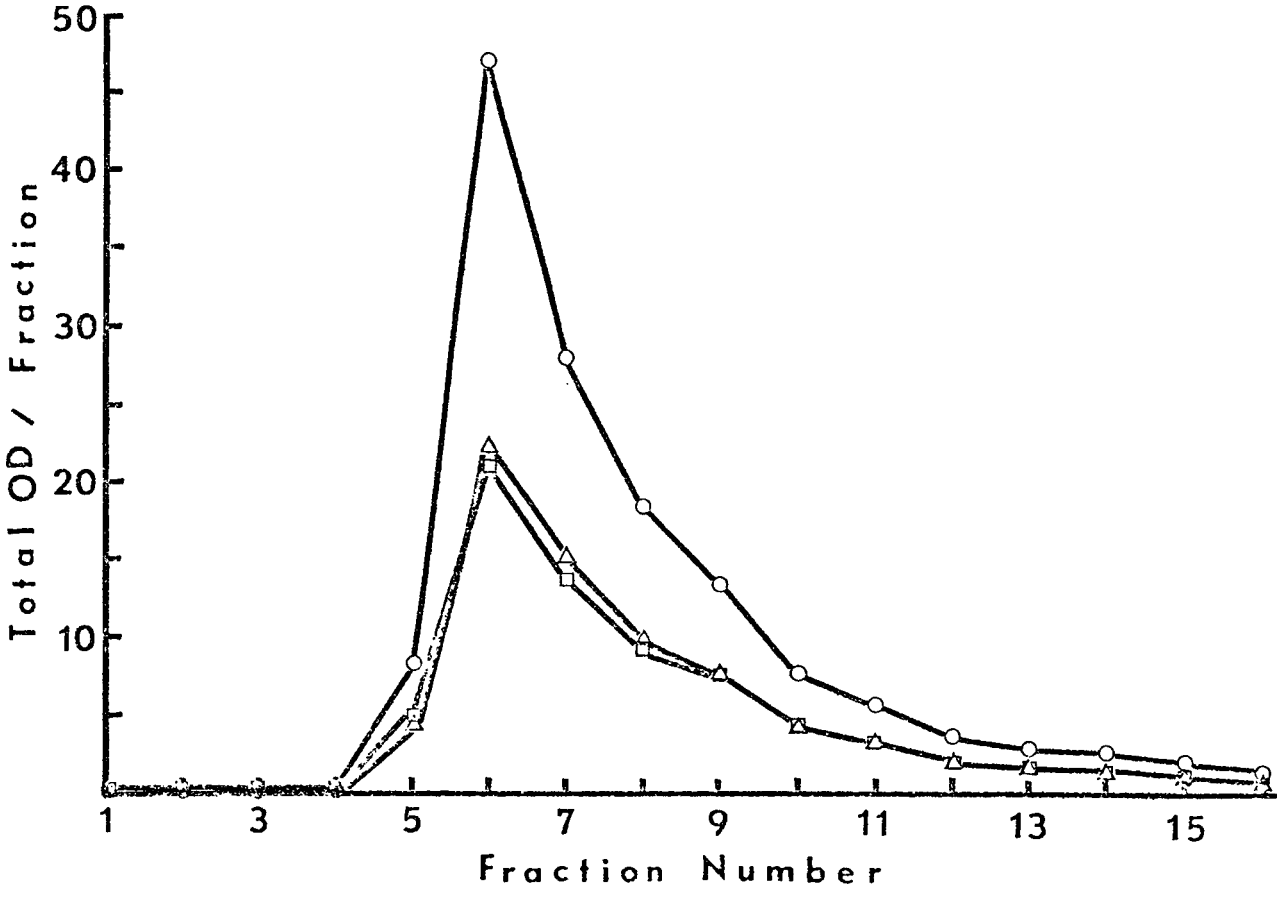

50
40
30
20
10
Total OD / Fraction
1
3
5
7
9
11
13
15
Fraction Number

Figure 18. Purity of Luzula purpurea DNA. Scanning was done in a spectrophotometer as described in Materials and Methods.

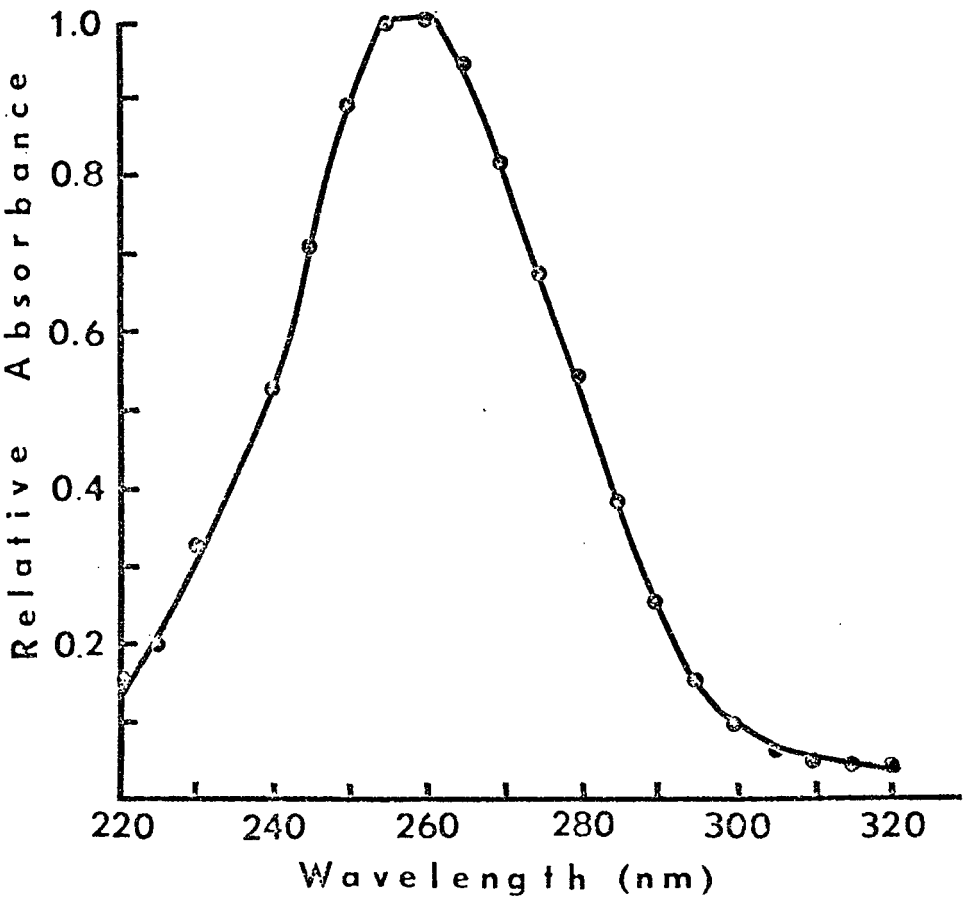

1.0
0.8
0.6
0.4
0.2
Relative Absorbance
220
240
260
280
300
320
Wavelength (nm)

Figure 19. HAP melting profile of Luzula purpurea DNA. Procedures are described in Materials and Methods. ▲——▲, L. purpurea sonicated DNA; ■——■, L. purpurea native DNA; ●——●, Escherichia coli B sonicated DNA.

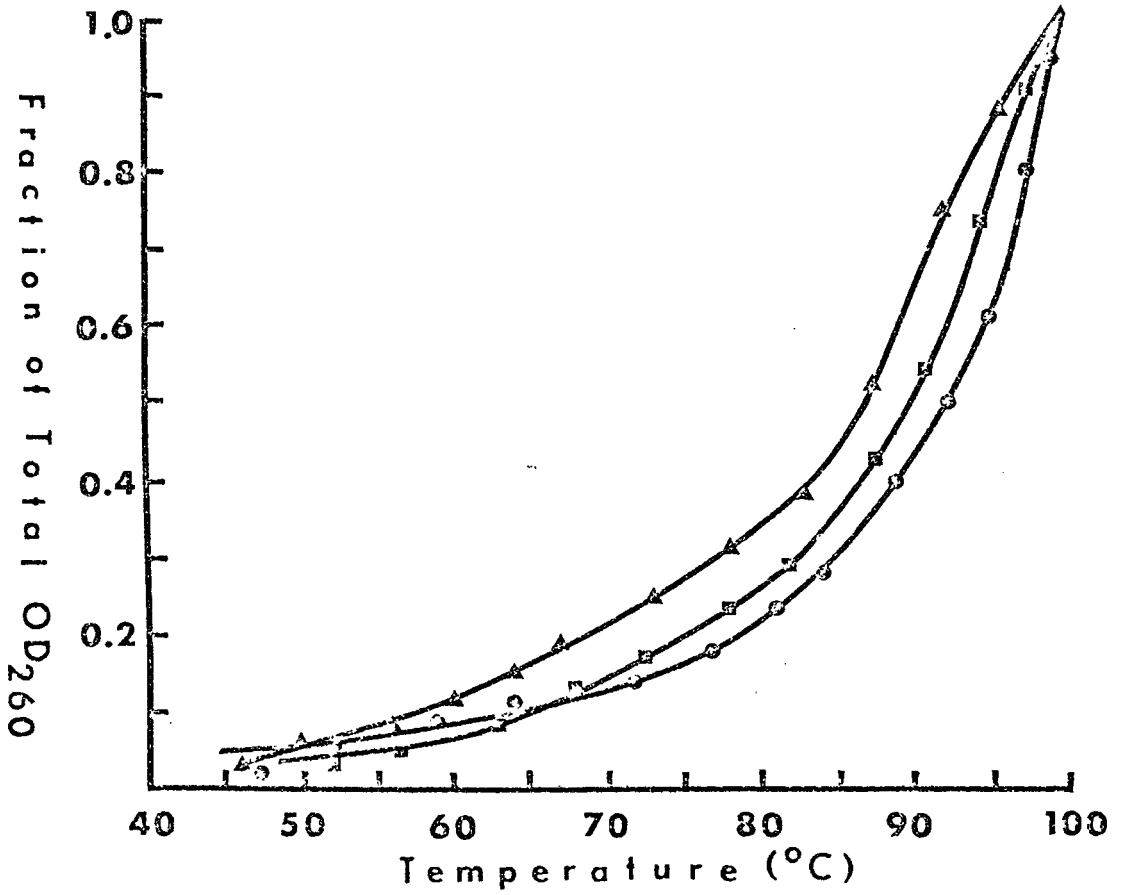

1.0
0.8
0.6
0.4
0.2
Fraction of Total OD260
40
50
60
70
80
90
100
Temperature (°C)

Figure 20. Spectrophotometric melting profile for Luzula purpurea DNA. Methods are described in Materials and Methods. ■——■, L. purpurea DNA isolated by HAP-urea method; ▲——▲, L. purpurea DNA isolated by the Bendich and Bolton method; ●——●, Escherichia coli B DNA.

