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DISTRIBUTION AND PROPERTIES OF NUCLEAR SATELLITE DNA

by

Graham George Pearson

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"Now, as far as the satellite itself is concerned, that does not raise my apprehensions, not one iota."

President Eisenhower.

4th October, 1957.

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ABBREVIATIONS

A	adenine
C	cytosine
DNA	deoxyribonucleic acid
HMB-DNA	homogeneous mainband deoxyribonucleic acid
rDNA	deoxyribonucleic acid containing the cistrons for ribosomal ribonucleic acid
sat.DNA	satellite deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
G	guanine
OD_{260}	optical density at 260nm.
PAS	4-aminosalicylic acid
PBD	2-phenyl-5-biphenyl-oxadiazole
ρ	buoyant-density
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNase	ribonuclease
r.p.m.	revolutions per minute
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
T	thymine
T_m	temperature corresponding to a 50% increase in hyperchromicity
TCA	trichloroacetic acid
TNS	sodium tri-iso-propyl naphthalene sulphonate
Tris	tris(hydroxymethyl)aminomethane
UV	ultra-violet
vol.	volume

SUMMARY

The aims of this thesis were fourfold:

a. to study the distribution of satellite DNAs (sat.DNAs) within a wide variety of plant species and to see whether there was any meaningful taxonomic correlation;

b. to determine whether sat.DNAs were quantitatively and qualitatively constant within different tissues and during development of a species;

c. to determine the properties of such DNAs in terms of buoyant-density, renaturation rate and homogeneity;

d. to consider possible functions of the sat.DNA in relation to rRNA cistrons and B-chromosomes.

Before examining the distribution and properties of sat.DNA, it was necessary to clearly define "sat.DNA" since other workers had used the term variously. In the context of this thesis, "sat.DNA" was normally defined as a minor component resolved in a neutral CsCl gradient.

Sat.DNA was shown to be distributed throughout a wide range of dicotyledon orders ranging from the primitive Magnoliales to the advanced Asterales. Although one species in a genus might contain a sat.DNA, it did not necessarily follow that every species in that genus would have a satellite. It was observed that only species with $\geq$2-3pg. DNA per 2C nucleus contained a sat.DNA. Sat.DNAs were not found in any species of monocotyledon examined. However, these all contained $>$2-3pg. DNA/2C nucleus. Most sat.DNA was found in plants which had been highly bred or cultivated e.g. melon, orange. Not every highly bred plant, however, contained sat.DNA e.g. pea. Certainly, no uncultivated plant contained $>$10% of sat.DNA.

Concerning the origin of sat.DNA, it was suggested that the Solanaceae at least must have individually developed sat.DNAs, and if this procedure was possible in one family, then presumably it was possible in others.

Normal sat.DNAs were shown to be plant nuclear in origin, unlike stress-satellite DNAs (sat.DNAs appearing during conditions of physiological stress) which were shown to be bacterial in origin. Like animal sat.DNAs, plant satellites renatured rapidly following denaturation and consisted of probably many (certainly at least 5-6) tandem copies of a relatively short sequence $\sim 2 \times 10^6$ daltons in length. The 'ancestral' sequence length was probably about the same as the present length. Plant sat.DNA appears to be more homogeneous than mainband DNA, particularly when of high molecular weight.

It was found that during development of melon and cucumber plants, the different tissues varied in both the percentage and buoyant-density of sat.DNA, meristematic tissues containing the highest percentage (e.g. seed and root tip) whereas differentiated tissues (e.g. fruit, mature leaves and cotyledons) contained least. As fruit, cotyledons and leaves aged, the percentage sat.DNA decreased, and in fruit, the buoyant-density increased. It was shown that in developing leaves, at least, the sat.DNA had a high rate of turnover. It was postulated that the increase in sat.DNA density during fruit maturation was caused by either differential synthesis of various fractions within the sat.DNA or by the degree of methylation of cytosine bases within new sat.DNA being less than in the old sat.DNA. The different amounts of sat.DNA in fruit and seed were correlated with different amounts of heterochromatin in these tissues. Furthermore, the higher percentage of sat.DNA in meristematic tissues suggested that the function of

sat.DNA might be associated with mitosis.

It was found that there was no enrichment of sat.DNA in the NaCl-insoluble fraction of chromatin from plant material.

There was no correlation between rDNA and sat.DNA nor, in the species examined, between B-chromosomes and sat.DNA.

SECTION 1: INTRODUCTION

Equilibrium centrifugation in neutral CsCl solution of DNA from two species of crab revealed that each contained two classes of DNA (Sueoka, 1961). Similarly it was observed that all tissues examined in mouse (Mus musculus) and guinea-pig (Cavia porcellus) contained two classes of DNA (Kit, 1961). Subsequently, the class of DNA present in the larger amount became known as the mainband DNA and the minor component as the satellite DNA (sat.DNA). Since 1961, several laboratories have made extensive studies on the mouse satellite DNA, the properties of which can serve as a reference with which to compare other less-well documented sat.DNAs.

The buoyant-densities of mouse DNAs were 1.700 $g.cm.^{-3}$ (mainband) and 1.690 $g.cm.^{-3}$ (satellite band). These buoyant-densities indicated DNAs containing 41% and 31% guanine+cytosine (GC content) respectively based on the linear relationship observed between buoyant-density and GC content (Schildkraut, Marmur and Doty; 1962). However, the compositions of these two DNAs were 40% and 41% GC when estimated from their thermal denaturation properties (Marmur and Doty, 1959). Direct chemical analysis showed respectively 40% and 35% GC (Corneo, Ginelli, Soave and Bernardi; 1968). Such results indicated peculiarities in the properties of the sat.DNA, since the relationships between buoyant-density and GC content and between thermal denaturation and GC content, which held for mainband DNA, were not applicable to the satellite component.

The sat.DNA comprised approximately 10% of the total cellular DNA (Kit; 1961, 1962). Furthermore, all mouse tissues and cultured mouse cells examined were shown to contain the sat.DNA in approximately

the same proportions (Kit; 1961, 1962). The proportion of satellite in DNA extracted from whole tissue was indistinguishable from that found in DNA prepared from purified nuclei (Corneo, Bianchi, Ginelli and Polli; 1966; Bond, Flamm, Burr and Bond; 1967).

Both satellite and mainband DNAs were double-stranded (Kit, 1962). Ultracentrifugation of sat.DNA in alkaline CsCl, under which conditions the two strands of the DNA separate, revealed two components (Flamm, McCallum and Walker; 1967) indicating that the two strands were of different buoyant-densities. After separation of the strands in an alkaline CsCl gradient, each component was purified and analysed on a neutral CsCl gradient. The densities were 1.698 and 1.727 $g.cm.^{-3}$ for the "light" and "heavy" strands respectively (Corneo, Ginelli, Soave and Bernardi; 1968; Flamm, McCallum and Walker; 1967). Chemical analysis of the purified strands showed that the purine:pyrimidine ratio of the "light" strand was 2.01 and of the "heavy" strand 0.49 (Corneo et al., 1968). There was, thus, a tremendous difference between the two complementary strands of the satellite. Alkaline CsCl centrifugation of mainband DNA yielded only a single peak. During alkaline denaturation of sat.DNA, titration of the N-H protons of the thymine and guanine residues occurs causing the two strands of the DNA to run at different densities on an alkaline CsCl gradient - the denser strand being G+T - rich and the less-dense strand A+C - rich (Vinograd, Morris, Davidson and Dove; 1963).

Neutral CsCl gradient analysis of heat denatured DNA showed that the buoyant-densities of both mainband and satellite increased by 14 $mg.cm.^{-3}$ When the denatured DNA was subjected to renaturing conditions, the sat.DNA renatured to within 3 $mg.cm.^{-3}$ of its initial density whereas the mainband DNA did not renature appreciably within

the same length of time (Waring and Britten, 1966). However, if denatured DNA was sheared at 800 atm., the sat.DNA renatured completely and the mainband renatured to within 4 $mg.cm.^{-3}$ of its initial density under identical conditions. When the renatured DNAs were melted, the sat.DNA showed a normal profile with full increase in hyperchromicity whereas the mainband DNA showed only a small increase in hyperchromicity and no distinct T_m, the temperature corresponding to a 50% increase in hyperchromicity. The rate of renaturation of sat.DNA closely followed that expected of a 2nd order reaction - twice the DNA concentration resulted in twice the rate of renaturation.

The renaturation rate of mouse sat.DNA was greater than that of any other type of DNA known at the time under similar conditions. It renatured 15x faster than SV40 DNA and was therefore composed of many copies of a short nucleotide sequence. Allowing that SV40 DNA contained 6,000 base-pairs, the length of the repeated sequence in mouse sat.DNA was 400 base-pairs or 264,000 daltons.

The mouse cell contained $5x10^{-12}$g. DNA i.e. $0.5x10^{-12}$g. sat.DNA. This was equivalent to $3x10^{11}$ daltons, indicating approximately 10^6 copies of such a DNA sequence. The sharpness of the satellite band of native DNA in neutral CsCl equilibrium centrifugation indicated that the molecular weight of the segment of DNA in which it occurred was at least several million daltons. (This was based on experiments in which DNA of varying molecular weights was centrifuged and the sharpness of the bands and the percentage of satellite were examined.) This suggested that the repeated sequences were clustered together. An estimate of the molecular weight of the clustered sat.DNA sequences indicated a minimum of 100 tandem copies of a sequence 300-400 base-pairs long. If these blocks were spread evenly throughout the genome,

there would be on average about 300 of such blocks per chromosome. If, on the other hand, the sequences were gathered in blocks up to the length of a chromosome, there would, in the mouse, need to be at least 4 chromosomes entirely composed of sat.DNA (Walker, Flamm and McLaren; 1969).

Subsequently, more-detailed studies on the melting of renatured sat.DNA showed that the T_m was 5^o lower than for the native satellite (Flamm et al., 1967). This decrease in thermal stability might be interpreted in terms of the occurrence of mismatching during renaturation; moreover, the complexity of the DNA was reduced from 300 to 120 base-pairs when allowance was made for mismatching (Sutton and McCallum, 1971). Consequently, it was not true to say that there were ca.10^6 copies of a sequence, but that there were ca.10^6 similar sequences.

The very large difference in base-composition between the two complementary strands of mouse sat.DNA greatly facilitated the determination of the sequence using pyrimidine tract analysis (Southern, 1970). It was proposed that the basic unit could be 8-13 base-pairs in length, as compared with the 300-400 base-pairs proposed by Waring and Britten (1966) or the 120 base-pairs after correction for mismatching (Sutton and McCallum, 1971).

The intra-cellular location of mouse sat.DNA was shown to be within the nucleus (Corneo, Bianchi, Ginelli and Polli, 1966; Bond, Flamm, Burr and Bond, 1967). Maio and Schildkraut (1969) showed that sat.DNA was present in the same proportion in isolated chromosomes of all sizes, but that it was restricted to the heterochromatic fractions. Schildkraut and Maio (1968) showed that there was an increase in the percentage of sat.DNA from preparations of isolated

nucleoli. Subsequently, Jones (1970) and Pardue and Gall (1970) showed, by *in situ* hybridization, that the sat.DNA was preferentially localized in the heterochromatic regions round the centromeres of metaphase chromosomes. This hybridization was specific since mouse sat.DNA hybridized *in situ* to rabbit cells gave no specific binding. Other workers have also produced evidence associating sat.DNA with heterochromatin (Yasmineh and Yunis, 1970; Mattocia and Comings, 1971; Madreiter and Osieka, 1971).

Satellite DNAs have been shown to be present in many animals other than mouse. The term "satellite DNA" has been extended to include fractions of DNA which are not necessarily detectable on neutral CsCl gradients. If heavy metal ions, such as Ag^{+} or Hg^{++}, were included in a Cs_2SO_4 gradient, components not apparent in a CsCl gradient were often resolved (Jensen and Davidson, 1966). Three such satellite components have been fractionated in guinea-pig DNA (Corneo, Ginelli, Soave and Bernardi; 1968) and three from human tissues (Corneo, Ginelli and Polli, 1967; Corneo, Ginelli and Polli, 1971a), their properties being very similar to those of the mouse satellite.

In addition, repetitious DNA which was not resolved by CsCl gradient centrifugation was also frequently referred to as satellite DNA. Britten and Kohne (1969) demonstrated that rapidly renaturing, repetitious DNA occurred in many species of plants and animals which showed no sat.DNA on a gradient. They showed that a general eucaryotic tissue contained the following DNAs: 10% of a fast-renaturing, highly repetitious DNA (10^4 copies); a very slow-renaturing, "unique-copy" DNA (1-10 copies); an "intermediate" fraction which renatured at a rate between the previous two fractions and which might have 10-1000

copies of a genome sequence.

There was also a fourth category of DNA which was sometimes called "satellite DNA", i.e. the DNA complementary to rRNA. If a CsCl gradient was loaded with Xenopus laevis oocyte DNA, a satellite DNA of density 1.723 $g.cm.^{-3}$ was observed, compared with mainband DNA of density 1.700 $g.cm.^{-3}$ This "sat.DNA" which contained the cistrons for rRNA was quite different from the other classes of sat.DNA, which do not contain the ribosomal cistrons. In the context of this thesis, sat.DNA will normally refer to a minor component resolved in a neutral CsCl gradient.

The three Ag^{+} sat.DNAs isolated from human DNA were similar to mouse satellite in that they were rapidly renaturing. An additional Hg^{++} satellite, termed the homogeneous mainband DNA (HMB-DNA), was isolated, which resembled the "intermediate" DNA of Britten and Kohne, 1969 (Corneo, Ginelli and Polli; 1971a). Like the mouse satellite, human Ag^{+} sat.DNA II was found to be restricted to the centromeric and other constitutive heterochromatic in chromosomes 1, 9, and 16 (Jones and Corneo, 1971). Human Ag^{+} sat.DNA III has been examined by Jones and Prosser (1973) who showed that it hybridized to the pericentromeric heterochromatin mainly on chromosome 9. Prosser (1974) demonstrated that human Ag^{+} sat.DNA I hybridized to a large block of heterochromatin on the distal arm of the Y-chromosome and the heterochromatin of a C-group chromosome, probably chromosome 9. In contrast, HMB-DNA did not hybridize to any specific sites on any of the chromosomes but had a heterodisperse distribution (Corneo, Ginelli and Polli, 1970b; 1971b).

Similar in situ hybridizations with Drosophila species, where giant salivary gland chromosomes allowed more detailed study, showed that, in general, the sat.DNA sequences were restricted to the

α - heterochromatin and were not present in the heterochromatin of the Y-chromosome.

In other mammals, the correlation between sat.DNA and heterochromatin has been shown in Cervulus muntjac (Comings, 1971), in Microtus agrestis (Arrighi, Hsu, Saunders and Saunders; 1970) and in Drosophila (Kram, Botchan and Hearst; 1972). The general procedure was to show by in situ hybridization that the sites in the chromosomes containing sat.DNA were also heterochromatic as revealed by staining with either Quinacrine fluorescence or Giemsa. A variant of this relationship was the demonstration by Gibson and Hewitt (1970) that in the grasshopper Myrmeleotettix maculatus those members of the species which contained a heterochromatic B-chromosome had a sat.DNA, whereas those without the B-chromosome had no sat.DNA.

To explain the origin of sat.DNA - the appearance of millions of copies of a short sequence, representing a considerable fraction of the genome - Britten and Kohne (1967) proposed "saltatory replication", multiplication of a sequence within a few cell divisions, possibly within one individual of the species. Mutagenic events during saltatory replication of this simple sequence have been proposed by Southern (1970) to account for the apparently longer sequence initially proposed by Walker. The sequences were then imagined to have spread throughout the whole species by breeding. Alternatively, the sequences might have accumulated slowly over a period of time comparable with the age of the species.

There was, therefore, some confusion as to the "ancestral sequence" complexity of mouse sat.DNA. Simple renaturation analysis suggested a complexity of 300-400 base-pairs. This might be reduced to 120 base-pairs by correcting for mismatching. Sequence determination

suggested the value could be as few as 8 base-pairs.

Studies on the possible origins of sat.DNAs led to phylogenetic taxonomies of their own. Southern (1970) proposed that the guinea-pig Ag^+ α-satellite might have originated as a sequence of only 6 base-pairs in length. This contrasted with larger estimates for the sequence length indicated from renaturation studies i.e. several hundred base-pairs (Corneo et al., 1970a). This discrepancy in apparent size could have arisen as a result of mutations introduced during the multiplication of this short sequence. From calculations involving possible mutation rates, Southern (1970) proposed that the guinea-pig could have diverged from the other rodents as long ago as 50×10^6 years. This date is in agreement with Simpson (1959) whose estimate was based on more-conventional, zoological data. Furthermore, within the rodents, Walker, Flamm and McLaren (1969) investigated the relationship between the highly repetitive DNAs in several species of rodent and concluded that guinea-pig (Cavia porcellus), European wood mouse (Apodemus sylvaticus), brown rat (Rattus norvegicus), and 2 species of N. American deer mouse (Peromyscus manipulatus and P. polionotus) contained less than 1 in 10^6 sequences like mouse (Mus musculus) sat.DNA, supporting the idea that divergence of satellite accompanied divergence of species.

Satellite DNA has also provided further phylogenetic and taxonomic information on the primates. Prosser, Moar, Bobrow and Jones (1973) showed that human Ag^+ sat.DNA III and chimpanzee sat.DNA A were virtually identical and hybridized to the same chromosome regions in human, chimpanzee, and orang-outang cells showing that the three primates contained very similar DNA sequences. Consequently, one could commence to date the origin of these sat.DNAs and/or the date of divergence of the species.