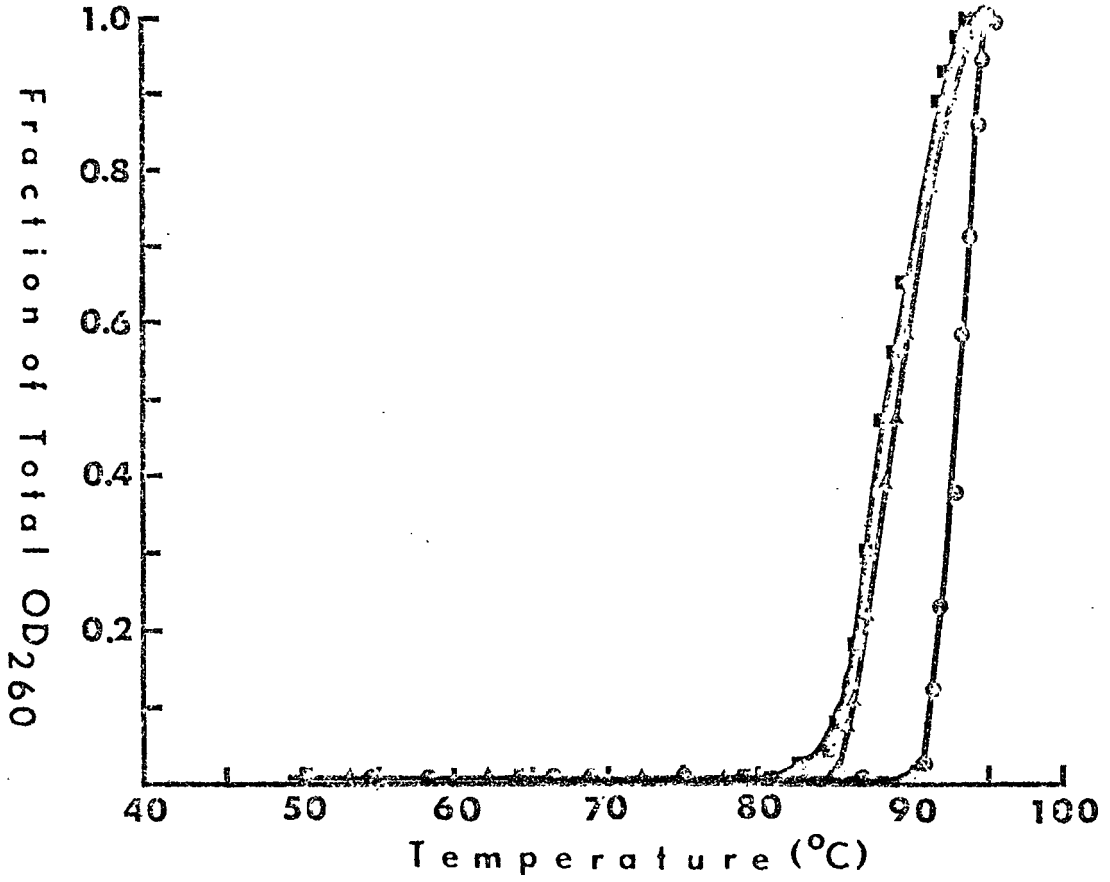

1.0
0.8
0.6
0.4
0.2
Fraction of Total OD260
40
50
60
70
80
90
100
Temperature (°C)

Table 2. Thermal stability of Luzula purpurea DNA. T_m and calculated %GC from both spectrophotometric and HAP melting data together with % hyperchromicity for the different DNA samples. Procedures are described in Materials and Methods.

* Bendich and Bolton (1967)

** Britten et al (1968)

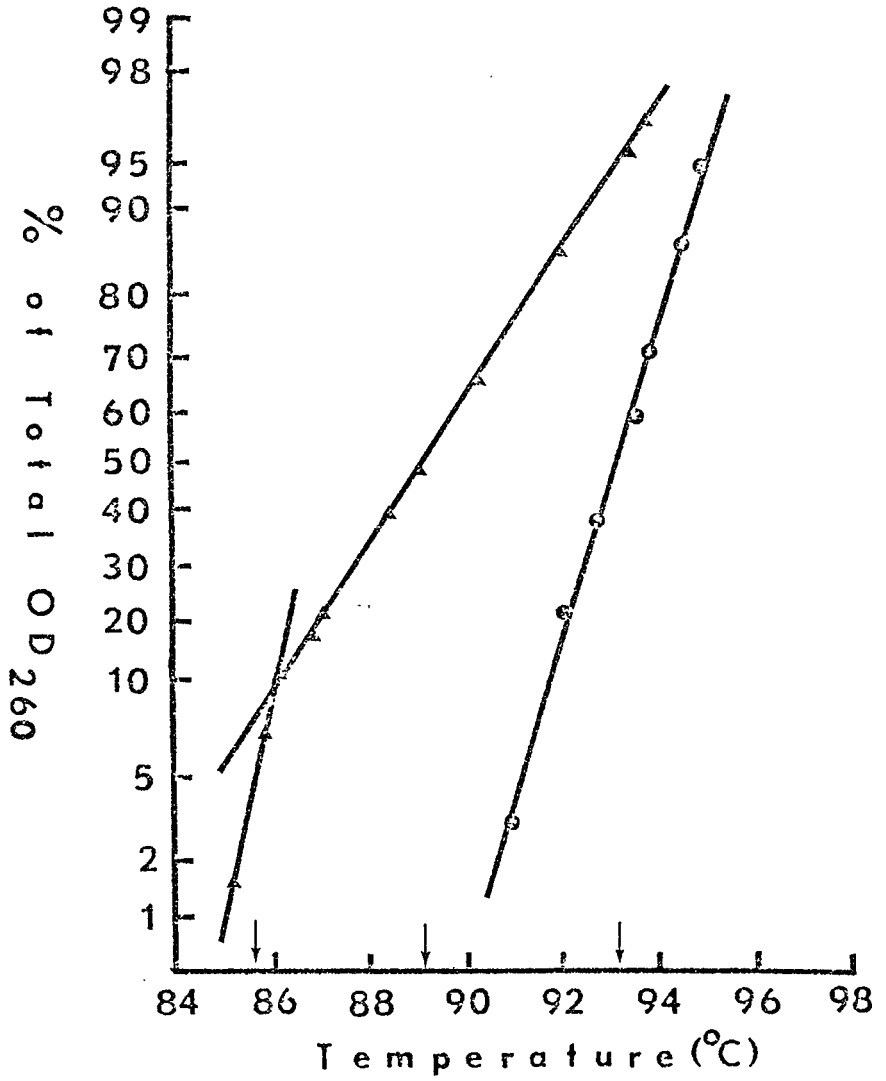
DNA	Thermal Stability				% Hyperchromicity
	Spectrophotometric		HAP		
	T_m	%GC	T_m	%GC	
Lucula purpurea					
Dendich and Dolton*	89.0° C	48.5%			35.2%
HAP-urea**					
Native	88.5° C	47.3%	90.0° C	50.9%	30.2%
Sonicated			87.5° C	44.2%	24.8%
Escherichia coli B					
Native	93.5° C	58.2%			31.7%
Sonicated			92.5° C	57.0%	24.8%

spectrophotometric melting profiles of the Luzula purpurea DNA samples were very similar to one another. The T_m for DNA isolated by the Bendich and Bolton method was 89° C while the T_m of HAP-urea-purified DNA was 88.5° C (Table 2). These T_m values corresponded to 48.5% and 47.3% GC, respectively.

Heterogeneous nucleotide distributions in DNA can be revealed by plotting melting profile data on normal probability paper (Knittel et al, 1968; McLean and Whiteley, 1973). If the nucleotide distribution is random, a plot of the data will be linear while a concentration of any base pair in any region of the DNA will produce a change in slope. Such a plot of L. purpurea data showed two different populations of molecules to exist in the DNA (Fig. 21). Approximately 10% of the DNA melted with a T_m of 85.5° C while 90% of the DNA melted with a T_m of 89° C. The corresponding GC contents were 39.9% and 48.5%, respectively. Escherichia coli B DNA melting profile data were plotted to show the linear profile obtained from random DNA nucleotide distributions.

Buoyant density determinations. The results of the buoyant density determinations revealed two interesting features of L. purpurea DNA (Fig. 22). The buoyant density of the main band of DNA isolated by the Bendich and Bolton method (Fig. 22a) was 1.6816 g/cm^3 when compared to Micrococcus luteus marker DNA (1.7245 g/cm^3). Main band DNA isolated by the HAP-urea method (Fig. 22c) had a buoyant density of

Figure 21. Normal probability plot of Luzula purpurea DNA spectrophotometric melting data. ▲——▲, L. purpurea DNA; ●——●, Escherichia coli B DNA.


99
98
95
90
80
70
60
50
40
30
20
10
5
2
1
% of Total OD260
84
86
88
90
92
94
96
98
Temperature (°C)

1.6822 g/cm^3, a value only 0.0005 g/cm^3 different from the buoyant density of DNA isolated by the Bendich and Bolton method. These DNA samples, although isolated by different methods, are obviously from the same DNA population. The calculated GC content of the main band DNAs was 28.0 - 28.5% (Table 3). In the DNA samples isolated by the Bendich and Bolton method, a shoulder was apparent on the heavy side of the main band DNA (Fig. 22a). This shoulder was much more obvious in scans made of similar DNA preparations with the scanner set at a greater sensitivity (Fig. 22b). The buoyant density of this satellite peak was 1.6928 g/cm^3 corresponding to a GC content of 39.2% (Table 3). No component with this buoyant density was seen in HAP-urea-purified DNA (Fig. 22c), but the excessive amount of shearing involved in its preparation possibly caused it to be obscured because of the increased width of the main band peak. A satellite having a buoyant density of 1.6972 g/cm^3 was found in HAP-urea-purified DNA (Fig. 22c). This density corresponds to a GC content of 43.6%, and the satellite probably represents chloroplast DNA as will be discussed later. In an attempt to determine if the discrepancies between the GC content of the DNA as determined by buoyant density measurements (28% GC) and that determined from the spectrophotometric melting profile (48% GC) was due to protein adhering to the DNA, the buoyant densities of the DNA samples were redetermined after incubation in the presence of nuclease-free pronase (Fig. 22d).

Figure 22. Buoyant density determinations of Luzula purpurea DNA. Methods are described in Materials and Methods.

a. L. purpurea DNA isolated by the Bendich and Bolton method.

b. Scan of a similar DNA sample as in 22a above using greater sensitivity settings.

c. L. purpurea DNA isolated by the HAP-urea method.

d. Recentrifugation of 22a above following pronase digestion.

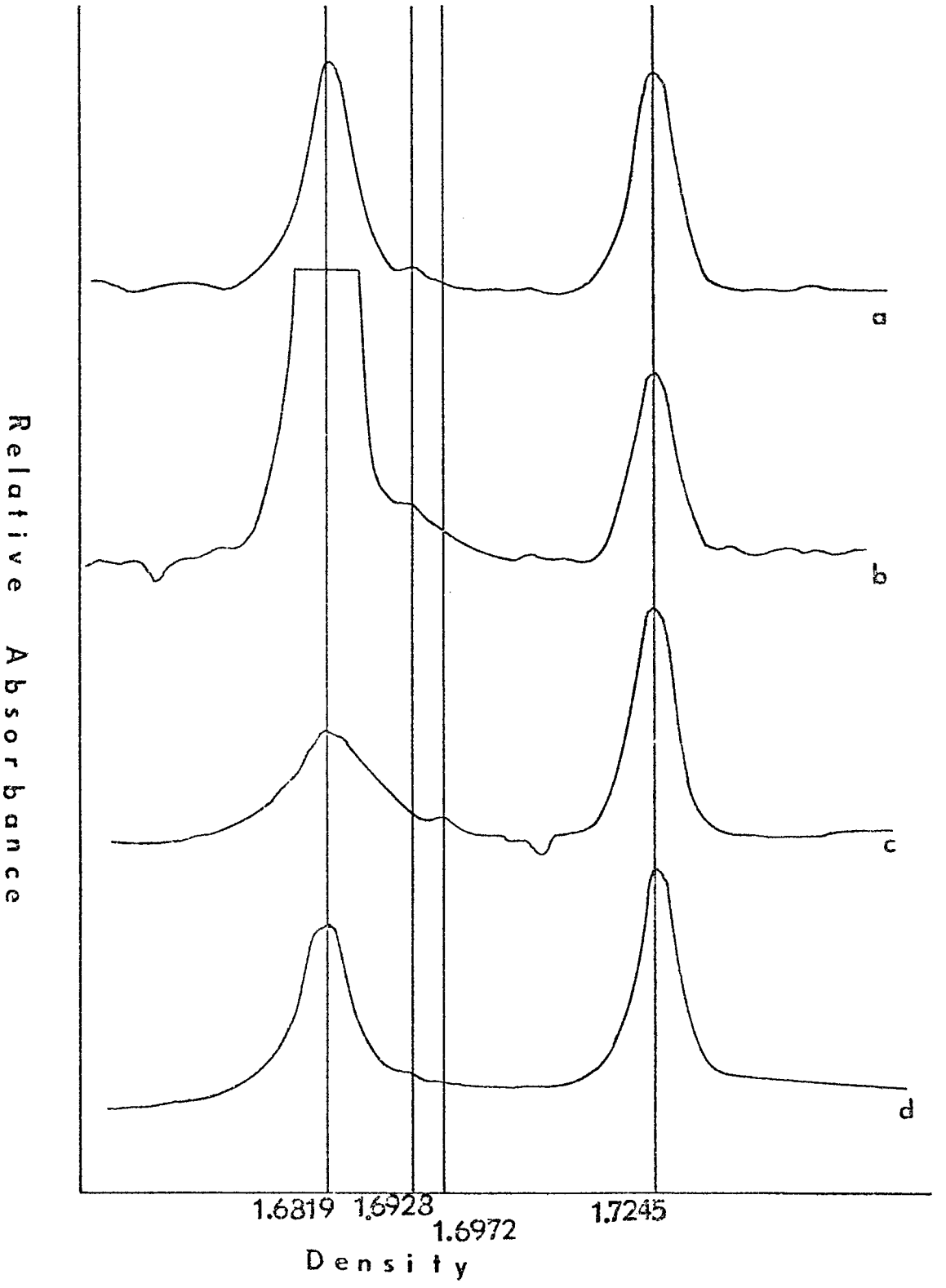

Relative Absorbance
a
b
c
d
1.6819
1.6928
1.6972
1.7245
Density

Table 3. Buoyant density of Luzula purpurea DNA. g/cm^3 and calculated %GC of main band and satellite DNA fractions of L. purpurea DNA isolated both by the HAP-urea and Bendich and Bolton methods. % hyperchromicity of the different DNAs are also included. Methods are described in Materials and Methods.

* Bendich and Bolton (1967)

** Britten et al (1968)

DNA	Buoyant Density (g/cm^3)				% Hyperchromicity
	Main Band	%GC	Satellite	%GC	
Luzula purpurea					
Bendich and Bolton*	1.6817	28.0%	1.6927	39.1%	33.0%
	1.6816	27.9%	1.6928	39.2%	35.2%
HAP-urea**	1.6822	28.5%	1.6972	43.6%	30.2%

A buoyant density identical to the original one was found (1.6818 $\pm$ 0.0004 g/cm^3). Thus, the discrepancy in GC content is real and not the result of protein contamination in the DNA.

DNA reassociation. Reassociation studies were conducted with Luzula purpurea DNA using Escherichia coli B DNA having both known kinetic complexity and size (4.5 X 10^6 nucleotide pairs) as a control. E. coli DNA reassociation occurred over a narrow Cot range (0.3 - 100) (Fig. 23), a characteristic of prokaryotic DNA consisting of unique nucleotide sequences. A $Cot_{1/2}$ of 7 was found which was as expected for DNA fragments consisting of 200 - 400 nucleotides, the size determined by boundary sedimentation of sonicated DNA.

L. purpurea DNA reassociated over a much broader Cot range (0 - 10,000) than E. coli (Fig. 24). This is characteristic for eukaryotic DNA consisting of both repeated and unique nucleotide sequences. The over-all $Cot_{1/2}$ was 200, but this gives little information about the complexity of the different fractions making up the DNA. For this reason, the over-all DNA reassociation profile was divided into different frequency classes (Britten and Kohne, 1968) (Table 4). Highly repeated DNA sequences reassociate by Cot 1; middle repetitive sequences reassociate by Cot 100; unique DNA sequences reassociate at Cots greater than 100. In this instance, the highly repeated DNA sequence class was divided

Figure 23. Escherichia coli B DNA reassociation profile. DNA concentration was approximately 2.0 denatured OD_{260}/ml. Procedures are described in Materials and Methods.