Suggestions for the function of sat.DNAs were based upon the correlation between sat.DNA and constitutive heterochromatin. The regions which hybridized with human Ag^+ sat.DNA II, i.e. the secondary constrictions on chromosomes 1, 9, and 16 (Jones, 1970), were heterochromatic on the basis of Giemsa and Quinacrine fluorescence staining (Craig-Holmes and Shaw, 1971; Ganner and Evans, 1971; Bobrow, Madan and Pearson, 1972; Saunders, Hsu, Getz, Simes and Arrighi, 1972). Moore and Abuelo (1971) further investigated these areas microscopically and found that they contained the largest number of DNA-containing inter-arm fibres - DNA strands linking the arms of the chromatids. They suggested that the inter-arm fibres might contain sat.DNA. Since the low level of complexity of sat.DNA indicated that it did not code for proteins, it appeared likely that it played a structural or organizational role - it could prepare the DNA for distribution to the daughter cells. The failure of fibres to separate might cause the 2 chromatids to go to the same daughter cell giving rise to one cell exhibiting trisomy. Walker (1971) suggested that sat.DNA might be important in the pairing of chromosomes during meiosis and that the satellite could be used in attachment of chromosomes to the spindle.

In plants, Suyama and Bonner (1966) demonstrated that turnip (Brassica rapa) nuclei contained 2 DNAs: a mainband of $\rho = 1.692$ g.cm.$^{-3}$ and a sat.DNA of $\rho = 1.700$ g.cm.$^{-3}$

Wells and Ingle (1970) examined a range of plants with mainband DNA varying from $\rho = 1.691 - 1.702$ g.cm.$^{-3}$ and found that the chloroplast DNA was constant at $\rho = 1.697 \pm 0.001$ g.cm.$^{-3}$ and the mitochondrial DNA at $\rho = 1.706 \pm 0.001$ g.cm.$^{-3}$ The sat.DNA in turnip (Suyama and Bonner, 1966) was, therefore, a true nuclear satellite.

Furthermore, it has been shown that in preparations of DNA from total-cellular material, chloroplast DNA was not resolved on the gradient. However, it could affect the percentage of sat.DNA, since the observed percentage of sat.DNA would be the satellite expressed as a fraction of the nuclear + chloroplast DNAs. Mitochondrial DNA, however, was resolved from the mainband but it was not observed since it was less than 0.1% of the total DNA. However, organelle DNAs could become the major components of DNA prepared from cell fractionations.

Satellite DNAs of plants were first investigated by Matsuda and Siegal (1967) who showed that purified nuclei from Chinese cabbage (Brassica pekinensis), French bean (Phaseolus vulgaris), and pumpkin (Cucurbita pepo) contained sat.DNAs. Furthermore, they found that the sat.DNAs, unlike the mainband DNAs, renatured rapidly following denaturation. They also observed that these 3 plants containing sat.DNAs had a higher percentage hybridization with rRNA than had wheat or tobacco, which contained no sat.DNA. This suggested that sat.DNAs were related to rRNA cistrons. The sat.DNA of pumpkin was fractionated from the mainband by preparative CsCl gradient centrifugation and was shown to contain 3.6% DNA complementary to rRNA, compared with 0.22% in the mainband. It has, however, been demonstrated that in fractionation of DNA from species which contained no sat.DNA, the rRNA cistrons occupied a position on the gradient of approximately 1.706 $g.cm.^{-3}$ - a density similar to that of the sat.DNA of pumpkin (Scott and Ingle, 1973). Consequently, the relationship between sat.DNA and rDNA in plants was not clear.

Beridze (1972) examined 6 different species of the genus Phaseolus and found that 3 had about 30% sat.DNA and 3 had less.

The former 3 species originated in Mexico and Central America, whereas the latter originated in S.E. Asia. Such a distribution suggested that the percentage of sat.DNA might be related to evolution.

A further type of sat.DNA appearing in response to certain physiological stress conditions has been described. When certain plants were either germinated in the dark on distilled water, wounded, bruised or decapitated, or seeds were incubated at 0 - 4°C for 15 days, a sat.DNA appeared that had a high buoyant-density, 1.722 $g.cm.^{-3}$ (Quétier and Guillé, 1968; Quétier, Guillé and Vedel, 1968; Guillé, Quétier and Cau, 1967; Guillé, Quétier and Huguet, 1968). Hybridization with rRNA showed that the stress-sat.DNA hybridized 100 times as much as the mainband DNA. Furthermore, from the small increase in density on denaturation, it was postulated that this DNA might be DNA in the course of transcription. From the similarity of density of stress-sat.DNA and the density of the rRNA cistrons in Xenopus, it was concluded that this stress-sat.DNA represented a massive amplification of the rDNA, analogous to the situation in X.laevis oocytes where several thousand copies of the rDNA were produced. It was also proposed (Guillé et al., 1968) that there might be some relationship between this stress-satellite and the so-called "conditioning" of plants during crown-gall infection. Some homology between the stress-sat.DNA, tumour-cell DNA, and DNA from the bacterium Agrobacterium tumefaciens was demonstrated (Quétier, Huguet and Guillé; 1969). A correlation between the appearance of the stress-satellite and the onset of tumourization was observed by Guillé and Quétier (1970) and an analysis of DNA from crown-gall infected tissues by $Ag^{+} - Cs_2SO_4$ density-gradient revealed a sat.DNA not present in healthy tissues and which appeared to be very similar to the stress-sat.DNA (Guillé and

Grisvard, 1971).

A similar dense sat.DNA has been described in Chlorella pyrenoidosa (Gense-Vimon, 1971; Rode and Bayen, 1970) and in Porphyridium sp. (Gense-Vimon, 1971). It was shown that the dense-sat.DNA of C.pyrenoidosa had several variable factors. In one experiment it was not detected under conditions which normally favoured its appearance and its density was found to vary from 1.7235 to 1.727 $g.cm.^{-3}$ under identical conditions. It was proposed that the time of sampling of the culture could be responsible for this variation (Bayen and Rode, 1970). Since this satellite appeared during the first nuclear division, it was suggested that it might have had a function in cell division.

Our knowledge of sat.DNA in plant cells was less than our knowledge of sat.DNA from animal tissues. Moreover, several kinds of observation related plant sat.DNA with rRNA cistrons, a relationship which certainly did not hold for the more-extensively studied animal sat.DNAs. The appearance of stress-satellites suggested a more physiological role, again not indicated from animal studies.

The aims of this thesis were as follows:-

(a) to study the distribution of sat.DNAs within a wide variety of plant species and to see whether there was any meaningful taxonomic correlation;

(b) to determine whether sat.DNAs were quantitatively and qualitatively constant within different tissues and during development of a species;

(c) to determine the properties of such DNAs in terms of buoyant-density, renaturation rate and homogeneity;

(d) to consider possible functions of the sat.DNA in relation to rRNA cistrons and B-chromosomes.

SECTION 2: MATERIALS AND METHODS

Material

Material was grown in the Departmental garden or greenhouse or obtained commercially.

Methods

1. Preparation of total DNA.

Small quantities (0-10g) of tissue were homogenized directly with a pestle and mortar in a detergent medium (4ml/g tissue) containing 1% TNS, 6% PAS, 50mM sodium chloride, 10mM Tris-HCl pH7.4, 6% n-butanol, and 10mM EDTA pH7.4.

Larger amounts of tissue (10-100g), particularly fruit which was difficult to grind in the pestle and mortar, were homogenized in the cold in the 'VirTis' or 'Polytron' in 50mM NaCl, 10mM Tris-HCl pH7.4, 6% n-butanol, and 10mM EDTA pH7.4. Detergents were immediately added to give final concentrations of 1% TNS and 6% PAS.

The NaCl concentration was then increased to 0.5M, and an equal volume of 96% chloroform/4% amyl alcohol added. Vigorous shaking was followed by centrifugation for 5-10 min. at 1500xg and $0^{o}C$.

The aqueous layer containing the nucleic acids was removed and further deproteinized with an equal volume of phenol mixture (phenol containing 10% m-cresol, 0.1% 8-hydroxyquinoline, and saturated with 10mM Tris-HCl pH7.4). After centrifugation, the aqueous layer was removed and the nucleic acids precipitated by the addition of 2 volumes of absolute ethanol and storage at $0-4^{o}C$ for 16-20 hr. The precipitate, containing the RNA and DNA, was collected by centrifugation and dissolved in a small volume of 0.5% SDS (B.D.H. "specially pure")

and 0.15M sodium acetate. The nucleic acids were reprecipitated by the addition of 2 volumes of ethanol and storage at 0°C for 2 hr. The precipitate was collected by centrifugation and washed by suspension in 80% ethanol-0.2% SDS. The nucleic acid pellet was dissolved in a small volume of 0.1xSSC (SSC=standard saline citrate: 0.15M NaCl, 0.05M sodium citrate, pH7.4) and then the concentration was increased to 1xSSC by the addition of 10xSSC. Ribonuclease (Type 1-A Bovine pancreas, Sigma), which had been treated at 80°C for 5 min. to inactivate any DNase, was added to give a final concentration of 50µg/ml and the solution was incubated at 37°C for 2 hr. Pronase (B.D.H. Chemicals Ltd.), which had been previously self-digested at 20°C for 10 min., was then added (final concentration 400µg/ml) and incubation continued at 37°C for 2 hr. to remove RNase and any protein remaining in the preparation. The solution was cleared of any particulate matter by centrifugation at 10,000xg for 10 min. and the DNA recovered by centrifugation at 125,000xg for 16 hr. at 0-10°C.

The pellet was dissolved in 0.1xSSC and the concentration of DNA calculated from the Optical Density at 260nm., assuming 40µg of DNA in 1ml has an OD_{260} of 1.0 in a 1cm. light-path.

Radio-active labelling of leaf DNA

Spanish Winter Melon seeds were germinated in Vermiculite. After 7 days, the seedlings were transferred to liquid culture (0.25xHoagland's solution) with 8 seedlings held in a blackened 50ml beaker. Growth continued with 8 hr. days (1800 foot candles illumination) at 20°C, with aeration provided by bubbling air through the culture solution. After 4 days, by which time the first leaf was 3-5mm. in length, the medium was removed and replaced by distilled water. Twenty-four hours later, 2mCi of ^{32}P-orthophosphate in $5x10^{-5}$M

^{31}P-orthophosphate (100 x phosphate concentration of ^{32}P) was added. Seedlings were harvested after a further 24 hr. growth and DNA was prepared from the first leaves. A sample of DNA was analysed on the Model E to determine the percentage of satellite DNA. The remainder of the DNA was fractionated by preparative CsCl centrifugation (see page 24) and 0.1ml samples collected. Each fraction was diluted to 1ml with 0.1xSSC and its degree of radioactivity determined by the Cerenkov radiation counting method on the "Intertechnique" Liquid Scintillation Counter Model SL30 (^{3}H-preset channel).

To further reduce any background due to RNA nucleotide contamination, each fraction was precipitated by TCA (5% final concentration) in the cold, and the DNA was collected onto an "Oxoid" membrane (3cm., standard grade). The Cerenkov tubes were then washed out with cold 5% TCA which was passed through the membranes which were finally washed with 1ml 0.5% TCA. The filters were dried and counted in 10ml of Toluene-Butyl PBD scintillation fluid by the SL30 (^{32}P-preset channel).

2. Preparation of nuclear DNA

Tissue was homogenized (1g tissue per 4 ml medium) at 0^{o}C in the 'VirTis' homogenizer in Honda medium (Honda et al., 1966) supplemented with magnesium (0.25M sucrose, 2.5% w/w Ficoll (molecular weight 400,000), 5% w/w Dextran (molecular weight 90,000), 0.025M Tris-HCl pH7.8, 10mM magnesium acetate, and 2mM calcium chloride). The homogenization was at 45,000 r.p.m. for as short a period as possible in order to break the maximum number of cells with minimal nuclear disruption. In practice, very few nuclei survived, as shown by microscopic examination, and the result was a chromatin preparation. The homogenate was filtered by squeezing through a nylon gauze (pore

diameter 25μ) and the filtrate centrifuged at 1000xg for 10 min. The pellet was resuspended in 0.2M sucrose, 0.01M Tris-HCl pH7.8, and 2mM calcium chloride (0.1 x volume used for homogenization) and treated with Triton X-100 (4% final concentration) to solubilize chloroplast and mitochondria membranes. If the pellet, collected by centrifugation at 2500xg for 15 min., was still green, the Triton treatment was repeated. DNA was prepared from the pellet by shaking in detergent medium, followed by deproteinization and purification as described for total DNA.

3. Properties of DNA

a. Buoyant density

The DNA was analysed by equilibrium centrifugation in neutral CsCl on the 'Beckman' Model E analytical ultracentrifuge. Samples containing 2.4μg DNA, 0.8g CsCl and 0.62ml 0.1xSSC were prepared. DNA of the bacterium Micrococcus lysodeikticus (buoyant density 1.731g.cm^{-3}) was added as internal marker (20μl containing 1μg DNA). The refractive index of the solution was checked to be within the range 1.3990 to 1.4010, corresponding to initial densities of 1.6959 to 1.7175 g.cm^{-3} respectively (Schildkraut et al., 1962). The samples were centrifuged within either the 2 or 4 place rotors at 44,000 r.p.m. for 20 hr. at 25°C. The positions of the DNA bands within the CsCl gradient were then determined from photographs taken with UV light using 'Ilford' N4E50 film, which was developed for 2 min. in 'Ilford' ID2 developer and fixed for 2 min. in 'Ilford' Ilfofix. Ten minutes washing in running water followed and then the film was allowed to dry.

The photographs were scanned with a Double-beam recording

microdensitometer, Mk.IIIC (Joyce, Loebl & Co. Ltd., Gateshead-on-Tyne). The buoyant-density of the sample DNA was normally calculated from its position in the gradient relative to the M.lysodeikticus marker DNA. In certain cases, it was more convenient to use Xenopus laevis or T_4 DNA as marker. The gradients were initially calibrated by the centrifugation of 2 marker DNAs (M.lysodeikticus and Xenopus) in solutions of varying initial density, which resulted in the DNAs banding in different regions of the cell. The difference in distance between the bands, known to be equivalent to 31 $mg.cm^{-3}$, was thereby related to their position within the cell, defined by the position of the M.lysodeikticus band relative to the reference marker at the bottom of the cell (Outside Marker) (fig.1).

The position of the M.lysodeikticus DNA relative to the bottom of the cell, therefore, gave the relevant value of the density gradient, which, when multiplied by the distance between the marker and sample DNAs, gave the difference in density between the two DNAs.

Error involved in DNA preparation and Model E analysis

Six preparations of total-cellular DNA were made from samples of 20 seeds obtained from one melon (S1 to S6). Each sample was analysed by Model E centrifugation, and analysis of S1 DNA was repeated at 6 different centrifugations, and of S2 and S3 at 2 different centrifugations each. The buoyant densities of the mainband and satellite DNAs and the satellite DNA expressed as a percentage of the total DNA for each centrifugation were determined (Table 1).

It was found that for S1, the mean density of the mainband DNA was 1.6921 ± 0.0002 $g.cm^{-3}$. The satellite was present as $24.4 \pm 1.0\%$ of the total DNA.

FIGURE 1

Microdensitometer scan of Model E photograph.

Micrococcus lysodeikticus (1.731 $g.cm.^{-3}$) and Xenopus laevis (1.700 $g.cm.^{-3}$) DNAs were centrifuged in a neutral CsCl gradient at 44,000 r.p.m. for 20 hr. at 25°C in the Model E as described in text. UV photographs were taken and a microdensitometer scan made.

O.M. = outside marker of the Model E cell.

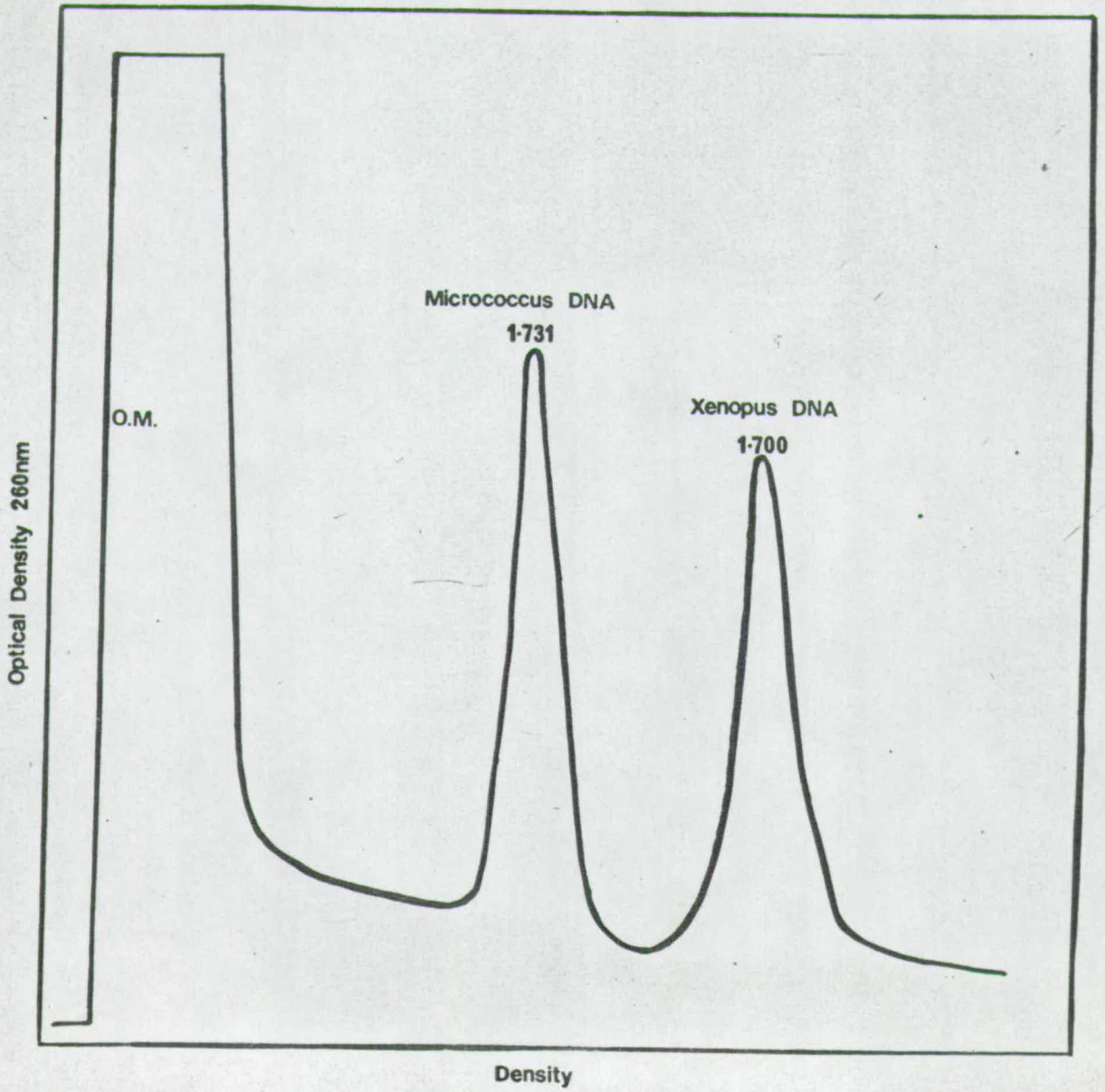

Optical Density 260nm
O.M.
Micrococcus DNA
1·731
Xenopus DNA
1·700
Density

Table 1

The accuracy of buoyant-density and percentage satellite determinations.

Sample	Buoyant-density ($g.cm.^{-3}$)		Satellite
	Mainband	Satellite	(% of total)
S1	1.6919*	1.7055	24.9
	1.6924	1.7059	24.6
	1.6922	1.7057	23.2
	1.6921	1.7056	23.1
	1.6921	1.7054	25.2
	1.6919	1.7055	25.6
S1 mean	1.6921 ± 0.0002	1.7056 ± 0.0002	24.4 ± 1.0
S2	1.6918	1.7055	27.0
S3	1.6924	1.7057	26.6
S4	1.6922	1.7057	23.3
S5	1.6923	1.7057	24.2
S6	1.6923	1.7058	23.9
S1-6 mean	1.6922 ± 0.0002	1.7057 ± 0.0002	25.0 ± 1.5

* Value used for the S_{1-6} analysis.

Six preparations of total DNA were made from samples of 20 seeds obtained from one melon (S1 to S6). Each sample was analysed by Model E centrifugation, and analysis of S1 DNA was repeated at 6 different centrifugations.

For the six samples (S1 to S6), the mean mainband density was 1.6922 ± 0.0002 $g.cm^{-3}$ and the mean satellite density 1.7057 ± 0.0002 $g.cm^{-3}$. 25.0 ± 1.5% of the total was satellite DNA.

The results indicate that the variance within S1 is not significantly different from the overall variance (S1 - 6), i.e. the error involved in DNA preparation is no greater than the error involved in Model E data analysis.

b. Determination of molecular weight

The molecular weight of the DNA preparation was determined using the "spillover" technique (Studier, 1965). When necessary, the molecular weight of the DNA was reduced to approximately $1-2x10^6$ daltons by repeated rapid passage through a syringe needle. DNA size could be further reduced by sonication with an M.S.E. "Ultrasonic Disintegrator, 60W Model" at a frequency of 20 kHz.

Approximately 30µl of DNA (60-80µg/ml) were placed in the spillover well in the centre-piece of the Model E cell. The rest of the cell was filled with 1.0M NaCl/0.05M sodium citrate, a small air-space being left at the top of the cell in order to give a visible meniscus on the photographic film. When centrifugation started (5000-10,000 r.p.m.), solution from the well spilled over onto the top of the liquid in the cell, which was equivalent to layering the sample on the top of a gradient. The sample was centrifuged at 26,000 or 30,000 r.p.m., depending on the size of the DNA, and UV photographs were taken every 4 min. The photographs were then scanned and the distance between the meniscus and the DNA band determined. The rate of sedimentation of the DNA, in $cm.s^{-1}$, was calculated and from this the absolute rate of sedimentation in the cell was calculated by dividing by the magnification factor involved in making the scan (18.1x).

This value was then divided by a constant of 42.624×10^6 (for 26,000 r.p.m.) or 56.7936×10^6 (for 30,000 r.p.m.) and the quotient multiplied by 10^{13} to give the sedimentation coefficient, s^o, which was then multiplied by 1.0761 (for double-stranded DNA) to give the $s^o_{20,w}$ (the value that would be obtained in water at 20°C). The molecular weight was then calculated from the relationship (Studier, 1965):

$$\log_{10} (\text{Molecular weight}) = \frac{\log_{10} s^o_{20,w} + 1.0545}{0.346}$$

c. Denaturation and Renaturation of DNA.

DNA (either mainband or satellite), which had been fractionated by preparative CsCl centrifugation (page 25), was recovered by centrifugation at 125,000xg for 16 hr. at 0-10°C and dissolved in 0.1xSSC.

The DNA was hand-sheared to approximately 1.2×10^6 daltons and denatured by heating at 100°C for 10 min. The solution was then cooled quickly under the tap and kept in ice. Essentially no renaturation of separated strands occurred under these conditions. The salt concentration was then increased to 1xSSC and a sample containing 2µg removed for Model E analysis. The rest of the sample was incubated at $(T_m - 25)$°C to allow renaturation to occur. Samples were removed after various periods for analysis. The T_m, temperature of 50% denaturation, was calculated from the base composition of the DNA (Marmur et al., 1959) which was in turn derived from the buoyant-density (Schildkraut et al., 1962).

d. Fractionation of DNA

Flamm et al. (1966) gave reasons for the higher order of resolution obtained by using a fixed-angle, rather than a swing-out,

rotor.

DNA was fractionated in CsCl gradients formed in 10ml tubes in the M.S.E. 10x10 rotor. For normal preparative fractionation, 7g CsCl were dissolved in 5.4ml 0.1xSSC containing 50-500µg DNA, plus 40µg M.lysodeikticus marker DNA. The refractive index was adjusted to 1.4000 ($1.7067 g.cm.^{-3}$) and samples were centrifuged in the M.S.E.50 ultracentrifuge at 25°C and 35,000 r.p.m. (80,000xg) for 66 hr. The rotor was allowed to slow without braking and the gradient collected by pumping 60% sucrose solution into the bottom of the tube. The OD_{254} was continually recorded with an L.K.B. Uvicord I and fractions were collected every 7.5s. or 15s. (0.1 or 0.2ml respectively). The exact position of the peak of the M.lysodeikticus DNA was located by determination of the OD_{260}, by the Unicam SP500 spectrophotometer, of the relevant fractions after dilution to 0.5ml with 0.1xSSC.

Hybridization of rRNA to DNA

The DNA in each fraction obtained from a preparative centrifugation was diluted to 0.5ml with 0.1xSSC and denatured by the addition of 0.5ml 1N NaOH. After 10 min. at room temperature, each fraction was neutralized (and simultaneously buffered and made to high salt) by the addition of 2ml of "acid" solution containing 0.25M Tris pH8.0, 0.25M HCl, and 0.75M NaCl. The denatured, neutralized DNA was then fixed to a "Millipore" filter (HA, 0.45µ pore diameter, 13mm filter diameter), which had been previously numbered and soaked for at least 2 hr. in 6xSSC, by allowing the 3ml to run through the filter at atmospheric pressure. The filter was washed with 1ml of the "neutral", buffered salt solution (4 vol. 1M Tris pH8.0, 4 vol. 1N HCl, 8 vol. 3M NaCl, 1 vol. 1N NaOH, and 1 vol. 0.1xSSC) and air dried on absorbent tissue in Petri dishes. The DNA was fixed to the

filter by heating at 80°C *in vacuo* for 2 hr. immediately prior to hybridization.

The DNA filters were incubated with artichoke (*Helianthus tuberosus*) ^{3}H-rRNA (1.3×10^{6} daltons), of known specific activity, in 6xSSC and 3 times in 2xSSC. RNase was added to the final 2xSSC wash at a concentration of 10µg/ml for 15 min. at room temperature. The filters were finally rinsed in 2xSSC, dried on tissue and then at 80°C *in vacuo* for 30 min., after which they were counted in toluene-butyl P.B.D. scintillation fluid by the "Intertechnique" SL30 Liquid Scintillation Counter using the pre-set ^{3}H-channel.

4. Determination of the degree and nature of bacterial contamination

0.5g of material was homogenized in 1ml of sterile water with a sterile pestle and mortar. A dilution series (1 to 10^{-5}) was made from the homogenate and 0.1ml of each was spread onto a nutrient agar medium (28g/l). Plates were scored for viable colonies after 2 or 3 days growth at 25°C.

In order to obtain pure bacterial strains from the contaminating population, the method of dilution streaking was used to obtain isolated colonies, each containing a pure strain. These colonies were then transferred to fresh plates and incubated for several days at 25°C. The bacterial growth was then scraped off the agar; DNA was isolated by shaking the bacteria in detergent medium, followed by deproteinization and purification as described for total DNA.

5. Cytology

a. Determination of amount of DNA per nucleus

Values for the amount of DNA per nucleus were obtained by comparative densitometry following staining with leuco-basic fuchsin

(Darlington et al., 1960; McLeish et al., 1961).

The samples, root tips or thin slices of artichoke tuber, were fixed in ethanol:acetic acid (3:1) for 1 hr. They were then washed three times in 95% ethanol and passed through an ethanol dilution series to water. After washing, the samples were hydrolysed for 6 min. at 60°C in 1N HCl followed by a rinse in water and staining in leuco-basic fuchsin for 2 hr. at 20°C. Excess stain was removed by three washes in SO_2-water at pH1.7, followed by a rinse in water, blotting, maceration in glycerol and squashing on a microscope-slide. The total amount of stain per nucleus was determined on an integrating microdensitometer (Barr and Stroud Ltd., Glasgow and London) Type GN2 by measurement of the OD_{565}. The DNA content of the tissues was related to pea roots containing 10×10^{-12}g. DNA/telophase nucleus.

b. Preparation of chromosome squashes.

Buds of Tradescantia virginiana (var. Purple Dome) were taken from several different clones growing in the Departmental garden.

Anthers were squashed with a blunt brass rod in a drop of lacto-propionic orcein on a glass slide and then covered with a cover-slip. Gentle pressure was applied to the cover-slip to spread out the cells, but all sidewards movement was avoided as this would have broken the cells.

The cells were examined on a Carl Zeiss Photomicroscope, and cells exhibiting diakinesis were photographed on 'Ilford' Pan-F film.

Table 2.

Species Distribution of Satellite DNAs

*Order No.	Family	Species	Common name	Buoyant-density ($g.cm.^{-3}$) Main	Sat.	% Sat.	
A. Dicotyledons							
1	Magnoliaceae	Magnolia soulangiana		1.699	-	0	(1)
	Winteraceae	Drimys piperita		1.698	1.709	7	(1)
9	Ranunculaceae	Ranunculus acris	Meadow buttercup	1.699	-	0	(1)
		Ranunculus ficaria	Lesser celandine	1.696	-	0	(1)
		Helleborus niger	Christmas rose	1.694	-	0	(1)
		Anemone coronaria	de Caen	1.696	-	0	(1)
		Pulsatilla vulgaris	Pasque flower	1.699	-	0	(1)
		Clematis montana		1.700	-	0	(2)
		Trollius europaeus	Globe flower	1.698	-	0	(1)
		Aconitum napellus	Monkshood	1.697	-	0	(1)
16	Hamamelidaceae	Hamamelis mollis	Witch hazel	1.695	1.706	6	(1)
27	Caryophyllaceae	Dianthus barbatus	Sweet William	1.694	-	0	(1)
	Chenopodiaceae	Beta vulgaris	Swiss chard	1.693	-	0	(n)
		Spinacia oleracea	Spinach	1.695	-	0	(1)
32	Paeoniaceae	Paeonia officinalis	Paeony	1.692	-	0	(1)
35	Passifloraceae	Passiflora antioquiensis	Passion-flower	1.700	-	0	(2)
36	Cucurbitaceae	Cucumis melo	Melon	1.692	1.706	25	(n)
		Cucumis sativus	Cucumber	1.694	(1.702	28	(n)
					(1.706	16	
		Cucurbita pepo	Marrow	1.696	1.706	18	(n)
			Pumpkin	1.695	1.707	16	(2)
			Squash	1.695	1.706	17	(4)

Table 2 (continued)

*Order No.	Family	Species	Common name	Buoyant-density ($g.cm.^{-3}$) Main	Sat.	% Sat.	
A. Dicotyledons							
		Citrullus vulgaris	Watermelon	1.693	1.708	3	(2)
		Bryonia dioica	White bryony	1.696	1.706	5	(2)
		Momordica charantia	Balsam pear	1.695	-	0	(3)
		Lagenaria vulgaris	Bottle gourd	1.692	1.707	9	(2)
		Luffa cylindrica		1.696	1.707	6	(1)
38	Cruciferae	Brassica rapa	Turnip	1.696	1.704	21	(n)
		Brassica pekinensis	Chinese cabbage	1.695	1.703	17	(1)
		Raphanus sativus	Radish	1.700	-	0	(1)
		Lobularia maritima		1.695	(1.688	23	(3)
					(1.706	9	
40	Salicaceae	Populus nigra	Black poplar	1.693	-	0	(1)
50	Leguminosae	Vicia faba	Broad bean	1.694	-	0	(2)
		Vicia benghalensis	Purple vetch	1.694	-	0	(1)
		Pisum sativum	Pea	1.695	-	0	(n)
		Phaseolus coccineus	Runner bean	1.693	1.702	24	(n)
		Phaseolus vulgaris	French bean	1.693	1.703	19	(2)
		Phaseolus aureus	Mung bean	1.692	1.705	5	(1)
56	Rutaceae	Citrus sinensis	Sweet orange	1.694	1.712	23	(4)
		Citrus limon	Lemon	1.694	1.711	23	(3)
		Citrus paradisi	Grapefruit	1.693	1.711	23	(2)
		Citrus reticulata	Tangerine	1.693	1.712	19	(1)
		Fortunella sp.	Kumquat	1.693	1.712	24	(1)

Table 2 (continued)

*Order No.	Family	Species	Common name	Buoyant-density ($g.cm.^{-3}$) Main	Sat.	% Sat.	
A. Dicotyledons							
		Choisya ternata	Mexican orange	1.694	-	0	(1)
		Skimmia japonica		1.695	-	0	(1)
58	Linaceae	Linum usitatissimum	Flax	1.699	1.689	15	(8)
		Linum grandiflorum rubrum	Red flax	1.698	-	0	(1)
70	Solanaceae	Solanum tuberosum	Potato	1.695	1.707	4	(2)
		Solanum crispum	Tree tomato	1.698	1.710	6	(2)
		Solanum capsicastrum	Christmas orange	1.693	-	0	(1)
		Lycopersicon esculentum	Tomato	1.694	1.705	8	(n)
		Atropa belladonna	Deadly nightshade	1.694	-	0	(1)
		Nicotiana tabacum	Tobacco	1.697	-	0	(1)
		Petunia hybrida	Garden petunia	1.696	-	0	(1)
71	Labiatae	Perilla frutescens		1.695	-	0	(1)
		Coleus blumei	Coleus	1.696	-	0	(2)
72	Compositae	Tagetes erecta gigantea	African marigold	1.692	-	0	(2)
		Calendula officinalis	Pot marigold	1.692	1.705	11	(4)
		Bellis perennis	Daisy	1.694	-	0	(1)
		Helianthus tuberosus	Jerusalem artichoke	1.695	-	0	(7)
		Hieracium aurantiacum	Orange hawkweed	1.696	-	0	(1)

Table 2 (continued)

*Order No.	Family	Species	Common name	Buoyant-density ($g.cm.^{-3}$) Main	Sat.	% Sat.	
B. Monocotyledons							
79	Liliaceae	Lilium regale	Regal lily	1.698	-	0	(1)
		Hyacinthus orientalis	Hyacinth	1.700	-	0	(n)
		Puschkinia libanotica		1.699	-	0	(3)
		Chlorophytum elatum variegatum	Spider plant	1.693	-	0	(1)
	Amaryllidaceae	Allium cepa	Onion	1.691	-	0	(6)
86	Commelinaceae	Tradescantia virginiana		1.695	-	0	(2)
89	Gramineae	Secale cereale	Rye	1.702	-	0	(9)
		Zea mays	Maize	1.701	-	0	(n)
		Triticum aestivum	Wheat	1.703	-	0	(7)
		Hordeum vulgare	Barley	1.701	-	0	(1)
92	Lemnaceae	Lemna minor	Duckweed	1.703	-	0	(1)

*Order No. is taken from the classification of Takhtajan.

Total DNA was prepared from leaf tissue except in the case of lemon and grapefruit where seeds were used. The DNA was prepared and analysed by neutral CsCl equilibrium centrifugation as previously described. The number of preparations analysed is shown in parentheses ($n > 10$).

SECTION 3: RESULTS

1. The Distribution of Satellite DNAs

A. Species range

Total DNA was prepared from a range of plant species. The buoyant-densities of the mainband and satellite (where present) DNAs are given in Table 2, together with the amount of sat.DNA expressed as a percentage of the total.