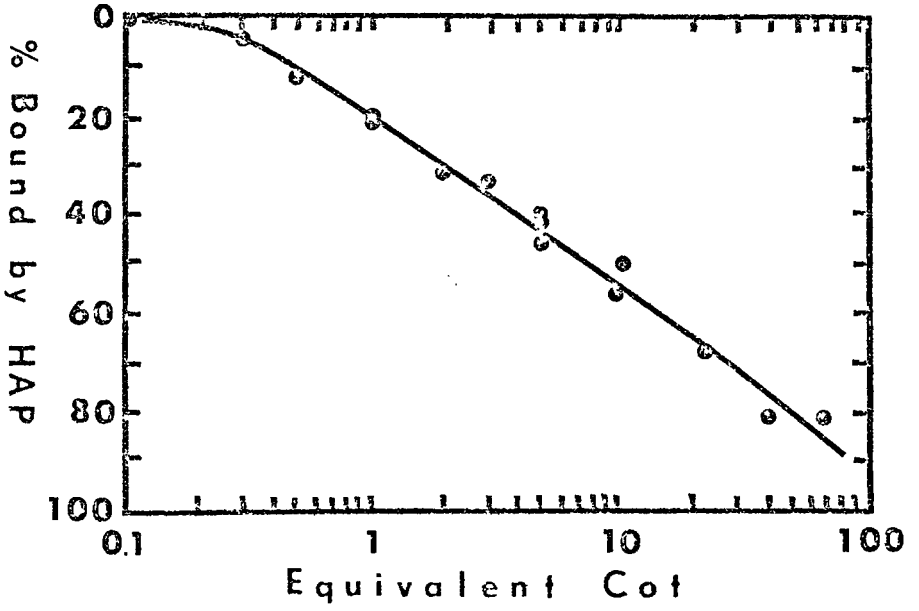

0
20
40
60
80
100
% Bound by HAP
0.1
1
10
100
Equivalent Cot

Figure 24. Luzula purpurea DNA reassociation profile. DNA concentrations and PB molarities were: ▲, 0.685 denatured OD_{260}/ml in 0.06 M PB; ●, 2.0 denatured OD_{260}/ml in 0.12 M PB; ■, 10.30 denatured OD_{260}/ml in 0.24 M PB; □, 11.55 denatured OD_{260}/ml in 0.48 M PB. Procedures are described in Materials and Methods.

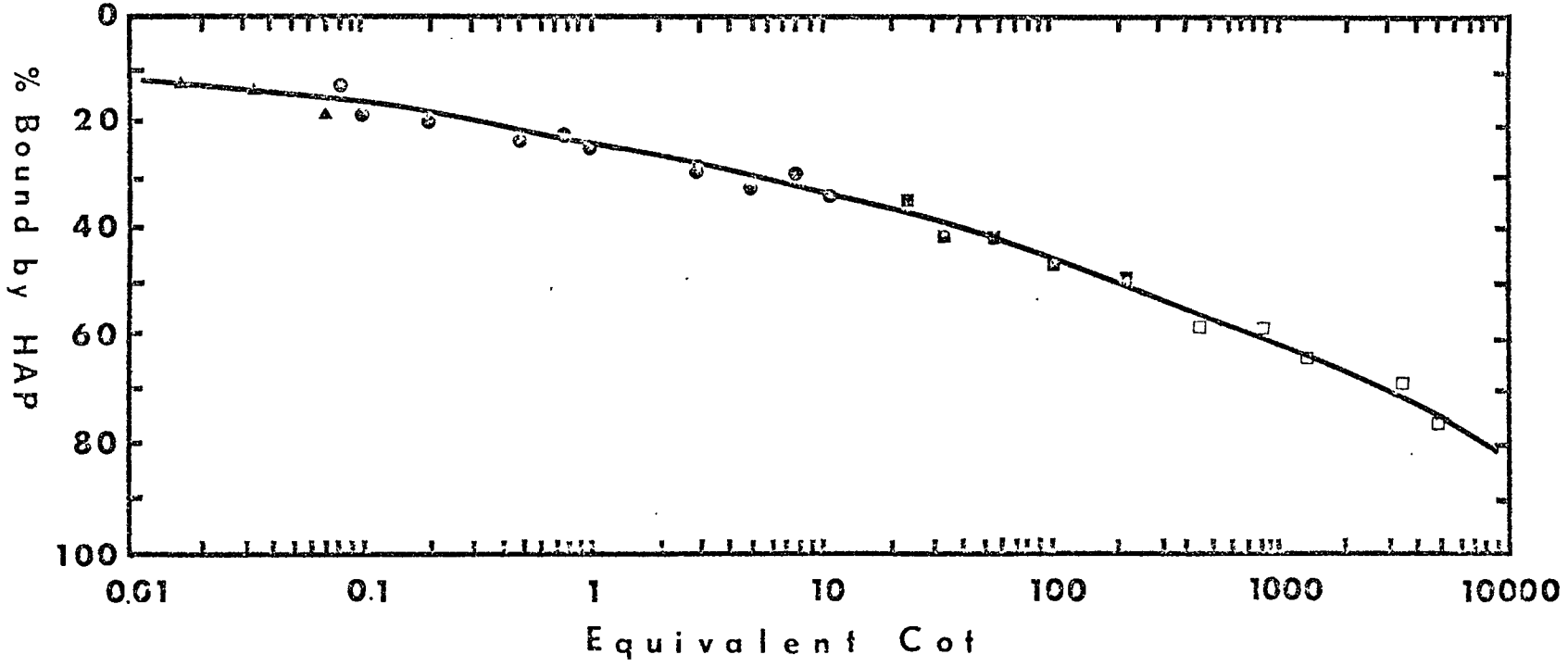

% Bound by HAP
0
20
40
60
80
100
0.01
0.1
1
10
100
1000
10000
Equivalent Cot

Table 4. Kinetic complexity of Luzula purpurea DNA. $Cot_{1/2}$ values and fractions of DNA are taken directly from Figure 24. The repetition frequencies of the different fractions are calculated assuming the Cot greater than 100 reassociating fraction consists of unique DNA sequences.

DNA Fraction	% of Total DNA	$Cot_{1/2}$	Frequency of Repetition
Highly repetitive sequences (Cot 0 - 1)	25%	0.01	4×10^5
Cot 0 - 0.01	12%	0.01 - 0.001	$4 \times 10^5 - 4 \times 10^6$
Cot 0.01 - 1.0	13%	0.15	2.7×10^4
Middle repetitive sequences (Cot 1 - 100)	21%	10.5	3.8×10^2
Unique DNA sequences (Cot 100)	54%	4000	1

into DNA reassociating by Cot 0.01 and DNA reassociating between Cots 0.01 - 1 (Ranjekar et al, 1974).

Highly repeated nucleotide sequences comprised 25% of the total genome with 12% of this frequency class being reassociated by Cot 0.01 ($Cot_{1/2}$ 0.001 - 0.01). Middle repetitive sequences made up 21% of the DNA ($Cot_{1/2}$ 10.5) while the majority of the nucleotide sequences, 54%, were unique ($Cot_{1/2}$ 4000). The degree of repetition of each frequency class was determined by comparing its $Cot_{1/2}$ value with that of the unique DNA sequence class. The middle repetitive sequence class was composed of sequences repeated an average of 3.8×10^2 times while the highly repeated DNA sequence class consisted of sequences repeated an average of 4×10^5 times. When the highly repeated DNA sequence class was divided into the Cot 0 - 0.01 and Cot 0.01 - 1 fractions, the former fraction was shown to be made up of sequences repeated from 4×10^5 - 4×10^6 times while the latter had sequences repeated 2.7×10^4 times.

DISCUSSION

Cytological Studies

The different cytological techniques used in the study permit homologous chromosomes to be recognized and comparisons to be made of their differential staining and DNA late-replication patterns. Such a comparison of *Vicia faba* chromosomes is shown in Figure 25. In the chromosome complement of normal squash preparations (Fig. 25a) the only chromosome pair that can be indisputably recognized is the pair of M chromosomes (pair 1). Not only are they larger than the other S chromosomes, but they also exhibit a secondary constriction in addition to that of the centromere. The S chromosomes are approximately equal-sized and have centromeres located in essentially the same regions making their alignment as pairs possible only on the basis of their measured lengths. C-banding patterns (Fig. 25b) permit each chromosome pair to be recognized. The M chromosome pair (pair 1) has a single C-positive region on the side of the centromere on which the secondary constriction is located and two C-banded regions lying adjacent to one another near the centromere on the other chromosome arm. Chromosome pairs 2, 3, and 6 each have two C-positive regions, but in each chromosome pair the C-banded appearance is different. In chromosome pair 4 a single C-band exists while in pair 5 each chromosome has three closely positioned C-bands. The DNA late-replicating regions (Figs. 25c; 25d)

Figure 25. Comparison of Vicia faba chromosomes treated by different cytological techniques. Procedures are described in Materials and Methods.

a. Chromosome complement from normal squash preparation arranged according to their size.

b. Pairing of homologous chromosomes according to their C-banding patterns.

c - d. Arrangement of chromosomes according to their DNA late-replicating regions.

e. Arrangement of chromosomes according to their Q-banding patterns.