None of the monocotyledons examined contained sat.DNA, although the density of the DNA varied from 1.691 $g.cm.^{-3}$ to 1.703 $g.cm.^{-3}$, and the species ranged from the primitive Liliaceae to the highly evolved Gramineae. In the dicotyledons, sat.DNA was distributed throughout the orders from the most primitive to the most advanced, as illustrated by Drimys (Magnoliales) and Calendula (Asterales) respectively. However, the occurrence of sat.DNA was more marked in some families, for example Cucurbitaceae and Rutaceae, whereas other families contained no sat.DNA in the range of species examined e.g. Ranunculaceae. Even in families where a large proportion of the species examined contained a sat.DNA, there were members which did not e.g. Momordica (Cucurbitaceae) and Choisya (Rutaceae).

The presence of sat.DNA was frequently restricted to certain genera within a family. In the Leguminosae, 3 species of Phaseolus contained sat.DNA whereas Pisum and 2 species of Vicia did not.

There was, possibly, a tendency for the more highly bred or cultivated plants to contain more sat.DNA than wild plants. In the Cucurbitaceae, Bryonia, the only wild species examined,

contained 5% sat.DNA. A small percentage of sat.DNA was also present in Luffa and Lagenaria, which although cultivated have not been subjected to intensive breeding like the melon and the cucumber which contained >20% sat.DNA. Similarly in the Rutaceae, the Citrus genus, which has been intensively bred for the fruit quality, contained >20% sat.DNA, whereas Choisya and Skimmia, which have not been similarly selected, contained no satellite.

Certainly, of the dicotyledon species examined, all those containing >10% sat.DNA had been subjected to intensive breeding.

B. Variation in buoyant-density and amount of satellite

The buoyant-density of both mainband and satellite DNA varied, with the satellite range being greater than that of the mainband.

Sat.DNAs varied from 1.688 to 1.712 $g.cm.^{-3}$ (a range of 24 $mg.cm.^{-3}$) whereas the mainband DNAs of species examined were all within a 12 $mg.cm.^{-3}$ spread from 1.691 to 1.703 $g.cm.^{-3}$. The density of the sat.DNA appeared to be independent of that of the mainband, since flax and Solanum crispum, both with similar mainband densities, had satellites with densities of 1.689 and 1.710 $g.cm.^{-3}$ respectively.

In the majority of plants analysed, the satellite was denser than the mainband, flax and Lobularia being the only examples where the sat.DNA was less dense than the mainband.

The separation of the satellite component from the mainband DNA varied from 19 $mg.cm.^{-3}$ (Fortunella) to 8 $mg.cm.^{-3}$ (turnip), when the sat.DNA appeared as little more than asymmetry of the peak. Components separated by less than 8 $mg.cm.^{-3}$ were,

FIGURE 2

The range of plant satellite DNAs.

Total DNA prepared from leaf tissue was analysed by neutral CsCl equilibrium centrifugation at 44,000 r.p.m., 25°C for 20 hr. The buoyant-densities were calculated relative to the internal marker DNA of *Micrococcus lysodeikticus* (1.731 $g.cm.^{-3}$).

a, *Fortunella* DNA, 1.712 and 1.693 $g.cm.^{-3}$; b, Melon DNA, 1.706 and 1.692 $g.cm.^{-3}$; c, Cucumber DNA, 1.706, 1.702 and 1.694 $g.cm.^{-3}$; d, Runner bean DNA, 1.702 and 1.693 $g.cm.^{-3}$; e, Turnip DNA, 1.704 and 1.696 $g.cm.^{-3}$; f, Flax DNA, 1.699 and 1.689 $g.cm.^{-3}$


a
b
c
d
e
f
E_{265}
20
10
0
10
20
DENSITY DIFFERENCE FROM MAIN BAND (mg cm^3)

therefore, not resolved (fig.2).

The largest amount of sat.DNA observed was 44% of total DNA in Cucumis sativus seeds when at least 2 satellite components were present. A single satellite component of 20 - 25% of the total DNA was found in melon, Citrus, Phaseolus, and turnip.

The lower limit of detection depended on the density difference between the satellite and mainband, the size of the DNA being fractionated, and the sensitivity of the analytical method. Consequently, although the 3% satellite peak in Citrullus was quite distinct (15 $mg.cm.^{-3}$ difference in density from mainband), in Bryonia the 5% satellite was present as only a shoulder. In general, components less than 5% were not recorded as a satellite unless particularly well resolved.

The majority of plants examined contained one satellite which was denser than the mainband although cucumber had two dense satellites. Flax and Lobularia both contained one less-dense satellite but Lobularia also had a sat.DNA which was denser than the mainband.

C. Heterogeneity of the satellite DNA

The heterogeneity of the plant DNAs, both mainband and satellite, was examined by re-running on the 'Model E' fractions taken throughout the relevant peaks from a preparative neutral CsCl fractionation.

FIGURE 3

Preparative fractionation and reconstitution of cucumber var. Kariha DNA and of marrow DNA.

Cucumber fruit DNA and marrow leaf DNA were fractionated by preparative centrifugation in the MSE 10x10 angle rotor at 35,000 r.p.m., 25°C for 66 hr. M. lysodeikticus DNA (1.731 $g.cm.^{-3}$) was included as internal marker. The gradient was dripped from the tube and collected as fractions. Each fraction from the mainband and satellite was analysed by Model E centrifugation, and the fractionation reconstituted as a histogram on the basis of the percentage distribution of the components after analysis and the amount of DNA in each initial fraction. a, Marrow leaf DNA; b, Cucumber fruit DNA.

For comparison, the continuous line shows Model E analyses of the 2 DNAs.

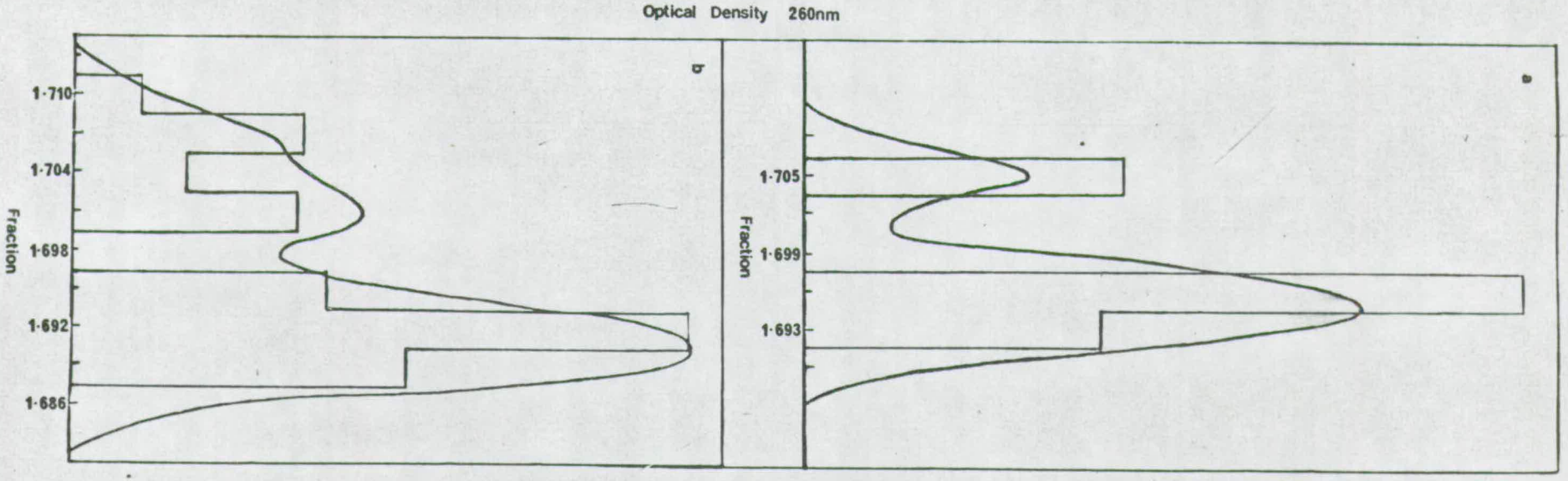

Optical Density 260nm
b
a
1·710
1·704
1·698
1·692
1·686
Fraction
1·705
1·699
1·693
Fraction

Table 3

Homogeneity of Mainband and Satellite DNAs

Marrow				Cucumber			
Initial density	Re-run densities			Initial density	Re-run densities		
1.688	1.692			1.685	1.688		
1.691	1.693			1.688	1.690		
1.694	1.695			1.691	1.691		
1.697 M	1.696			1.692 M	1.692		
1.700	1.696			1.696	1.694		
1.702	1.697	1.706		1.699	1.692	1.700	
1.705 S	1.697	1.706		1.701 S	1.694	1.702	1.707
1.708	1.695	1.706		1.704	1.691	1.704	1.706
1.711		1.706		1.707 S	1.691		1.706
1.714		1.706		1.710			1.708
				1.712			1.709

DNA (50 to 100 µg.) was fractionated by preparative CsCl preparative CsCl equilibrium centrifugation and the average buoyant-density of each fraction estimated from the known densities of the mainband and M. lysodeikticus DNAs. Approximately 2µg. of DNA from each fraction was analysed by Model E CsCl centrifugation. The positions of the mainband and satellite peaks in the preparative fractionation are indicated by M or S respectively. Where two or more components were resolved on the re-run the major component is underlined.

The relatively broad mainband of marrow, with a peak at 1.696 g.cm.$^{-3}$, consisted of components of densities of 1.692 - 1.697 g.cm.$^{-3}$, since fractions of initial density 1.688 - 1.700 g.cm.$^{-3}$ re-ran to give peaks corresponding to these values (Table 3; fig.3a). The width of the mainband peak, therefore, resulted partly from the heterogeneity of its component DNA and partly from the size of the DNA analysed.

Mainband DNA of density 1.696 g.cm.$^{-3}$ was also present throughout the sat.DNA region up to a density of 1.708 g.cm.$^{-3}$ In contrast to the mainband, the satellite peak was homogeneous, since samples taken from the preparative gradient at densities from 1.702 to 1.714 g.cm.$^{-3}$ all re-ran to 1.706 g.cm.$^{-3}$

In cucumber, the mainband was again heterogeneous, but in contrast to marrow, samples from the satellite region indicated a range of components with densities from 1.700 to 1.709 g.cm.$^{-3}$ (Table 3; fig.3b).

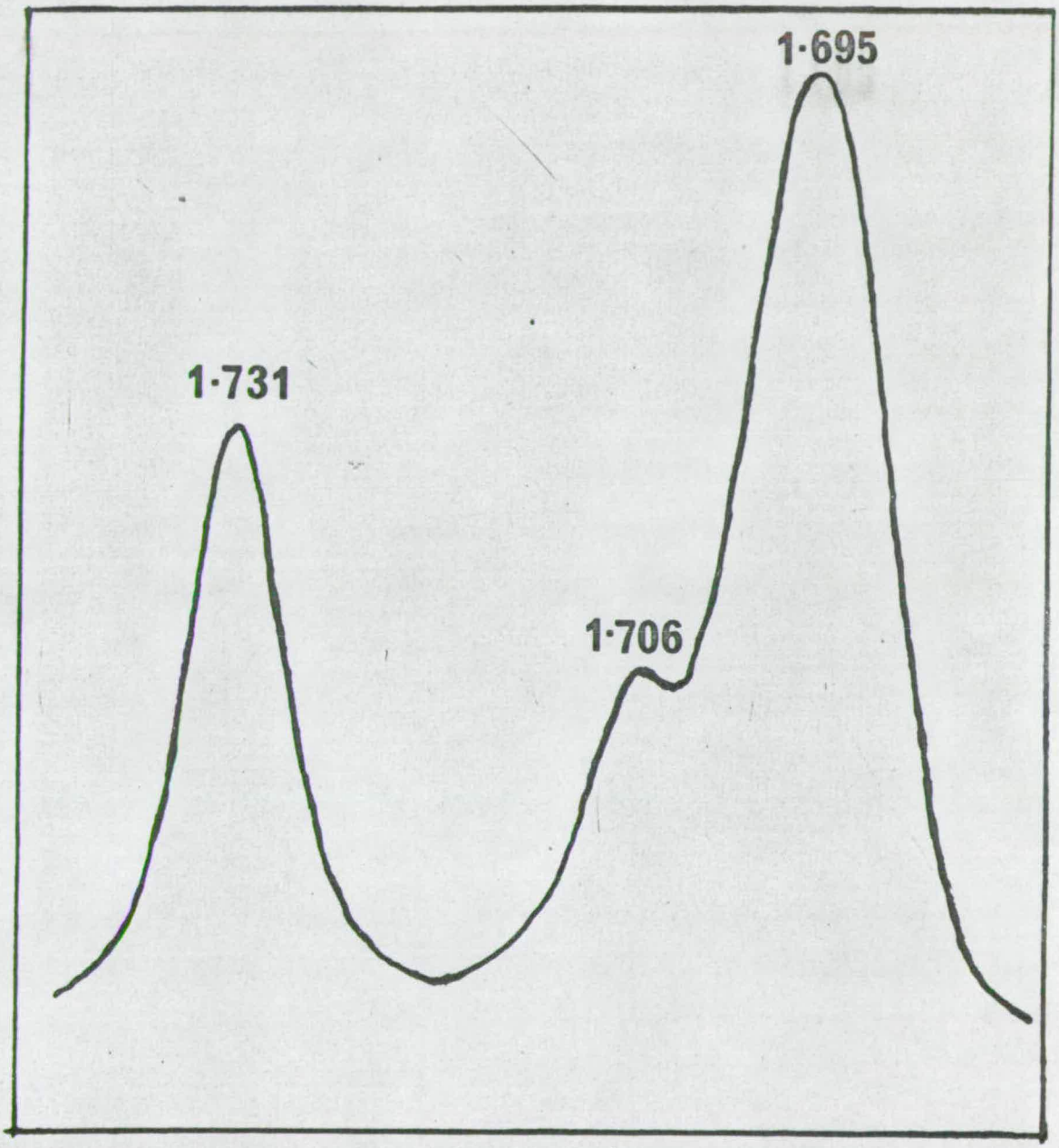
2. Cellular Location of Satellite DNA

The analyses of the species previously described involved total cellular DNA. It was necessary, therefore, to show that the satellite, when present, was nuclear. Plant cells contained two organelles: chloroplasts and mitochondria. The density of chloroplast DNA had been shown to be constant at 1.697 g.cm.$^{-3}$ in the species studied and it would not be resolved from mainband DNA. Satellite DNAs were, by definition, resolved from the mainband. Mitochondrial DNA with a density of 1.706 g.cm.$^{-3}$ had a density similar to many of the satellite components. However, mitochondrial DNA appeared to be present in very small amounts (<1% of the total DNA). Onion roots, which presumably had a

FIGURE 4

Marrow DNA from a nuclear preparation.

A crude nuclear preparation was made as described in the text (1,500xg pellet) and subsequently washed with Triton X-100 to solubilize mitochondria and chloroplasts. Insoluble material was pelleted by centrifugation at 2,500xg for 20 min. at 0°C and DNA prepared and analysed on the Model E.


1·695
1·731
1·706

normal number of mitochondria, contained no component at 1.706 g.cm.$^{-3}$, which would be completely resolved from the mainband of density 1.691 g.cm.$^{-3}$ Sat.DNAs were not, therefore, of chloroplast or mitochondrial origin.

It was possible that satellites were not of plant origin at all, since material from the greenhouse or garden was not sterile. It was necessary, therefore, to ensure that sat.DNAs were not bacterial. The number of bacteria present in a sample of tissue was determined by dilution plating and varied from 10^4 to 10^5 bacteria/g. The total amount of bacterial DNA present was calculated on the basis of there being 10^{-14}g. DNA/bacterium. The 10^5 bacteria/g. of tissue contributed 10^{-3}µg. DNA compared with ~10µg. sat.DNA per gram of tissue. Furthermore, the interior of the fruits of melon and cucumber were sterile, as were the seeds from these fruits. Nevertheless, they contained the full amount of sat.DNA. Therefore, with healthy plant material, although bacteria were present, bacterial DNA could account for no more than 0.01% of the sat.DNA, and therefore it was concluded that sat.DNA was not of bacterial origin.

The analysis of DNA from a nuclear preparation, which had been washed with Triton X-100 to solubilize chloroplasts and mitochondria, contained the sat.DNA (fig.4). In some instances there was a slight enrichment of satellite relative to a total-cellular preparation. This was consistent with contamination of mainband by chloroplast DNA in a total-cellular preparation reducing the percentage of sat.DNA. Because of the relatively low yield of nuclear material (1% from many of the tissues studied) and since these preparations contained broken nuclei with the

possibility of preferential loss of chromatic fractions, total-cellular DNA was routinely prepared for analysis.

3. Complexity of Satellite DNA

One of the most characteristic properties of animal sat.DNA is its rapid rate of renaturation owing to the repetitious nature of the DNA. The rate and extent of renaturation of plant sat.DNA was therefore determined.

Citrus sinensis (Sweet orange) leaf sat.DNA (1.712 $g.cm.^{-3}$) was purified by a preparative CsCl gradient, denatured, and then subjected to conditions favouring renaturation. Renaturation was followed by 'Model C' analysis. On denaturation, the density of the satellite increased to 1.722 $g.cm.^{-3}$ After 5 minutes at 3µg./ml. in 1×SSC, the DNA had renatured to 1.713 $g.cm.^{-3}$ (fig.5). From this, the time for 50% renaturation could be estimated to be <2 min., indicating that orange satellite was similar to melon sat.DNA which was 50% renatured in 1.5 min. (2µg./ml. in SSC.). Under identical conditions of renaturation, T_4 DNA at 2µg./ml. took 80 min. for 50% renaturation. The kinetic complexity of T_4 DNA was 140×10^6 daltons. Orange and melon satellites would, therefore, be much less complex i.e. $\frac{1.5}{80} \times 140 \times 10^6 = 2.6 \times 10^6$ daltons. This was sharply contrasted with the mainband DNA of melon which renatured hardly at all (Table 4).

FIGURE 5

Rate of renaturation of orange leaf satellite DNA.

DNA was denatured in 0.1xSSC by heating at 100°C for 5 min. and then quickly cooled. A sample of the denatured DNA was analysed by Model E CsCl centrifugation. The salt concentration was then increased to 1xSSC, and the temperature raised to T_m-25°C. Samples were analysed in the Model E after various time intervals. a, native satellite DNA: 1.712 $g.cm.^{-3}$; b, denatured satellite DNA: 1.722 $g.cm.^{-3}$; c, satellite DNA renatured for 5 min.: 1.713 $g.cm.^{-3}$ Marker DNAs: a, M. lysodeikticus 1.731 $g.cm.^{-3}$; b, c, T_4 1.700 $g.cm.^{-3}$

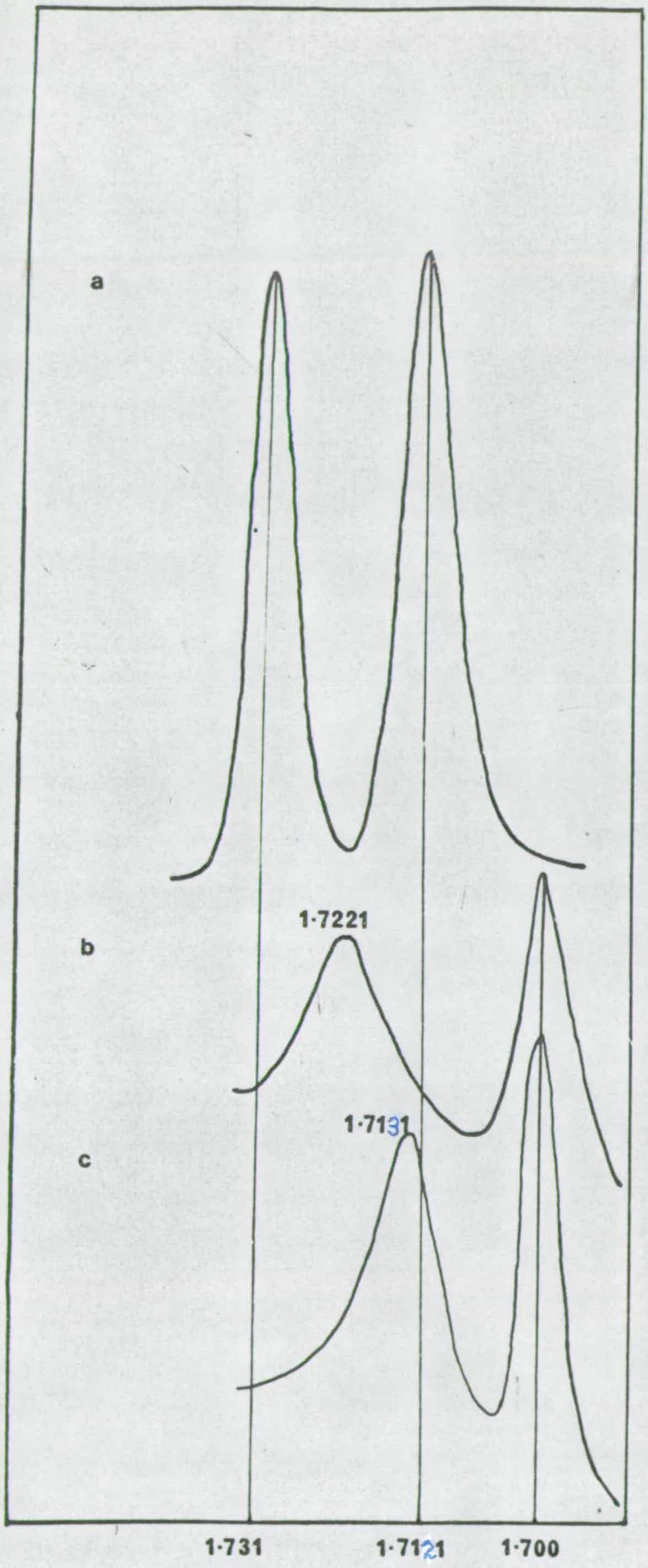

a
b
c
1·7221
1·7131
1·731
1·7121
1·700

Table 4

Renaturation of Satellite DNAs

DNA	Time for 50% renaturation (min.)	Extent of renaturation*	Complexity relative to T_4
T_4	80	13/15	1
E. coli	900	12/13	11
Melon stress-satellite	1500	6/10	19
Melon mainband	-	2/16	-
Orange satellite	2.5	9/10	0.03

All DNA samples were hand-sheared to approximately 2×10^6 daltons double-stranded fragment, and renaturation results have been corrected to a concentration of $2 \mu g./ml.^{-1}$ The time for 50% renaturation was estimated from Model E centrifugation analyses as described in the text.

*Expressed as the decrease in buoyant-density during 22 hr. renaturation relative to the increase in buoyant-density observed on denaturation ($mg.cm.^{-3}$).

The telophase nucleus in orange contained 2×10^{-12} g. DNA of which 20% was sat.DNA i.e. 4×10^{-13} g. sat.DNA/2C nucleus. The total satellite was therefore equivalent to $4 \times 10^{-13} \times 6 \times 10^{23}$ daltons = 2.4×10^{11} daltons.

The orange therefore contained $\frac{2.4 \times 10^{11}}{2.6 \times 10^{6}} = 10^{5}$ copies of the reiterated sequence per genome.

4. Constancy of Satellite DNA during Development

Initial analyses of different tissues of a particular species, such as leaf, fruit, root tip, seed, showed that when satellite was present it was found in all tissues, but there was some suggestion that the amount of sat.DNA varied a little. The amount and buoyant-density of sat.DNA was, therefore, determined in different tissues of an individual plant to confirm whether or not such differences were significant. The appearance of additional satellite components had been reported during certain conditions of physiological stress. This phenomenon was also further investigated.

A. Stress-satellite DNA

Plants were subjected to the conditions of physiological stress which had been reported to give rise to the stress-sat.DNA (Quétier, Guillé and Vedel, 1968; Guillé, Quétier and Huguet, 1968). The stress conditions used by these workers included growing seedlings on water in the dark for long periods, wounding dark-grown seedlings by bruising, slitting, or decapitation, or storing seeds for 2 - 3 weeks at $4^{\circ}C$.

In preliminary experiments designed to confirm the

observations of Quétier, Guillé and Vedel (1968), tomato and cucumber seedlings were germinated in the dark at 20°C for 20 days, and seeds from two melons were stored at 4°C for 15 days. Analysis of the DNA prepared from the hypocotyl tissue of 7 and 20 day old cucumber seedlings showed only the normal nuclear DNA components - the mainband at 1.693 $g.cm.^{-3}$ and the double sat.DNA peaks at 1.702 and 1.707 $g.cm.^{-3}$ (fig.6). After 14 days etiolated growth, the tomato hypocotyl contained a trace of the stress-sat.DNA (1.720 $g.cm.^{-3}$) in addition to the normal mainband (1.694 $g.cm.^{-3}$) and satellite (1.705 $g.cm.^{-3}$) DNAs (fig.7).

Melon seeds stored for 15 days, however, contained large amounts of the stress-sat.DNAs, which were not present in the fresh seeds (fig.8a). Honeydew melon seeds contained one stress-sat.DNA at 1.718 $g.cm.^{-3}$ in addition to the normal components at 1.693 and 1.707 $g.cm.^{-3}$ (fig.8b), whereas the Hero of Lockinge seeds contained stress-sat.DNAs at 1.698, 1.715, 1.719 and 1.726 $g.cm.^{-3}$ (fig.8c). Further studies were therefore concentrated on the appearance of these stress-satellites during the cold-storage of seeds of Cucurbitaceae species.

Several properties of the stress-sat.DNA prepared from cold-stored seeds of Honeydew melon were determined. All of the components in the CsCl gradient (fig.8b) were completely removed by treatment with deoxyribonuclease. Analysis of the CsCl gradient with Schlieren optics showed no evidence of polysaccharide contamination of the peaks (Edelman, Swinton, Schiff, Epstein and Zeldin; 1967). The stress-sat.DNA (1.720 $g.cm.^{-3}$), isolated from a preparative CsCl gradient of

FIGURE 6

DNA of dark-grown cucumber seedlings.

Cucumber seedlings were grown in the dark at 20°C and the DNA was prepared from the hypocotyls after 7 days (a) and 20 days (b). The DNA was analysed by Model E centrifugation.

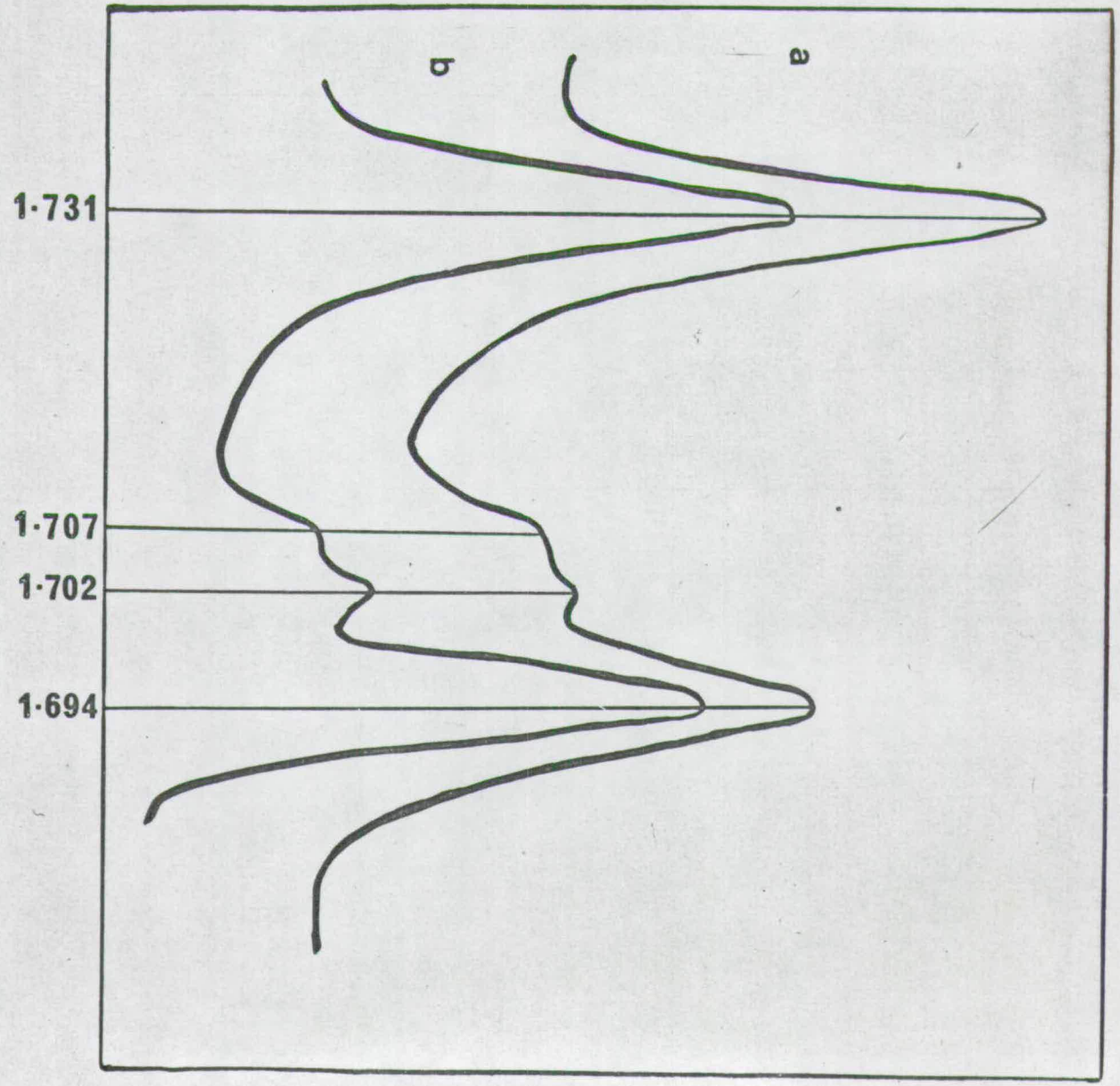

d
a
1·731
1·707
1·702
1·694

FIGURE 7

DNA of dark-grown tomato seedlings.

Tomato seedlings were grown in the dark at 20°C for 14 days. DNA prepared from the hypocotyls was analysed by Model E centrifugation.

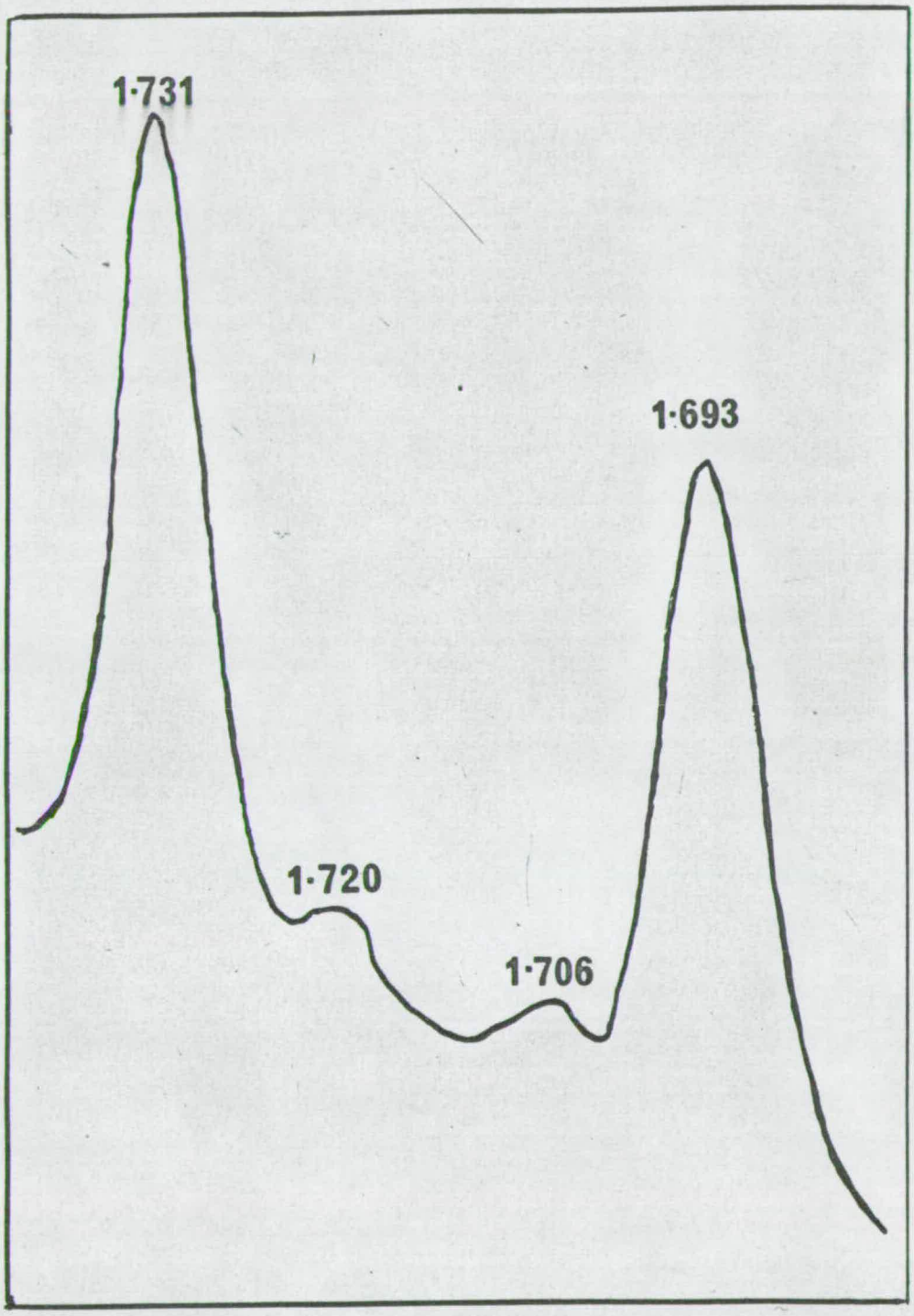
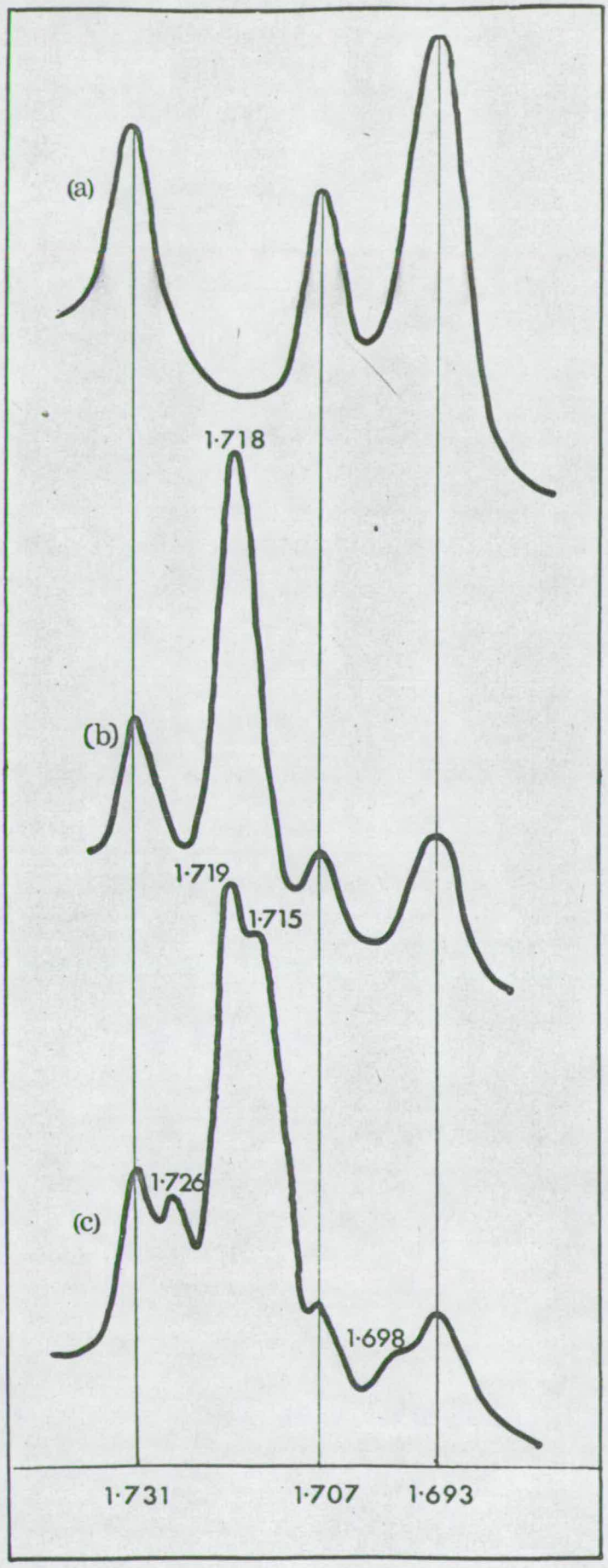