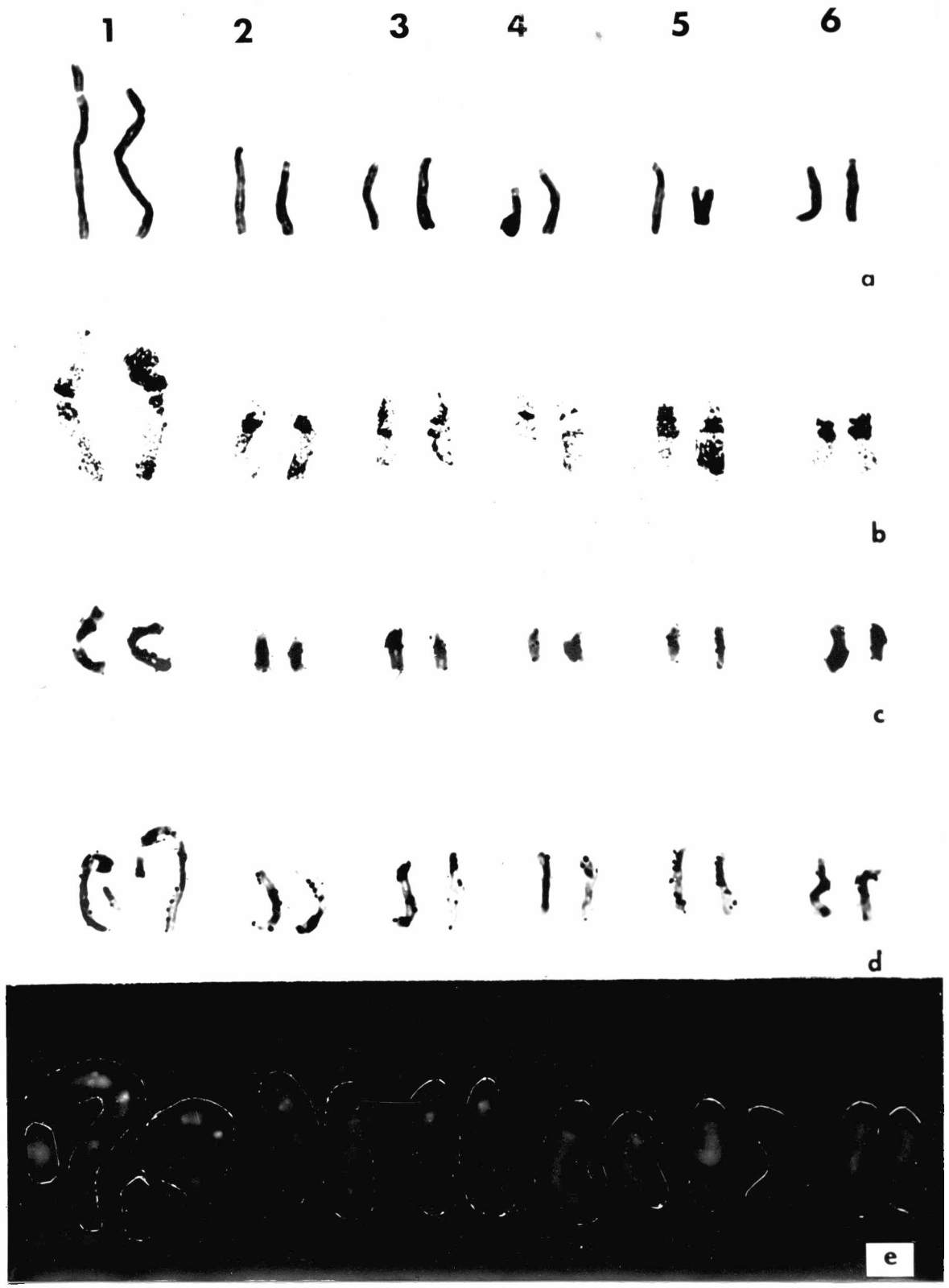

1
2
3
4
5
6
a
b
c
d
e

exhibit essentially the same chromosome distributions as those of the C-bands although DNA late-replicating regions are additionally located just proximal and distal to the secondary constriction of the M chromosome (pair 1). The Q-positive chromosome regions lie adjacent to the centromeres (Fig. 25e) as the C-bands did. Unlike the C-positive regions, however, the two Q-positive regions are located on the arm of the centromere possessing the secondary constriction and one Q-positive region is found on the other chromosome arm. An additional interstitial Q-positive region is present on the chromosome arm lacking the secondary constriction. Each of the S chromosome pairs has a Q-positive region adjacent to the centromere on the long arm.

The differential staining profiles reported here are similar to those determined by other workers using similar or different techniques. Multiple C-positive regions have been found in Vicia faba utilizing urea (Dobel et al, 1973), NaOH (Takehisa and Utsumi, 1973b), acetic acid (Schweizer, 1973), and saturated $Ba(OH)_2$ at room temperature (Vosa and Marchi, 1972; Klasterska and Natarajan, 1975). Minor differences exist between each of the published C-banded profiles as they do between each of the profiles and the one reported here. These differences are apparently real and probably reflect the fact that different varieties of V. faba were used in each of the studies. Similar differences in C-banded profiles have been reported in rye (Sarma and Natarajan, 1973; Verma and Rees, 1974; Gill and Kimber,

1974a; Vosa, 1974) and wheat (Natarajan and Sarma, 1974; Gill and Kimber, 1974b), and these discrepancies have also been attributable to the use of different seed varieties as the chromosome source. Vicia faba DNA late-replicating regions similar to those reported here were previously shown by Evans (1964). The DNA late-replicating regions were on either side of the centromere of the M chromosomes and near the centromeres in the long arms of the S chromosomes. The reason for the lack of complete correspondence between the Q-positive chromosome regions and those of C-bands and DNA late-replicating regions is not apparent. The Q-positive regions around the centromere of the M chromosomes are identical to those previously reported (Caspersson et al, 1968; 1969a; 1969b; Zech, 1973). Multiple rather than single Q-positive sites have been seen in some of the S chromosomes (Caspersson et al, 1969a; Zech, 1973) although these are generally fainter than those of the M chromosomes. The single Q-positive regions found here may be a reflection of the difficulty encountered in the photographic reproduction of Q-banding patterns or they may indicate that more than one type of DNA-protein interaction occurs in heterochromatin and that the differences in Q- and C- banding patterns are a result of the specificity of quinacrine mustard and Giemsa for particular types of nuclear proteins. A previous indication of the occurrence of different types of heterochromatin was found in V. faba (Schweizer, 1973) where different banding patterns were produced depending on the pretreatment used.

Differences in DNA-protein interactions in heterochromatin could also account for the different C-banded patterns produced when slides are stored for different periods of time prior to C-banding (Fiskesjo, 1974b) or when different denaturing techniques (hot PB vs $Ba(OH)_2$) are used (Stack, 1974; Stack et al, 1974).

A comparison of Luzula purpurea chromosomes subjected to different cytological techniques shows distinct patterns to be produced in each chromosome pair (Fig. 26). The one exception occurs in the chromosomes of normal squash preparations (Fig. 26a). Their lack of primary constrictions and approximately equal size makes pairing of them rather arbitrary. When squash preparations are subjected to continuous labeling with ^{3}H-thymidine (Fig. 26b), C-banding (Fig. 26c), ^{3}H-RNA in situ hybridization (Fig. 26d), and Q-banding (Fig. 26e), however, not only does each chromosome pair exhibit unique reactive regions with a given technique, but the same chromosome regions are similarly affected with each of the other techniques. Just as one chromosome pair has three DNA late-replicating regions (pair 1), another chromosome pair has two DNA late-replicating regions (pair 2), and the final pair has DNA late-replicating regions scattered throughout its length (pair 3) (Fig. 26b), the C-banding patterns (Fig. 26c) are also present in three regions (pair 1), two regions (pair 2), and all along the chromosomes (pair 3). The C-banded regions are replicated around the middle or very early in the late portion of the S stage of interphase. This is

Figure 26. Comparison of Luzula purpurea chromosomes treated by different cytological techniques. Procedures are described in Materials and Methods.

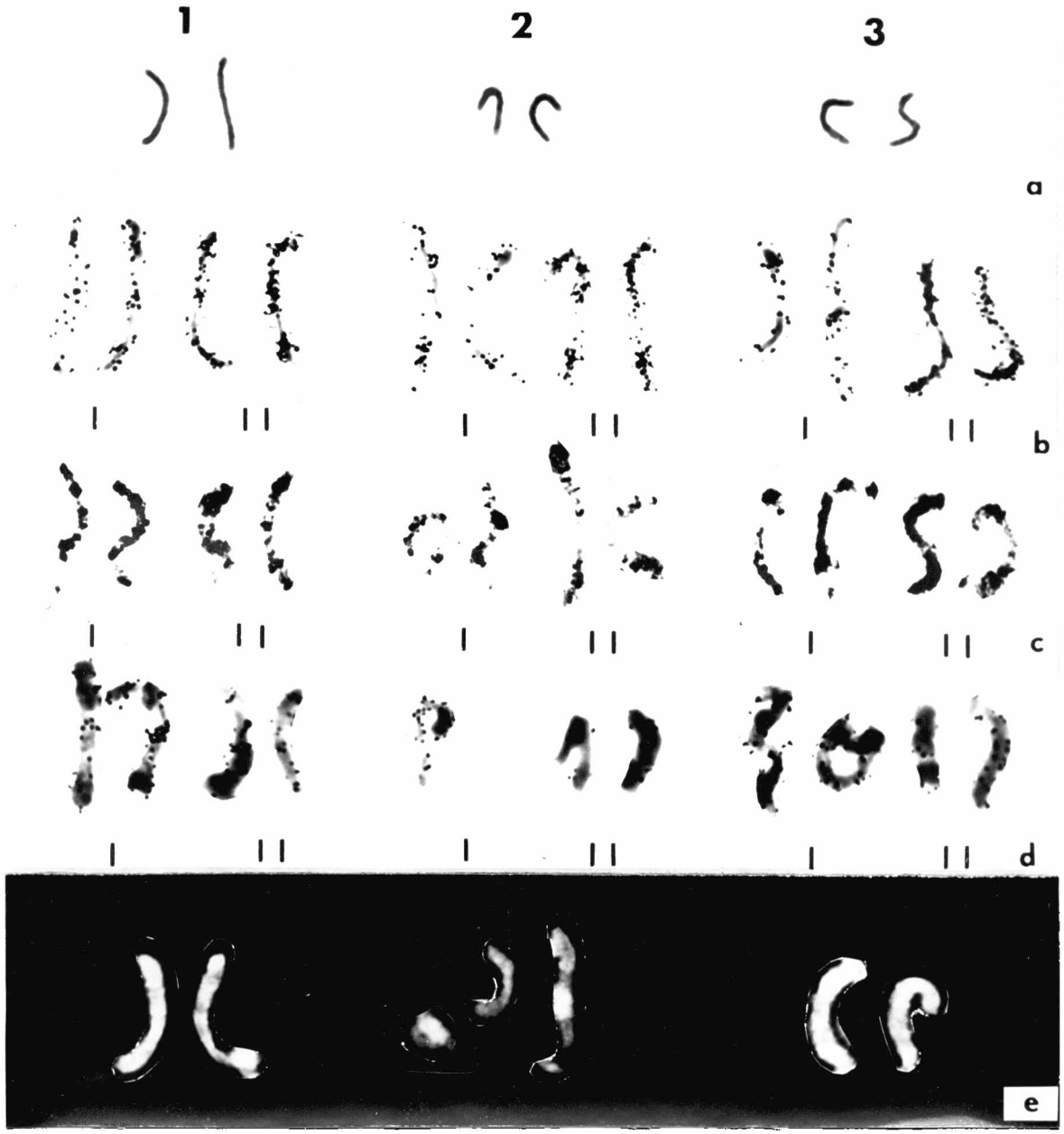
a. Chromosomes from normal squash preparations arbitrarily arranged in pairs.

b. Two different stages in the DNA replication cycle. The set of chromosomes on the left (I) shows late replicating patterns. The set of chromosomes on the right (II) is from nuclei exposed to ^{3}H-thymidine about the middle of the DNA replication cycle.

c. C-banding patterns in the paired chromosomes from two different cells (I and II). One of the chromosomes of pair 2 (I) is missing.

d. ^{3}H-RNA *in situ* hybridized chromosome regions in the chromosomes from two different cells (I and II).

e. Arrangement of chromosomes in pairs on the basis of their Q-banding pattern.


1
2
3
a
b
c
d
e

shown by comparing the C-banded patterns (Fig. 26c) with those of DNA replication (Fig. 26b) where two different stages in the DNA replication cycle are shown for each chromosome pair. The chromosome pair on the left (I) in each case represents DNA late-replication patterns while those on the right (II) are from chromosomes that were in the middle of their replication cycle when ^{3}H-thymidine was added. The C-banded regions are not identical to either of these patterns but clearly fall between them. The ^{3}H-RNA *in situ* hybridized chromosome regions in the two different sets of chromosomes (I and II) (Fig. 26d) also exhibit three hybridized chromosome regions, two hybridized regions with more label on one chromosome end, and over-all label distribution, respectively. A similar arrangement of Q-positive regions was also found (Fig. 26e) although the banding patterns were much less distinct than those obtained with the other techniques.

Multiple C-banding patterns like those found here for *Luzula purpurea* have not been reported in any other organisms. They closely resemble the G-banding patterns that have been produced in animal chromosomes when Giemsa stain is applied following trypsin digestion (Seabright, 1971; Wang and Federoff, 1972). Like G-bands, the number of C-bands in *L. purpurea* chromosomes changes as the chromosomes become more condensed with many more C-bands being observed in prophase chromosomes than metaphase ones (Figs. 10; 11). Similar correlations between chromosome length and the

number of G-bands have been reported (Rohme, 1974; Yunis and Sanchez, 1975) suggesting that each of the G-bands in metaphase chromosomes is actually composed of several G-positive regions. Likewise, each of the C-bands of Luzula purpurea metaphase chromosomes is also probably made up of several C-positive regions. That the multiple bands of L. purpurea chromosomes are C-bands and not G-bands is shown by the fact that the same technique produces C-banding patterns in Vicia faba (Fig. 9) like those previously reported and by the very definition of C-bands--Giemsa positive chromosome regions following dissociation-reassociation procedures such as those used in this work.

C-bands have been shown to represent constitutive heterochromatin (Arrighi and Hsu, 1971) which is typically late-replicating (Schnedl, 1972; Voiculescu et al, 1972; Czaker, 1973; Parker et al, 1973; Pathak et al, 1973a; 1973b; Schnedl and Czaker, 1974) and composed of highly repeated DNA sequences which may or may not appear as a satellite (Arrighi et al, 1970a; Jones, 1970; Jones and Robertson, 1970; Pardue and Gall, 1970; Rae, 1970; Botchan et al, 1971; Gall et al, 1971; Jones and Corneo, 1971; Macgregor and Kezer, 1971; Brown and Jones, 1972; Saunders et al, 1972; Jones et al, 1973; Kurnit et al, 1973; Macgregor et al, 1973; Natarajan et al, 1973; Prescott et al, 1973; Ahnstrom and Natarajan, 1974; Brown and Wilmore, 1974; Jones et al, 1974; Kunz and Eckhardt, 1974; Natarajan and Raposa, 1974; Stefos and Arrighi, 1974; Gosden et al, 1975; Jones et al, 1975).

The DNA of the C-bands of Luzula purpurea is also replicated during the last half of the interphase S stage and consists of highly repeated nucleotide sequences. The Q-bands also correspond to C-bands as is characteristic for plants (Vosa and Marchi, 1972; Vosa, 1973a; 1973b; 1974).

Although no study similar to the one reported here has been conducted in plants, a comparison of the labeled chromosome regions resulting from the in situ hybridization of ^{3}H-RNA complementary to a rapidly reassociating satellite DNA fraction isolated by Ag^{+}-$CsSO_4$ ultracentrifugation (Timmis et al, 1975) with C-banded and Q-banded regions of Scilla siberica (Vosa, 1973a) shows that the DNA of C- and Q- banded regions are composed of highly repeated nucleotide sequences. These regions are also cold sensitive which is characteristic for many plant heterochromatins (Vosa and Marchi, 1972; Timmis et al, 1975). Therefore, just as constitutive heterochromatin of animal C-banded chromosome regions is composed of highly repeated DNA sequences that may or may not exist as satellite DNA fractions on centrifugation to equilibrium in CsCl, this also appears to be the case for the constitutive heterochromatin in the C-bands of plant chromosomes.

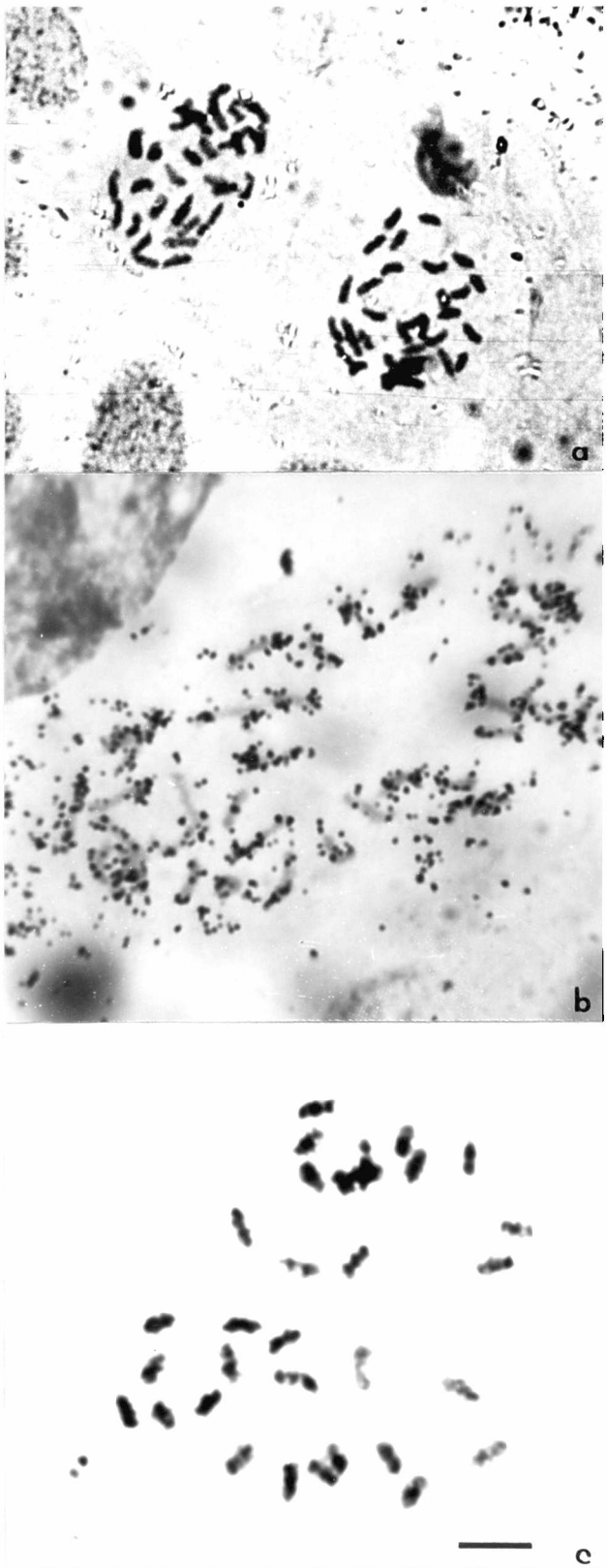
Multiple late-replication and C-banding regions are also present on L. multiflora chromosomes (Fig. 27). As was the case with L. purpurea chromosomes, the chromosomes of L. multiflora are also indistinguishable from one another due both to their small size and the lack of primary

Figure 27. Comparison of Luzula multiflora chromosomes treated by different cytological techniques. Procedures were carried out according to those listed in Materials and Methods. Bar represents 5 μ.

a. Prophase chromosomes in normal squash preparations of two different cells.

b. DNA late-replicating patterns in prophase chromosomes.

c. C-banding patterns in prophase chromosomes.


a
b
c

constrictions (Fig. 27a). The multiple DNA late-replicating regions (Fig. 27b) and C-banded regions (Fig. 27c) are positioned on the chromosomes in such a way that distinct patterns are formed. Although the tendency of the small chromosomes to clump precludes observation of the full chromosome complement in any one cell, there are many similarities in the C-banding and DNA late-replicating patterns suggesting that the multiple C-bands of Luzula multiflora are late-replicating just as those of L. purpurea are.