1·731
1·693
1·720
1·706

FIGURE 8

DNA from melon seeds stored for varying lengths of time at 4°C. a, DNA from fresh seeds of Honeydew melon. Mainband and satellite at 1.693 and 1.707 $g.cm.^{-3}$ respectively; b, DNA from 15-day cold-stored seeds of Honeydew melon. Mainband and satellite plus stress-satellite at 1.718 $g.cm.^{-3}$; c, DNA from 15-day cold-stored seeds of Hero of Lockinge melon. Mainband and satellite at 1.693 and 1.707 $g.cm.^{-3}$ respectively with stress-satellites at 1.698, 1.715, 1.719 and 1.726 $g.cm.^{-3}$


(a)
1·718
(b)
1·719
1·715
1·726
(c)
1·698
1·731
1·707
1·693

stored melon seeds, showed normal thermal melting with a T_m of 88.5°C corresponding to a 60% (G+C) (Marmur and Doty, 1959). On denaturation, the density of the DNA increased by 8 mg.cm.$^{-3}$ It was suggested by Quétier, Guillé and Vedel (1968) that the small increase in density on denaturation might be the result of the DNA being in the process of transcription, but it was probably due to the high (G+C) content of the DNA since Micrococcus lysodeikticus DNA (73%GC) increased from 1.731 to only 1.737 g.cm.$^{-3}$ on denaturation (Pearson and Ingle, 1972).

The stress-sat.DNA from an Ogen melon (1.711 g.cm.$^{-3}$) showed 50% renaturation after approximately 24 hours (1500 min.) in 1xSSC at a concentration of 2µg./ml. (fig.9) compared with 80 min. for T_4, 900 min. for E. Coli and 1.5 min. for melon satellite (1.706 g.cm.$^{-3}$) DNAs under identical conditions (Table 4). This stress-satellite was not, therefore, rapidly renaturing like the normal sat.DNA of melon, being more similar in complexity to E. Coli DNA.

These results confirmed that the stress-sat.DNA was indeed double-stranded, but its complexity (1000-fold greater than for the normal satellite) indicated that it was not composed of highly reiterated sequences.

The appearance of the stress-sat.DNA was quantitated during the storage of seeds from a Musk melon (fig.10) and a Honeydew melon (fig.11). The total DNA content was calculated from the OD_{260} of the purified DNA preparation and the individual amounts of the mainband, satellite, and stress-satellite DNAs estimated from the scans of the 'Model E' analyses. The amounts of the mainband and normal satellite DNAs remained

FIGURE 9

Renaturation of the stress-satellite DNA from an Ogen melon. The stress-satellite DNA was isolated and purified from a preparative CsCl density-gradient and then denatured and renatured as described in fig.5. Renaturation was allowed to proceed for 24 hr. n = native DNA density; d = denatured DNA density. Internal marker = T_4 DNA (1.700 $g.cm.^{-3}$).

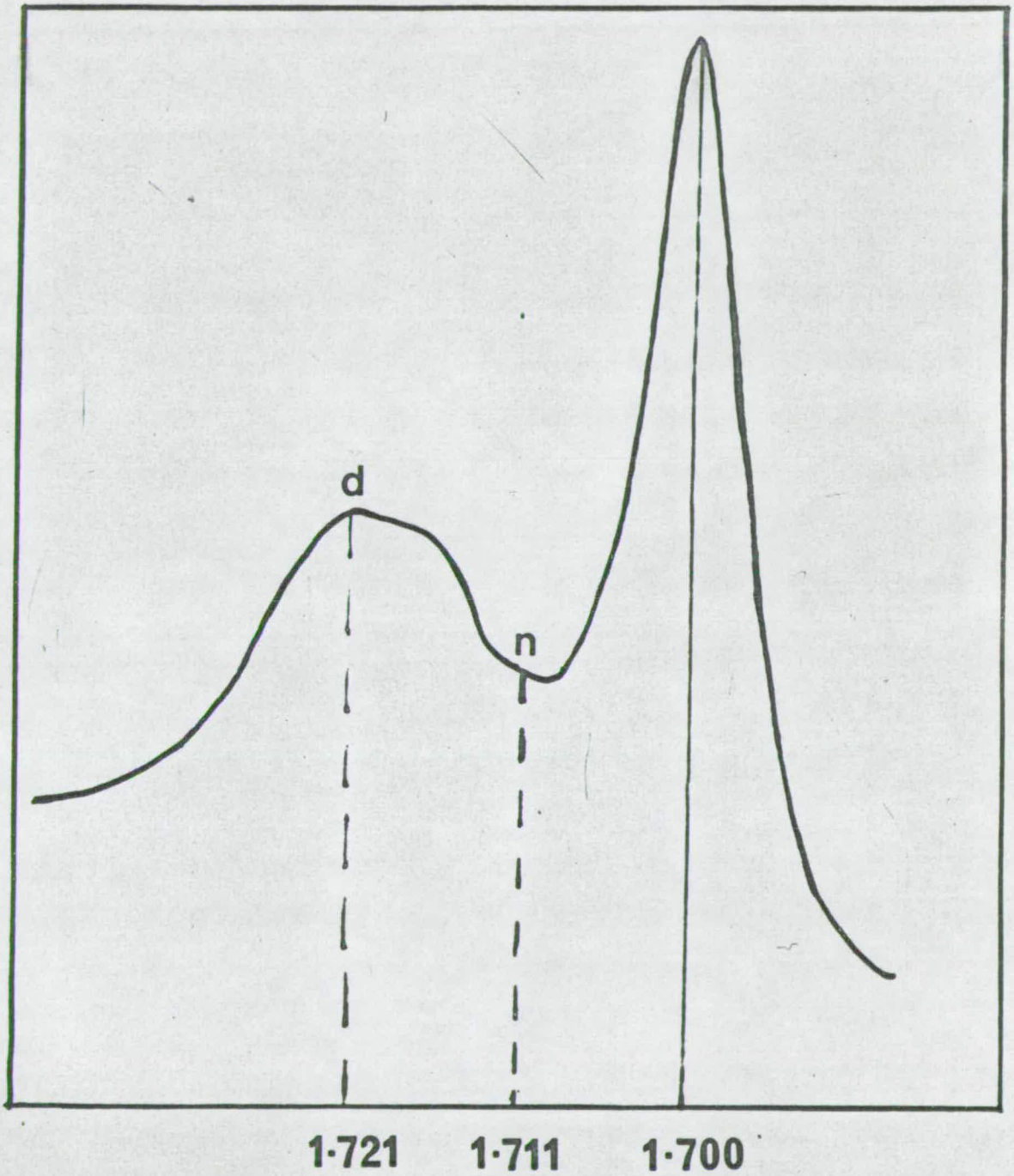

d
n
1·721
1·711
1·700

FIGURE 10

Appearance of stress-satellite during cold-storage.
Seeds of a Musk melon were stored at 0-4°C. The total DNA content of the seed was calculated from the purified DNA preparation. The amounts of the individual DNA components were determined from the Model E fractionations.

—■—■— Total DNA; —□—□— stress-satellite DNA (1.711 g.cm.$^{-3}$); —▲—▲ mainband DNA (1.693 g.cm.$^{-3}$); —▽—▽ normal satellite DNA (1.707 g.cm.$^{-3}$).

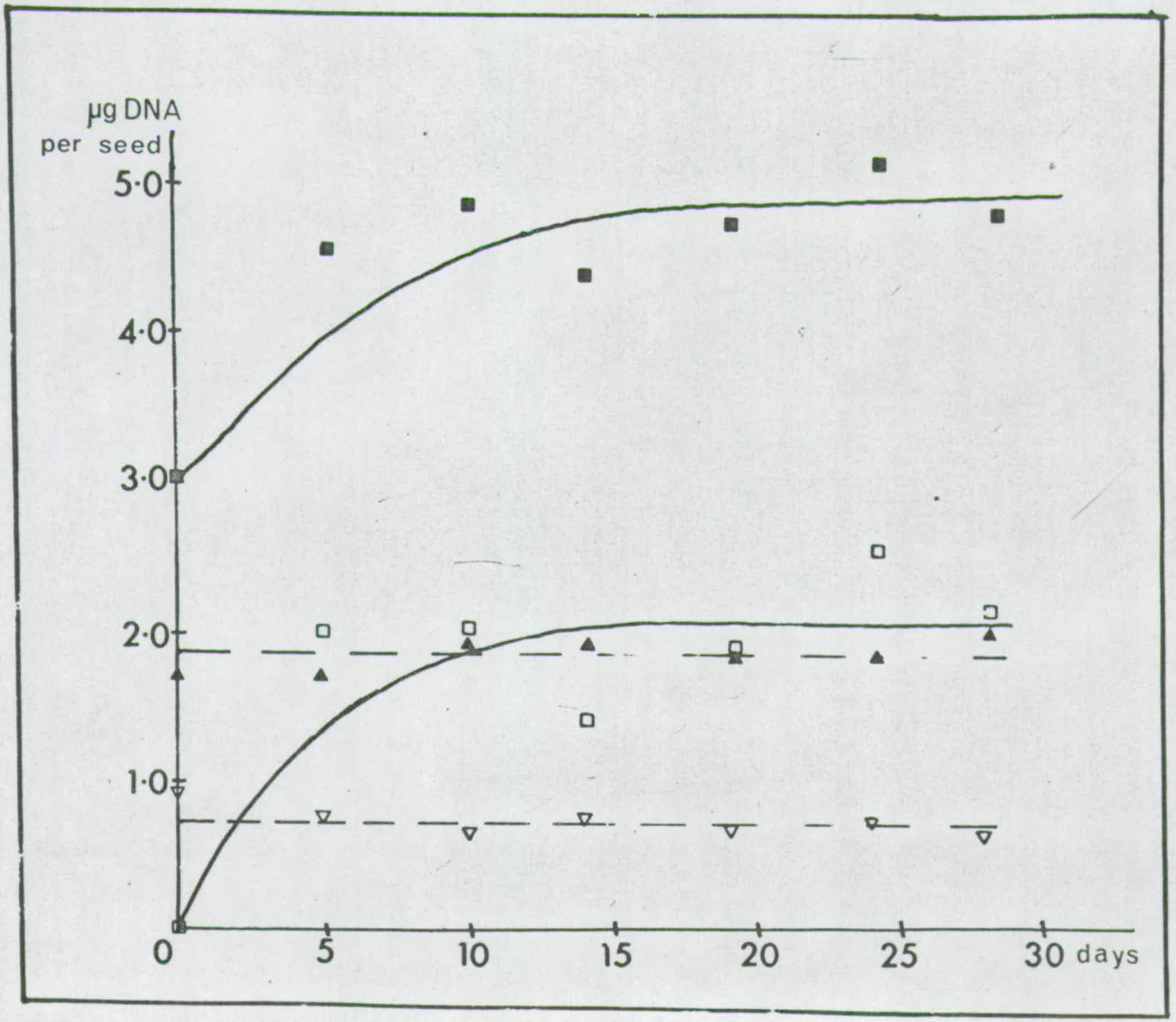
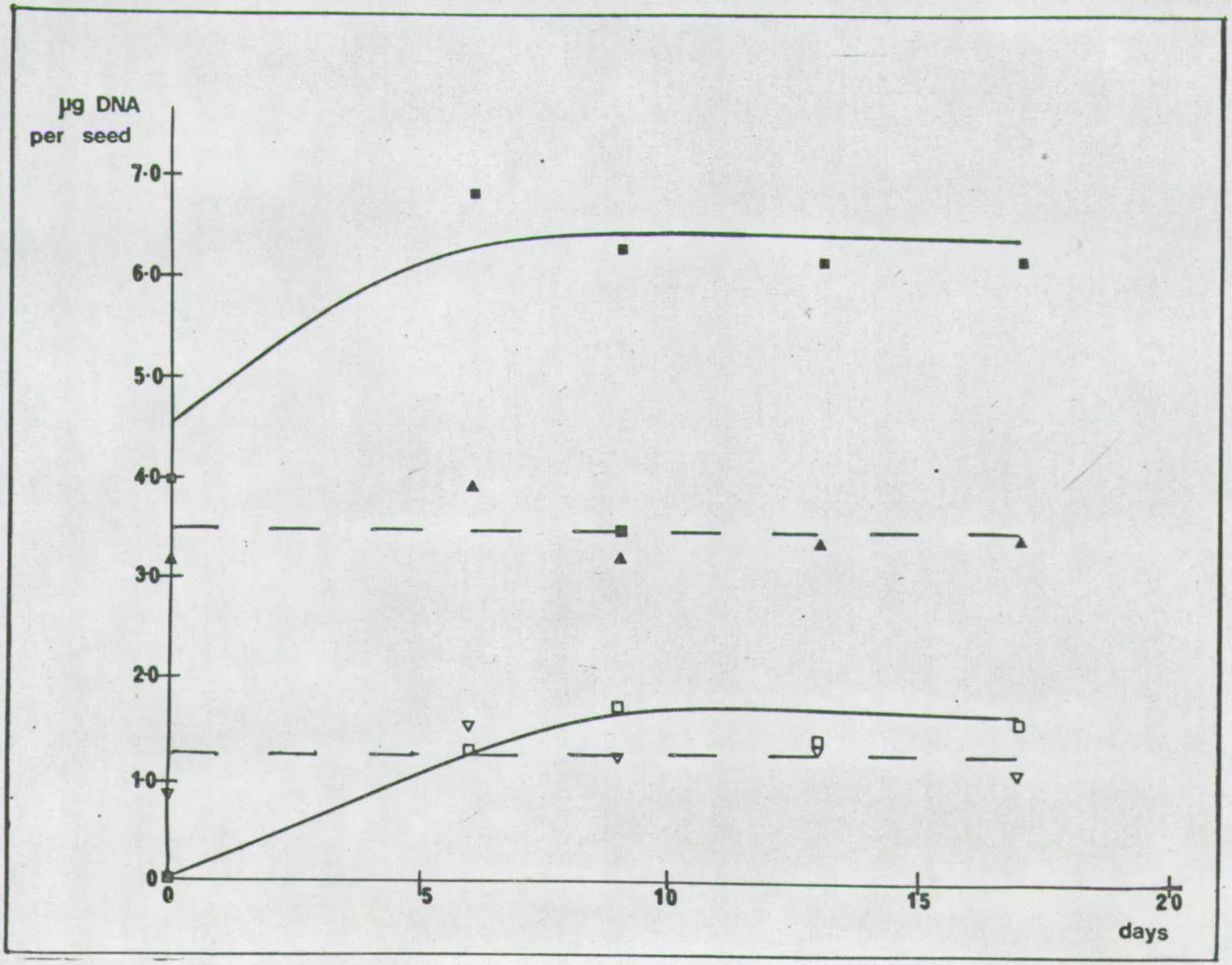

µg DNA
per seed
5·0
4·0
3·0
2·0
1·0
0
5
10
15
20
25
30 days

FIGURE 11

Appearance of stress-satellite DNA during cold-storage.

Seeds of a Honeydew melon were stored at 0-4°C. The total DNA content of the seed was calculated from the purified DNA preparation. The amounts of the individual DNA components were determined from the Model E fractionations.

—■—■— Total DNA; —⊟—⊟— stress-satellite DNA ($1.718\ g.cm.^{-3}$); —▲—▲ mainband DNA ($1.693\ g.cm.^{-3}$); —▽—▽ normal satellite DNA ($1.706\ g.cm.^{-3}$).