Multiple C-bands are a common feature of both Luzula species studied. An interesting point for consideration is why multiple C-positive regions are found in L. purpurea and L. multiflora chromosomes when no C-bands were detectable in the chromosomes of Tetranychus urticae (Pijnacker and Ferwerda, 1976), another organism lacking localized centromeres. It is possible that their attempts to detect C-bands were made with metaphase chromosomes. The detection of C-banding patterns in metaphase chromosomes in L. purpurea and L. multiflora was practically impossible due to the dark background staining in the condensed chromosomes. Only rarely were suitably stained metaphase figures found (Fig. 11g). Late prophase chromosomes were much more suitable for the study of C-banding patterns. Another possibility for C-bands not having been detected is the lack of perserverance on the part of the investigators. C-bands were frequently not present in chromosomes subjected to dissociation, reassociation, and Giemsa staining. A more likely reason can be

drawn from ultrastructural studies of plant and animal chromosomes lacking localized centromeres.

Mitotic chromosomes of Rhodnius prolixus (Buck, 1967) and Oncopeltus fasciatus (Comings and Okada, 1972) contain a layer of material assumed to be the kinetochore plate extending almost their entire length while the meiotic chromosomes of Philaenus sp. (Ris and Kubai, 1970), Bombyx mori (Friedlander and Wahrman, 1970), and O. fasciatus (Comings and Okada, 1972) totally lack any organized kinetochore plate. In contrast to the condition found in the chromosomes of animals with nonlocalized centromeres, the kinetochores of plants exist as discrete entities (Braselton, 1971; Lambert, 1971). The discrete kinetochores of Luzula purpurea (Braselton, 1971) and L. albida (Lambert, 1971) occur in recesses along the poleward surface of the chromosomes. Cyperus alternifolius (Braselton, 1971) chromosomes have the same kinetochore distribution patterns, but the kinetochores do not occur in recesses. Serial sections of L. purpurea metaphase chromosomes (Braselton, 1971) showed the kinetochores to be spaced at 0.2 - 0.3 μ intervals along their lengths. It is not clear whether each chromosome was entirely sectioned so it is not known whether each chromosome contains such a kinetochore distribution. There is also no way of determining if the observed C-positive regions represent kinetochore DNA. The fact that distinct C-banded regions can be seen in metaphase chromosomes (Fig. 11g; 11h) at least shows that the DNA of all kinetochore regions could

not be C-positive because their proximity to each other is at the limit of resolution of the light microscope. This would lead to the appearance of entirely C-positive chromosomes.

Many differences exist between the differential staining and DNA late-replication patterns of Vicia faba nuclei and chromosomes and those of Luzula purpurea and L. multiflora. Both the nuclei and chromosomes of V. faba exhibit a few DNA late-replicating regions with restricted distributions (Figs. 4a; 4b; 4e; 4f) while L. purpurea and L. multiflora DNA late-replicating regions are distributed throughout both nuclei (Figs. 5a; 8a) and chromosomes (Figs. 5b - 5e; 8b - 8d). The C-banding patterns are also distinctly different. C-positive regions are localized in restricted regions of V. faba interphase nuclei (Fig. 9a) and are found on either side of the centromere of the M chromosomes and adjacent to the centromere in the long arms of the S chromosomes (Figs. 9c - 9e). Although multiple C-positive regions frequently occur, they lie immediately adjacent to one another. In contrast to this localized arrangement, the multiple C-positive regions of L. purpurea and L. multiflora are distributed throughout the nuclei (Figs. 10a; 12a) and chromosomes (Figs. 10b; - 10f; 11; 12b - 12d). A final dissimilarity occurs in the Q-banding patterns. Q-positive regions of V. faba exhibit a typically restricted distribution pattern (Fig. 13) while those of L. purpurea are widely distributed throughout both nuclei and chromosomes (Fig. 14).

The differences in differential staining and DNA late-replication patterns of Vicia faba and Luzula purpurea and L. multiflora result from the localized nature of the V. faba chromosomal centromere and the nonlocalized nature of the centromeres of L. purpurea and L. multiflora chromosomes. In chromosomes with localized centromeres constitutive heterochromatin is restricted in its distribution while in chromosomes with nonlocalized centromeres constitutive heterochromatin is widely distributed.

Wide distributions of constitutive heterochromatin and highly repeated DNA sequences might be reflected in the DNA from organisms with nonlocalized centromeres. The biophysical characterization of L. purpurea DNA was undertaken to provide information on this hypothesis.

DNA Studies

The results of the DNA studies showed two unusual properties to be present in L. purpurea DNA. Firstly, there was a lack of correspondence in the GC content determined from melting profile data and that determined from buoyant density data, and secondly, a satellite fraction was present in the DNA.

The spectrophotometric T_m values for DNA isolated by both the Bendich and Bolton method and the HAP-urea method were very close to one another (88.5° C and 89.0° C, respectively) as they were to the HAP T_m value for HAP-urea isolated DNA (90.0° C) (Table 2). The GC content determined

from the melting profile data (47.3 - 50.9% GC) was within the range previously found in plants (38 - 50% GC) (Biswas and Sarkar, 1970). On the basis of the GC content of the DNA calculated from the melting profile data, the buoyant density for the DNA was expected to lie between 1.7008 - 1.7044 g/cm^3. The actual buoyant density of DNA isolated by both the Bendich and Bolton method and the HAP-urea method was determined to be 1.6819 $\pm$ 0.0003 g/cm^3 (Fig. 22; Table 3). That this lighter than expected buoyant density was the true one was indicated by the relative purity of the DNA as determined from the 260/280 and 260/230 ratios close to 2.0 and the absence of excessive contaminants in UV scans of the DNA samples (Fig. 18) and was proven by the fact that re-centrifugation of pronase-digested DNA resulted in the DNA's having a buoyant density of 1.6818 $\pm$ 0.0004 g/cm^3, a buoyant density identical to the previously determined one. The estimates of GC content determined from the melting profile data and buoyant density data differed by approximately 20%. Such differences in DNA GC content when determined by different methods are indicative of unusual bases being present (Mandel and Marmur, 1968).

Methylated bases have long been known to be present in DNA (Wyatt, 1951). The principal methylated base has been 5-methyldeoxycytidine. It has been found in organisms ranging from microorganisms to mammals. In animals the content has generally ranged from 0.6 - 2.61 mole % (Vanyushin et al, 1970; 1973) although Unger and Venner (1966) found

8 mole % in human and bull sperm. Microorganisms have an average 5-methyldeoxycytidine content of only 0.2 mole % (Mazin and Sulimova, 1973) while in plants the average 5-methyldeoxycytidine content varies from 2.3 mole % in algae to 5.9 mole % in monocots (Vanyushin et al, 1971; Mazin and Sulimova, 1973). Ferns, mosses, and gymnosperms have an average 5-methyldeoxycytidine content of 3.4 mole % while dicots average 4.9 mole %. The highest quantity of 5-methyldeoxycytidine has been 9.5 mole % in some members of the class Liliatae (Mazin and Sulimova, 1973).

The only other unusual base that has been detected in significant quantities has been 6-methylaminopurine (N^6-methyladenine). It has been reported in bull and human sperm DNA (Unger and Venner, 1966) and plant DNA (Vanyushin et al, 1971) with the content being 0.8 mole % in the sperm and less than 0.1 mole % in plants. Trace quantities of 1-methylguanine and N^2-dimethylguanine were found in human and bull sperm, respectively (Unger and Venner, 1966). The aforementioned methylated bases, as well as, 3-methylcytosine, N^2-methylguanine, and 7-methylguanine were reported in HeLa cell DNA (Culp et al, 1970) although these latter results were not reproducible (Lawley et al, 1972).

Kirk (1967) proposed that the increased volume of bases resulting from the added methyl group would overcome the increase in mass and result in a decrease in the buoyant density. Gill et al (1974) demonstrated that this was true for synthetic DNAs containing 5-methyldeoxycytidine as did Ehrlich

et al (1975) for Xanthomonas oryzae bacteriophage XP-12 DNA in which all the cytosine residues are 5-methyldeoxycytidine. The decrease in synthetic DNA buoyant density varied from 0.00045 - 0.00078 g/cm^3 per percent methylation while XP-12 DNA had a buoyant density of 1.710 g/cm^3, 0.16 g/cm^3 lower than that expected for DNA of comparable GC content. Just as the addition of methyl groups lowered the buoyant density, a loss of methyl groups in bacteriophage PBS2 DNA in which uracil replaces thymine increased the buoyant density (Takahashi and Marmur, 1963). Kemp and Sutton (1976) studied several plant DNAs in terms of their T_m values, buoyant densities, and base compositions and concluded that the presence of 5-methyldeoxycytidine in the DNA does not affect the T_m but that each 6.3 mole % 5-methyldeoxycytidine resulted in a 0.004 g/cm^3 decrease in buoyant density. They found it was possible to determine the 5-methyldeoxycytidine content, and probably the methylated base content in general, of DNA by combining the equations for T_m and buoyant density determination:

$$\text{5-methyldeoxycytidine (Methylated bases)} = \frac{1.6535 - \rho + 0.098\,\frac{T_m - 69}{41}}{0.064}$$

Using this equation the 5-methyldeoxycytidine content of Luzula purpurea DNA was determined to be 30.5 mole %. Since the GC content of the DNA is only 47 - 50%, no more than 23.5 - 25.0% of the methylated bases could be accounted for by 5-methyldeoxycytidine residues even if all deoxycytidines were substituted for in the DNA. Other minor bases

must clearly be present in the DNA, or the DNA must have some unusual structure that is not evident from the data.

A totally unexpected finding was the presence of a satellite fraction in the DNA of Luzula purpurea. Although the number of CsCl buoyant density determinations utilizing monocotyledonous DNA is admittedly small with only eleven having been reported (Green and Gordon, 1967; Ingle et al, 1973), no satellite fraction has been found that has not been attributable to either chloroplast, mitochondrial, or ribosomal DNA. The buoyant density of the satellite fraction (1.6928 g/cm^3) that is present in L. purpurea DNA isolated by the Bendich and Bolton method (Fig. 22a; 22b; Table 3) does not fit into any of these categories.

The buoyant density of higher plant chloroplast DNA has been found to be relatively constant at 1.697 ± 0.001 g/cm^3 (Wells and Ingle, 1970; Kirk, 1971; Herrmann, 1972). A similar situation was found for mitochondrial DNA from higher plants where the buoyant density has always been around 1.706 ± 0.001 g/cm^3 (Borst et al, 1967; Swift and Wolstenholme, 1969; Wells and Ingle, 1970). The high GC content of ribosomal RNA has also led to its DNA template frequently occurring as a satellite band. The DNA also has a high GC content and consequently has a buoyant density greater than 1.700 g/cm^3 (Birnstiel et al, 1971). In plants the satellite bands have had a buoyant density around 1.706 g/cm^3 (Matsuda and Siegel, 1967; Birnstiel et al, 1971).

Another factor favoring the reality of the satellite DNA fraction is the production of two different-sloped lines when the T_m data is plotted on normal probability paper (Fig. 21). Such changes in slope are indicative of nonrandom base arrangements in the DNA resulting from the clustering of particular sequences (Knittel et al, 1968; McLean and Whiteley, 1973). The low melting fraction comprising some 10% of the total DNA and melting with a T_m of 85.5° C corresponds to a DNA fraction containing 39.9% GC. Such a DNA fraction would be expected to have a buoyant density of 1.6930 g/cm^3 which is remarkably close to the one found (1.6928 g/cm^3). The satellite fraction does not correspond to 12% of the total DNA which is the fraction of extremely highly repeated DNA present in Luzula purpurea (Fig. 24; Table 4). This indicates that most of the sequences of the satellite fraction must be interspersed with the main band DNA and thus band with the main band DNA in CsCl. Such a distribution has also been indicated in the DNA of Oncopeltus fasciatus, another organism lacking localized centromeres, (Lagowski et al, 1973) where treatment of reassociated 800 nucleotide long fragments with S_1 nuclease to remove single-stranded regions resulted in a raising of the T_m, a lowering of the sedimentation value, and a decrease in the percent DNA that would then reassociate by low Cot values by an amount indicating that only 40% of the original reassociated fragments had been base paired. This suggests that 400 nucleotide long sequences are interspersed with unique ones

in Oncopeltus fasciatus DNA.

The reality of the satellite of Luzula purpurea is also indicated by the fact that approximately 12% of the DNA is reassociated by very low Cots (Cot 0.01) (Fig. 24), which is a characteristic of DNA sequences with a repetition frequency of 4×10^5 - 4×10^6 (Table 4). The close correspondency between the satellite GC content determined from T_m and buoyant density data (39.9% and 40.9%, respectively) shows that the satellite fraction lacks the unusual bases that are present in main band DNA. A similar lack of unusual bases is present in a satellite DNA associated with chloroplast DNA of Euglena gracilis (Ray and Hanawalt, 1964). However, mouse satellite DNA contains a several-fold greater 5-methyldeoxycytidine content than main band DNA (Salomon et al, 1969).

The additional satellite fraction present in L. purpurea DNA isolated by the HAP-urea method (1.6972 g/cm^3) (Fig. 22c) probably represents chloroplast DNA. Its presence in this DNA preparation and its absence in DNA isolated by the method of Bendich and Bolton probably resulted from the fact that the initial homogenization in the HAP-urea method was by high-speed blending resulting in a fragmentation of both the nuclear DNA and organelles such as chloroplasts.

Approximately 12% of the L. purpurea genome is reassociated by Cot 0.01 indicating these sequences to be highly repeated in the DNA. Similar portions of highly repeated DNA sequences have been reported in other plants that have

been studied (Ranjekar et al, 1974; 1976; Flavell et al, 1974). Included in these plants are several monocots although none of these has been found to have a satellite fraction when centrifuged to equilibrium in CsCl. When the total repeated sequences (Cot 0 - 100) are compared between Luzula purpurea and other plants, the L. purpurea genome at first appears to contain a far lower percentage of repeated sequences. L. purpurea DNA is 46% reassociated by Cot 100 while most other plant DNAs are 70 - 95% reassociated by this Cot value (Flavell et al, 1974; Smith and Flavell, 1974; Ranjekar et al, 1974; 1976). The average size of the DNA fragments used for these reassociation studies were more than double the average size used in the studies reported here (300 nucleotides long) averaging 700 nucleotides in length. When DNA fragments of a size comparable to that used here were used in reassociation studies of conifers, 40 - 50% of the genomes were found to be made up of repeated DNA sequences (Miksche and Hotta, 1973). This suggests that the high fraction of DNA reassociating by Cot 100 in the other studies was due to unique DNA sequences being interspersed with middle repetitive ones as has been found to be the case with middle repetitive DNA sequences of animals (Davidson et al, 1975).

The complexity of the L. purpurea genome was determined from the DNA reassociation data (Fig. 24; Table 4). The measured $Cot_{1/2}$ of the unique sequence class corrected for dilution by repeated DNA sequences (4000 X 0.54 = 2160)

gives a corrected $Cot_{1/2}$ value for unique DNA sequences of 2160. Comparison of the corrected $Cot_{1/2}$ value with the $Cot_{1/2}$ value for Escherichia coli ($Cot_{1/2}$ = 7) (Fig. 23) shows the Luzula purpurea genome to be 308 times as complex as the E. coli genome indicating the haploid DNA content to consist of approximately 1.4×10^9 nucleotide pairs (4.5×10^6 nucleotide pairs X 308).

The apparent wide distribution of the low melting satellite DNA fraction in the L. purpurea genome is very much like the wide distribution of the rapidly reassociating DNA sequences which correspond to the constitutive heterochromatin of C-bands. While it cannot be unequivocally concluded that the C-bands of L. purpurea are comprised of satellite DNA, the following information from the literature suggests that they possibly are. All satellite DNAs that have been studied consisted of highly repeated DNA sequences; satellite DNAs have been localized in constitutive heterochromatin of C-bands; satellite DNAs are generally late-replicating. These facts lend credence to the proposal that the late-replicating highly repeated DNA sequences associated with the C-positive and Q-positive regions of L. purpurea chromosomes are the same DNA sequences that make up the satellite DNA fraction observed in buoyant density studies. Whether in fact such is the case awaits the isolation, biophysical characterization, and cytological localization of the DNA sequences of the satellite.

SUMMARY

The cytological studies of Vicia faba, Luzula purpurea, and L. multiflora and the biophysical characterization of L. purpurea DNA revealed several interesting features in both the chromosomes and DNA of organisms lacking localized centromeres.

In V. faba, an organism with chromosomes containing localized centromeres, constitutive heterochromatin was restricted in its distribution both in nuclei and chromosomes as were the DNA late-replicating and Q-positive regions. A close degree of correspondence existed between C-positive, DNA late-replicating, and Q-positive regions. The major differences that were present occurred in the Q-positive and C-positive chromosome regions with more regions being C-positive than Q-positive indicating that different nuclear protein specificities might exist for the chromosomal binding of quinacrine mustard and Giemsa.

L. purpurea C-positive and DNA late-replicating regions were scattered throughout both nuclei and chromosomes as were C-positive and DNA late-replicating regions in L. multiflora nuclei and chromosomes. There was a close correlation between their chromosomal distributions indicating that constitutive heterochromatin is late-replicating in these organisms just as is generally the case with other organisms. The C-positive and DNA late-replicating regions in the chromosomes of L. purpurea also corresponded to the Q-positive

regions and the labeled chromosome regions resulting from in situ hybridization of ^{3}H-RNA complementary to highly repeated DNA sequences. The techniques permitted not only the identification of homologous chromosomes but also allowed comparisons to be made of chromosomes subjected to different cytological techniques.

Luzula purpurea DNA exhibited two unexpected characteristics. From the melting temperature of the DNA (88.5° C - 90.0° C) a buoyant density of greater than 1.7000 g/cm^3 was expected. The actual buoyant density was 1.6819 ± 0.0003 g/cm^3 indicating a high content of methylated bases to occur in the DNA (30.5 mole %). Two satellite DNA fractions (1.6928 g/cm^3 and 1.6972 g/cm^3) were found. The heavier satellite corresponds to chloroplast DNA (1.697 ± 0.001 g/cm^3), but the lighter one appears to be nuclear in origin being widely distributed in the DNA and lacking methylated bases. Reassociation experiments showed the DNA to consist of 25%, 21%, and 54% highly repeated, moderately repeated, and unique DNA sequences, respectively.

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*Original article not consulted.