µg DNA
per seed
7·0
6·0
5·0
4·0
3·0
2·0
1·0
0
0
5
10
15
20
days

constant at 1.8 and 0.7 µg./seed respectively in the Musk melon, while the stress-satellite increased from 0 to 2µg. in 5 days. In the Honeydew melon, the mainband and satellite were constant at 3.5 and 1.25 µg./seed respectively and the stress-sat.DNA increased from 0 to 1.8 µg. in 9 days. Consequently, the total DNA per seed increased by 50 - 100% during cold storage. These results indicated that the increase in stress-satellite represented de novo synthesis of DNA rather than a conversion of existing nuclear DNA. Such time course experiments were repeated with seeds from a variety of melons, and from cucumber and marrow seeds.

Table 5

The buoyant-densities of the stress-satellite DNAs produced during cold storage of seeds.

Seed *	Buoyant-densities of stress-satellite DNAs × ($g.cm.^{-3}$)			
Melon				
Hero of Lockinge	1.698	**1.716**	**1.719**	1.726
Honeydew a			1.718	
b		1.713	**1.718**	
c			1.718	
d	None			
Spanish Winter a			1.717	
b			1.718	
c			1.718	
Ogen a		1.711		
b	None			
Musk		1.711		
Cucumber				
Kariha a		**1.711**	1.719	
b			1.718	
Marrow				
Courgette a	1.695	1.714	**1.720**	
b	None			

* Melons a, b, c, d, etc. indicate seeds from 4 different fruits.

× The major components are underlined. Seeds were stored at 0-4°C for several weeks. Approximately 2µg. of DNA prepared from the seeds was analysed by neutral CsCl equilibrium centrifugation.

FIGURE 12

DNA from melon seeds stored at 0-4°C. a, Spanish Winter melon seeds stored for 28 days within the fruit *in toto*. Mainband and normal satellite DNAs only at densities of 1.692 and 1.707 $g.cm.^{-3}$ respectively; b, Musk melon seeds stored for 14 days. Washed seeds: stress-satellite DNA present (1.711 $g.cm.^{-3}$); c, Musk melon seeds stored for 14 days. Seeds stored in the mucilaginous placental material from the melon: stress-satellite absent. Mainband and normal satellite DNAs only at 1.692 and 1.707 $g.cm.^{-3}$ respectively.

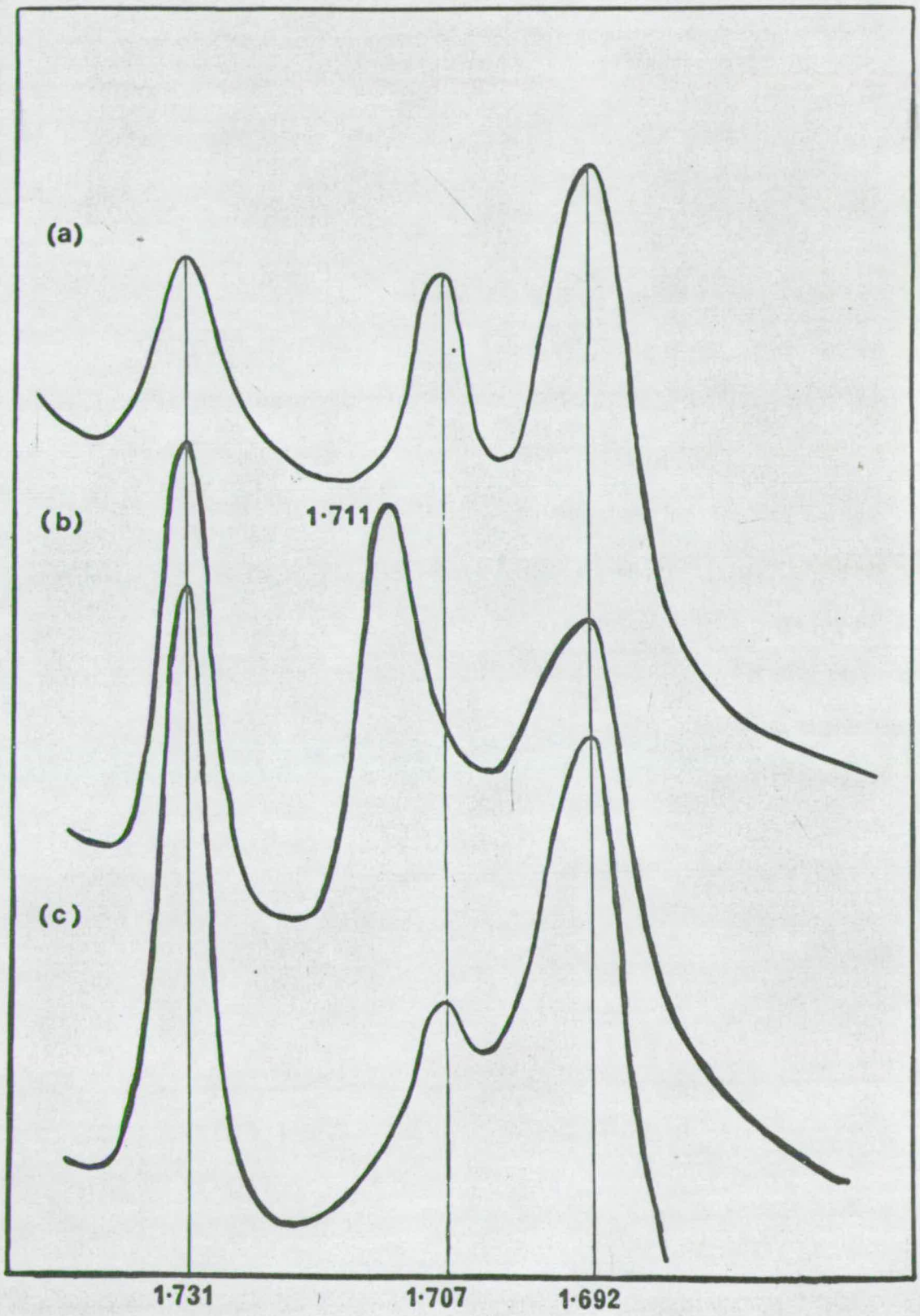
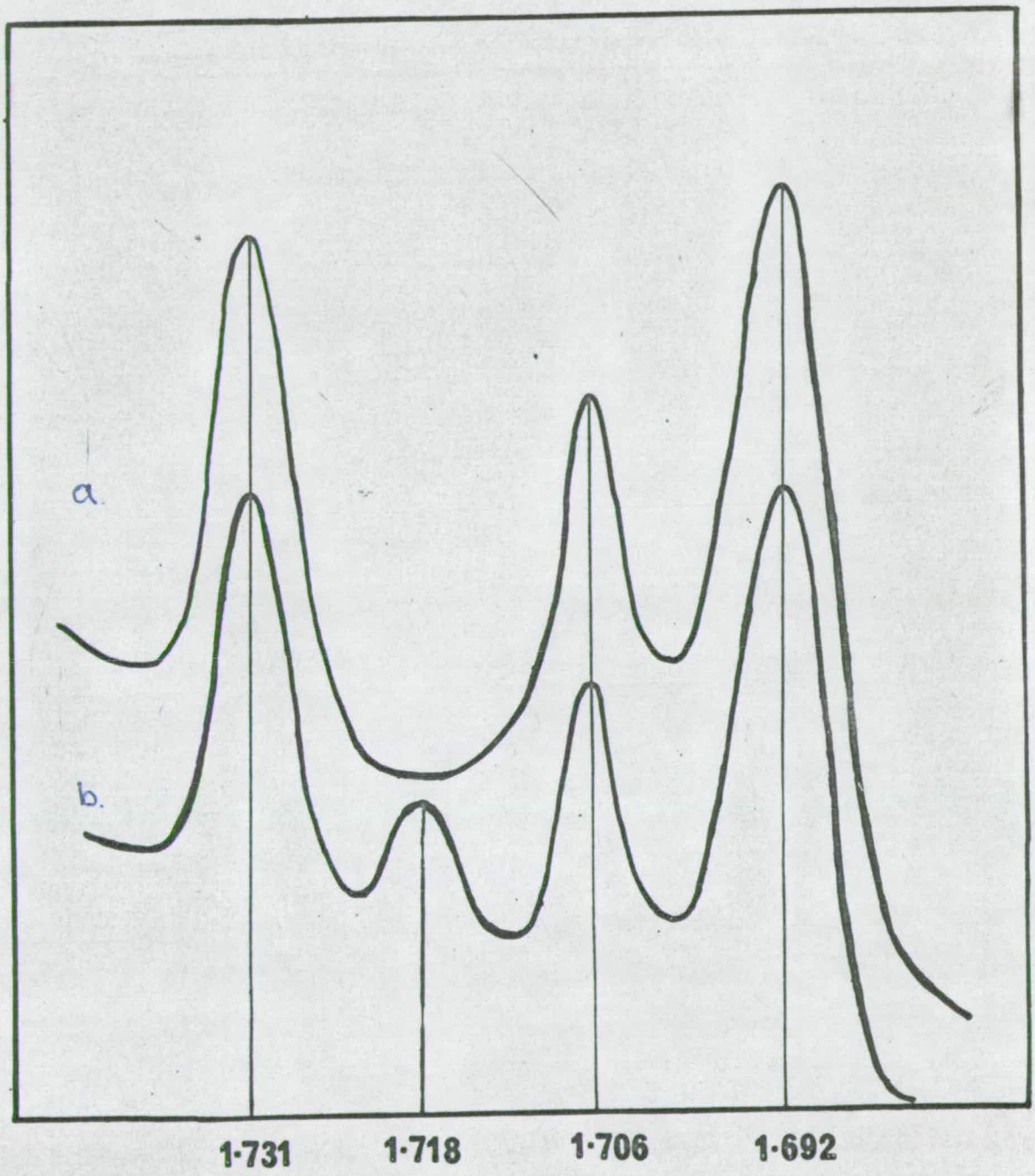

(a)
(b)
1·711
(c)
1·731
1·707
1·692

FIGURE 13

DNA from seeds of Honeydew melon stored for 14 days at 0-4°C. a, DNA prepared by tissue disintegration in the 'VirTis' homogenizer showing mainband and satellite DNAs only at 1.692 and 1.706 $g.cm.^{-3}$ respectively; b, DNA prepared by homogenization in a pestle and mortar showing mainband, normal satellite, and stress-satellite DNAs (1.692, 1.706, and 1.718 $g.cm.^{-3}$ respectively).


a.
b.
1·731
1·718
1·706
1·692

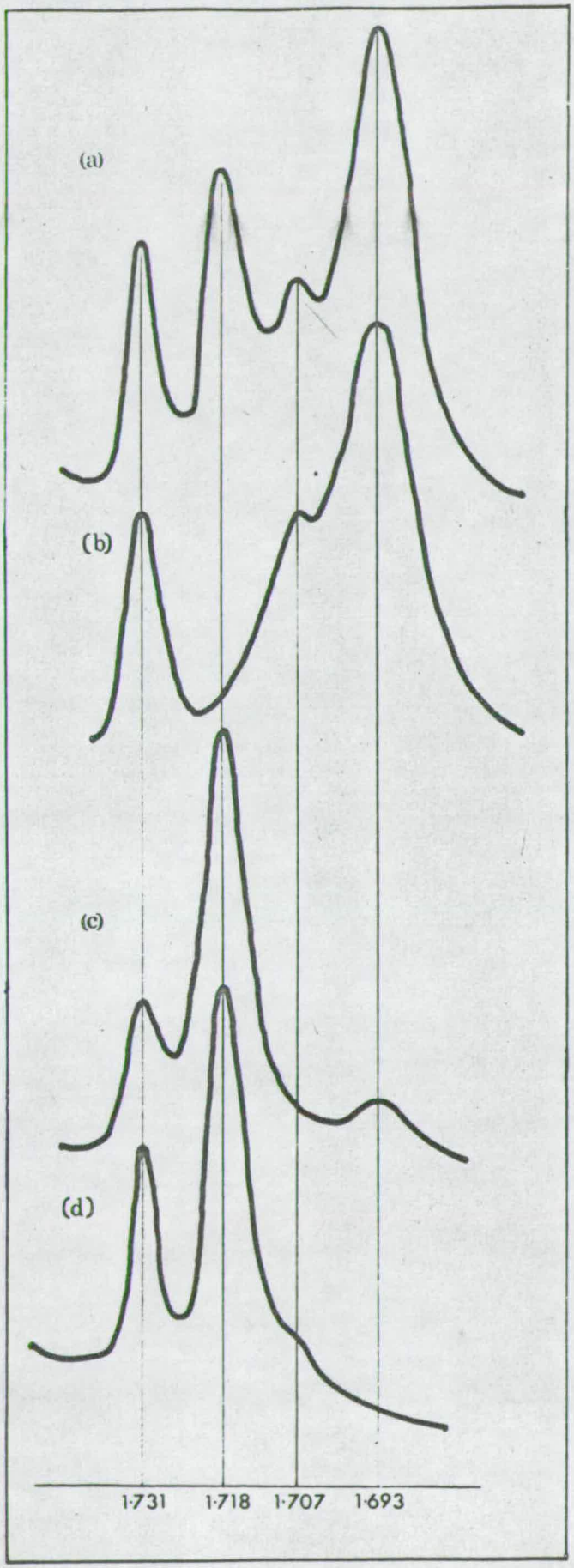
The density of the stress-sat.DNA varied with different species, with different varieties of the same species, and also to a certain extent for a given variety (Table 5). The time at which stress-satellite appeared and the final amount of stress-satellite also varied considerably. Moreover, with seeds from one Honeydew melon, one Ogen melon, and one marrow, no stress-sat.DNAs were produced (Table 5). It was also shown that no stress-sat.DNA was produced when the seeds were stored in the cold within the melon fruit in toto (fig.12a) and further there was no change in the DNA fractionation when the seeds were removed from the fruit but maintained in the mucilaginous placental material for the cold-storage period (fig.12c).

It was observed in preliminary experiments that the yield of the stress-sat.DNA, expressed as a percentage of the total seed DNA, varied considerably depending upon the homogenization method used in the DNA preparation. Homogenization with a pestle and mortar, which resulted in a very low yield of total DNA, produced a much higher percentage of the stress-sat.DNA than after a very thorough tissue disintegration in the 'VirTis' homogenizer (fig.13).

Following this indication that the stress-sat.DNA was more readily extracted than the normal DNA, cold-stored seeds were dissected into cotyledon and seed-coat tissue and DNA prepared from each fraction. Although the whole seed of Honeydew melon contained a large proportion of stress-sat.DNA (1.718 $g.cm.^{-3}$, fig.14a), the cotyledons contained only the mainband (1.693 $g.cm.^{-3}$) and normal satellite (1.707 $g.cm.^{-3}$) DNAs (fig.14b). Essentially

FIGURE 14

Localization of the stress-satellite DNA within the melon seed. Seeds of Honeydew melon were stored for 34 days at temperatures of 0-4°C and then separated into cotyledon and seed-coat tissue. DNA was prepared from these tissues, and from the complete seed, and analysed by Model E centrifugation. a, DNA from the complete seed; b, DNA from the cotyledon tissue; c, DNA from the seed-coat tissue; d, DNA prepared by washing the intact seeds in the detergent extraction medium.


(a)
(b)
(c)
(d)
1·731
1·718
1·707
1·693

all of the 1.718 g.cm.$^{-3}$ DNA was present in the seed-coat tissue, together with a trace of mainband DNA (fig.14c).

Similar results were obtained on dissection of marrow seed, where the total seed had a stress-sat.DNA (1.720 g.cm.$^{-3}$, fig.15) which was absent in the isolated cotyledons (1.695 and 1.705 g.cm.$^{-3}$). The seed-coat contained large amounts of the 1.720 and also 1.695 g.cm.$^{-3}$ DNAs. This, therefore, suggested that there was some stress-sat.DNA of a similar density to the mainband.

The restriction of stress-sat.DNA accumulation to the seed-coat, which was composed largely of dead cells, indicated that this DNA was probably not of plant origin, but was the result of bacterial contamination on the outside of the seed-coat. This was confirmed by "washing" cold-stored seeds in detergent extraction medium without any homogenization of the seed material. The DNA recovered from the "washing" was identical in buoyant-density (Melon: 1.718 g.cm.$^{-3}$, fig.14d; Marrow: 1.720 and 1.695 g.cm.$^{-3}$, fig.15d) and in yield (Table 6) to the stress-satellite component of total seed DNA.

FIGURE 15

Localization of the stress-satellite DNA within the marrow seed. Seeds were stored for 22 days at temperatures of 0-4°C and then separated into cotyledon and seed-coat tissue. DNA was prepared from these tissues, and from the complete seed, and analysed by Model E centrifugation. a, DNA from the complete seed; b, DNA from the cotyledon tissue; c, DNA from the seed-coat tissue; d, DNA prepared by washing the intact seeds in the detergent extraction medium.

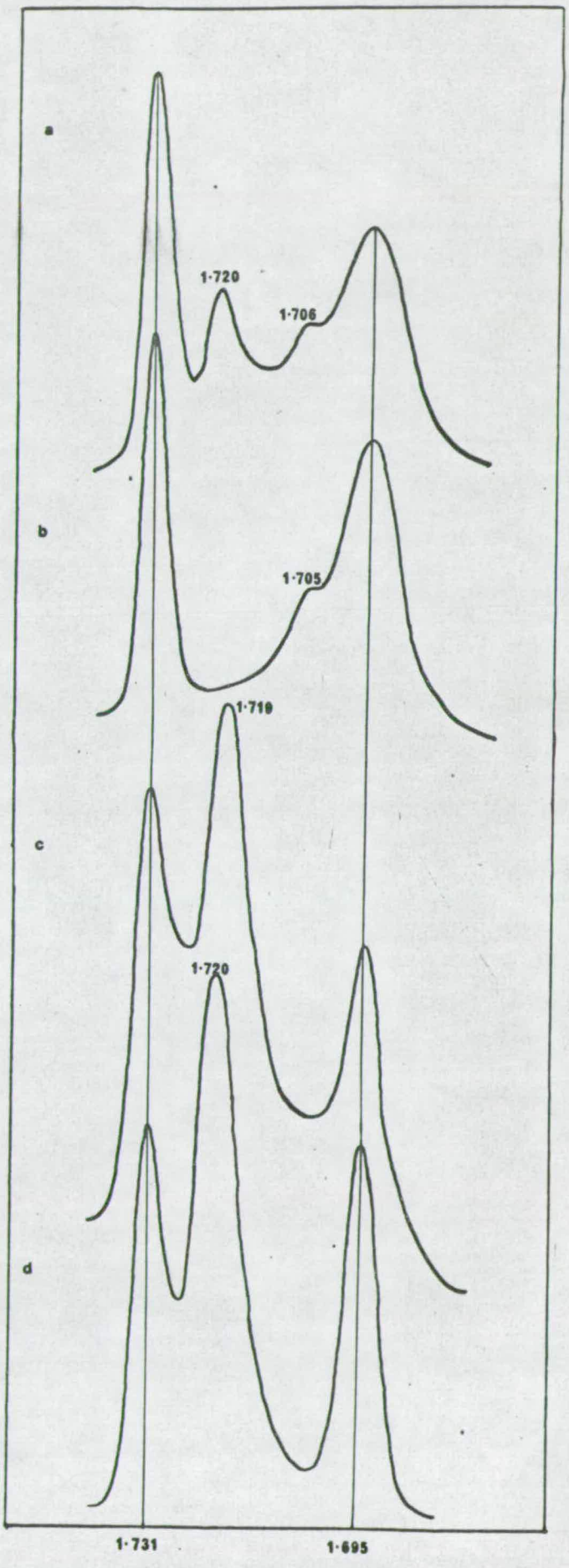

a
1·720
1·706
b
1·705
1·719
c
1·720
d
1·731
1·695

Table 6

The location of the stress-satellite DNA in the seed and its correlation with bacterial contamination.

Seeds	No. days cold-storage	µg. DNA/seed				No. bacteria per seed
		Stress-satellite	Seed Coat	Detergent Wash	Bacterial	
Honeydew melon c	0	0	0	0	-	-
	20	1.5	1.4	-	-	-
	22	-	-	1.8	2.0	2×10^8
	24	1.7	1.8	-	-	-
Honeydew melon d	15	0	-	-	0	40
	28	0	-	-	0	200
Courgette marrow a	0	0	-	-	0	20
	22	4.5	3.8	-	-	-
	46	8.8	6.8	6.1	>2	$>2 \times 10^8$

The amount of stress-satellite DNA was calculated from the Model E fractionation. The seed-coat DNA was the total amount of DNA prepared from the isolated seed-coats. The detergent-wash DNA was the total amount of DNA extracted by washing the intact seeds in the detergent medium. The bacterial DNA was calculated from the number of bacterial contaminants per seed on the basis of 10^{-14} g. per bacterium.

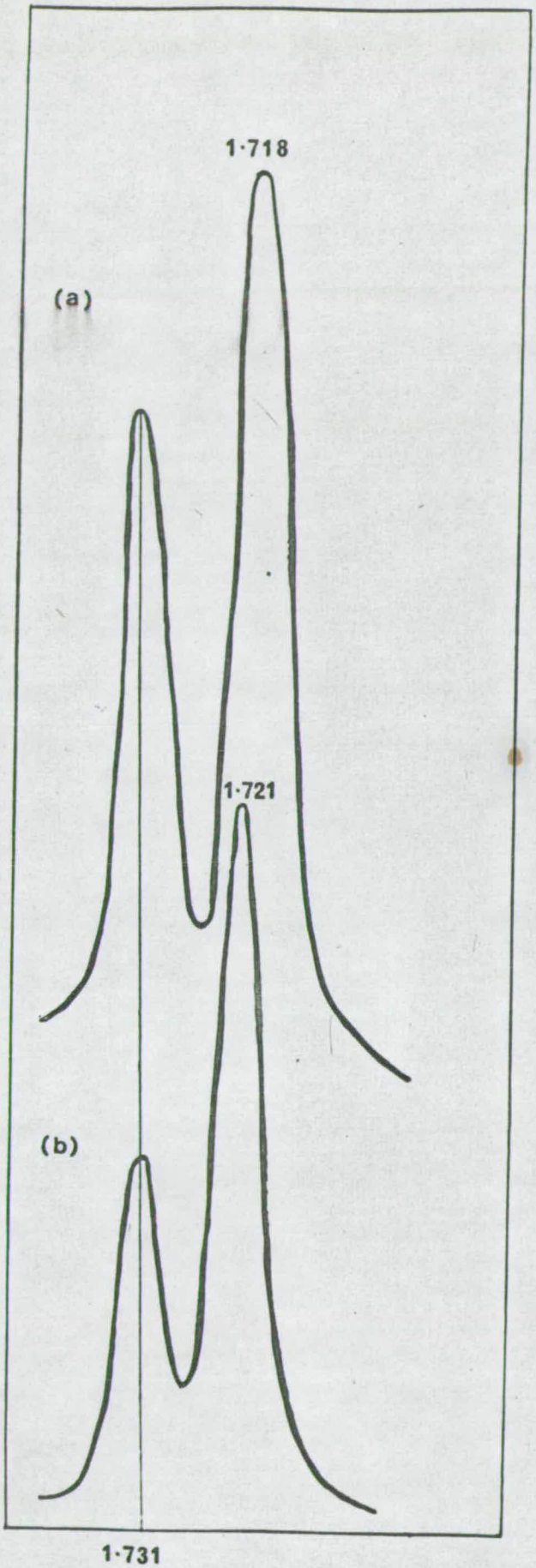
The presence and amount of the stress-sat.DNA was also well correlated with the extent of the bacterial contamination. The amount of bacterial DNA per seed, calculated on the basis of 10^{-14}g. of DNA per bacterium, was similar to the stress-satellite component of the total DNA, to the DNA content of the seed-coat, and to the DNA prepared by detergent washing of the seed (Table 6). Those seeds which produced no stress-sat.DNA (Honeydew melon d, Table 5) had very little bacterial contamination, even after long storage periods (Table 6). Isolates of bacteria from different seeds were cultured, and the DNAs prepared from such cultures were identical in density to the stress-sat.DNAs (fig.16). DNA from the bacterial strain isolated from cucumber (var. Karina) seeds was denatured and renatured. The DNA (10µg./ml.) was 50% renatured in 1xSSC after between 4 and 26 hr. This was equivalent to 50% renaturation after between 1,200 and 7,800 min. in 1xSSC at a concentration of 2µg./ml. and was of the same order as that of stress-sat.DNA and E. Coli DNA (Table 4). In conclusion, these results showed that the stress-sat.DNA which was produced in seeds of the Cucurbitaceae during cold-storage was bacterial in origin.

FIGURE 16

DNA of bacteria isolated from cold-stored seeds.

a, bacterial culture from cold-stored Honeydew melon seeds;

b, bacterial culture from cold-stored marrow seeds.


1·718
(a)
1·721
(b)
1·731

B. Differential DNA replication during normal growth and development.

(i) Difference in satellite DNA between tissues.

Table 7

Variation in the percentage of satellite DNA present in melon tissues.

Variety	Seed	Fruit	Mature Leaf	Mature Cotyledon	Root	Hypocotyl	Flower
Hero of Lockinge	26.1	17.1	15.6	14.4	25.8	19.7	21.5
Honeydew a	29.4			17.2		22.4	
Musk b	29.5	17.1	18.9	17.6			
Spanish Winter c	27.7	18.5		17.9		21.9	
Spanish Winter e	24.3	16.7	16.2	19.2			
Spanish Winter g	30.3	15.2		20.4	29.7	21.9	
Spanish Winter h	24.8	20.9	16.3	14.1			
Spanish Winter j	34.1	23.9		20.9			
Spanish Winter k	36.3		15.1	19.3			

The letter following the variety identifies an individual melon.

DNA was prepared from fruit tissue and seeds of commercially obtained melons. Seeds were then germinated and DNA prepared from the different tissues of the seedlings.

The percentage of sat.DNA in different tissues of melon plants was determined (Table 7). The seeds and roots contained a higher percentage of sat.DNA than the other tissues, whilst the fruit, mature cotyledons, and leaves had the least. Hypocotyls and flowers had intermediate percentages of sat.DNA. Typical scans of the DNA from four of these tissues are shown (fig.17). These differences between the various tissues were much greater than the errors involved in preparation and analysis of DNA (Table 1). Since the biggest differences seemed to be between fruit and seed, and since substantial amounts of DNA could be obtained from both fruit and seed of individual melons, further studies concentrated on these tissues.

FIGURE 17

Analysis of DNA prepared from melon tissues.

Total DNA was prepared from the seeds (a) and the fruit (b) of Spanish Winter melon g. Seeds of this melon were germinated and DNA was prepared from the root tip (c) and hypocotyl tissue (d). The DNAs were analysed by Model E centrifugation. The value indicates the amount of satellite as a percentage of the total DNA.

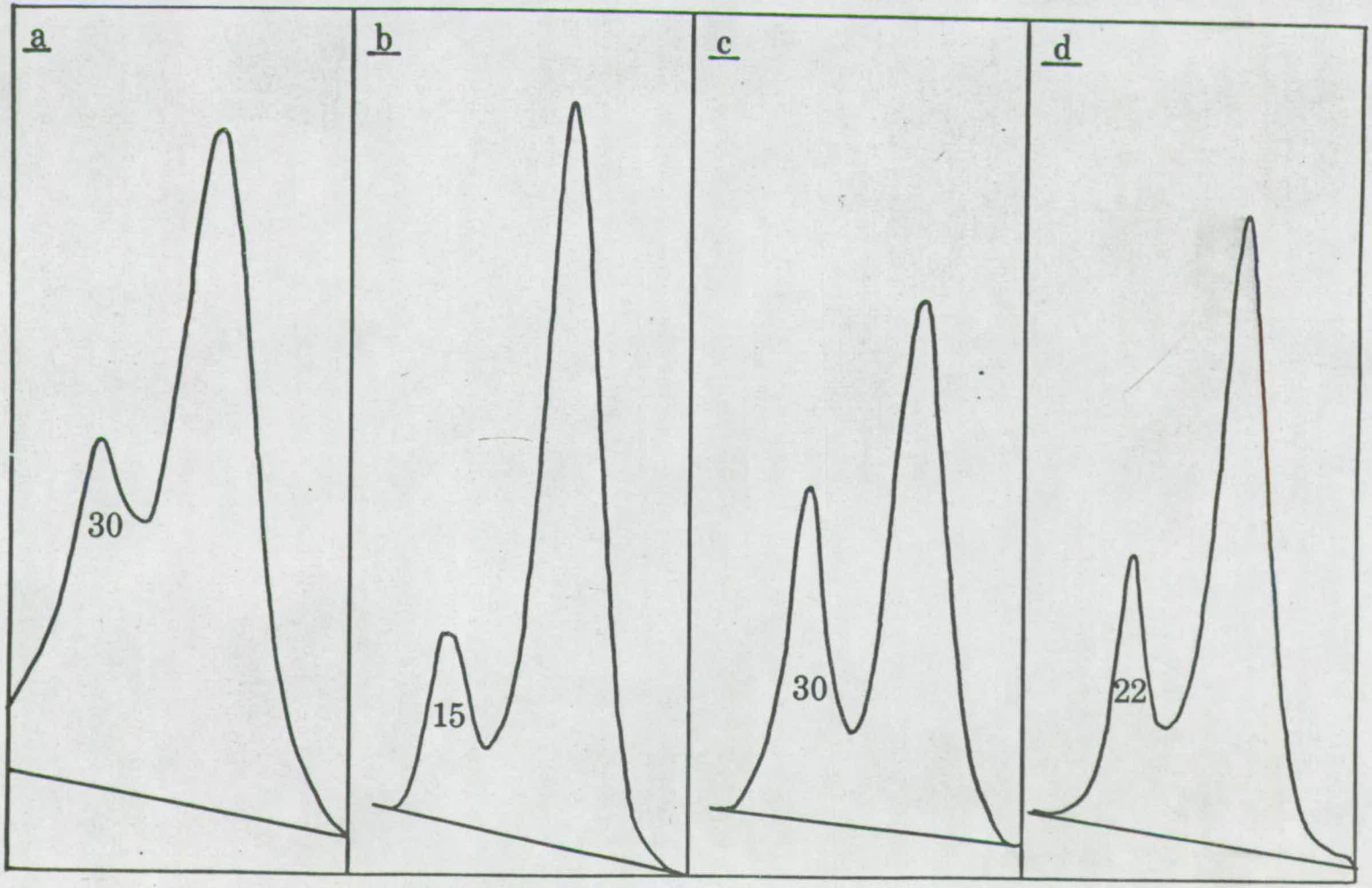

a
30
b
15
c
30
d
22

Table 8

Distribution and buoyant-densities of mainband and satellite DNAs from seed and fruit tissues.

Variety	% Satellite		Buoyant-density ($g.cm.^{-3}$) Mainband		Satellite	
	Seed	Fruit	Seed	Fruit	Seed	Fruit
Melon						
Hero of Lockinge	26.1	17.1	1.6924	1.6938	1.7058	1.7085
Honeydew e	29.2	21.8	1.6919	1.6933	1.7062	1.7076
f	24.7	18.4	1.6922	1.6936	1.7056	1.7079
g	23.4	18.1	1.6922	1.6926	1.7056	1.7066
h	25.5	17.3	1.6928	1.6939	1.7063	1.7082
Ogen a	30.6	18.2	1.6927	1.6937	1.7056	1.7088
c	24.9	19.1	1.6929	1.6930	1.7063	1.7067
Musk a	24.2	16.3	1.6923	1.6927	1.7057	1.7070
b	29.5	17.1	1.6926	1.6940	1.7056	1.7071
Spanish Winter c	27.7	18.5	1.6917	1.6931	1.7063	1.7075
e	24.3	16.7	1.6931	1.6932	1.7068	1.7076
g	30.3	15.2	1.6923	1.6928	1.7064	1.7079
h	24.8	20.9	1.6918	1.6928	1.7057	1.7070
j	34.1	23.9	1.6921	1.6925	1.7056	1.7069
Cucumber						
Kariha satellite 1	41	34	1.6939	1.6943	1.7011	1.7034
satellite 2					1.7060	1.7084

In cucumber, the data are the means from 7 samples of seed and fruit. Owing to lack of resolution of the 2 satellites in cucumber, the combined satellites are expressed as percentage of the total DNA.

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In every melon examined, the percentage of sat.DNA in the fruit was less than in the seeds, in some cases even half (Table 8). Differences between individual melons of the same variety might have resulted from the confused taxonomy of melons and from their different genetic backgrounds. The variation was not due to variability of extraction and analysis, since there were very small differences when 6 batches of seeds from a single melon were analysed (Table 1). Furthermore, in every melon examined it was observed that the densities of both mainband and satellite DNAs were less in the seed than in the fruit (Table 8). These results concerning the difference between fruit and seed DNA, both with regard to density and sat.DNA percentage, were highly significant ($p < 0.1\%$).

There was a similar difference in the amount of sat.DNA between cucumber fruit and seed (Table 8). For each of the 2 sat.DNAs of cucumber, the percentage present in DNA of fruit origin was less than in DNA of seed origin. Furthermore, the density of each sat.DNA was significantly greater in the fruit than in the seed (Table 8) as was the case in melon. However, unlike melon, cucumber mainband DNA was not significantly different in density in the fruit or seed (Table 8).

Owing to the very different natures of seed and fruit tissue, it was possible that these differences might result from differential extraction of mainband and satellite components. This possibility was checked by using a different method of DNA preparation, and by several consecutive extractions of tissue:

(a) SDS/Pronase procedure for DNA preparation.

Fruit and seed material from a Honeydew melon were homogenized in SSC. SDS (Sodium dodecyl sulphate) was added to a final concentration of 0.5%, and then pronase (self-digested for 10 min.) was added to give a final concentration of 800µg./ml. After incubation at 37°C for 16 hr., debris was removed by centrifugation and the supernatant was further deproteinized with phenol mixture (see total DNA preparation). The aqueous supernatant containing the nucleic acids was purified as normal by RNase and pronase treatments followed by high-speed centrifugation to yield the DNA. The DNA prepared by this method contained 25% sat.DNA in the seed and 17% in the fruit, with buoyant-densities of 1.6926 and 1.7064 $g.cm.^{-3}$ in the seed and 1.6940 and 1.7082 $g.cm.^{-3}$ in the fruit for the mainband and sat.DNA respectively, and was identical to that prepared by the normal detergent procedure (Table 8).

(b) Sequential extractions

Seed and fruit material from another Honeydew melon were homogenized in detergent as in the normal DNA preparation procedure. The aqueous layer was removed after treatment with chloroform. DNA was precipitated from it by the addition of 2 vols. ethanol. The chloroform layer and interface were re-extracted with more detergent and the above process repeated. DNA was quantitatively removed from seeds by 2 extractions and from fruit by 3. There were no significant changes in either sat.DNA percentage or buoyant-density in the sequential extractions. Fruit

and seed DNAs, however, were markedly different with regard to density and sat.DNA percentage (Table 9).

Table 9

Distribution and buoyant-densities of mainband and satellite DNAs of sequential extractions from seed and fruit of a Honeydew melon.

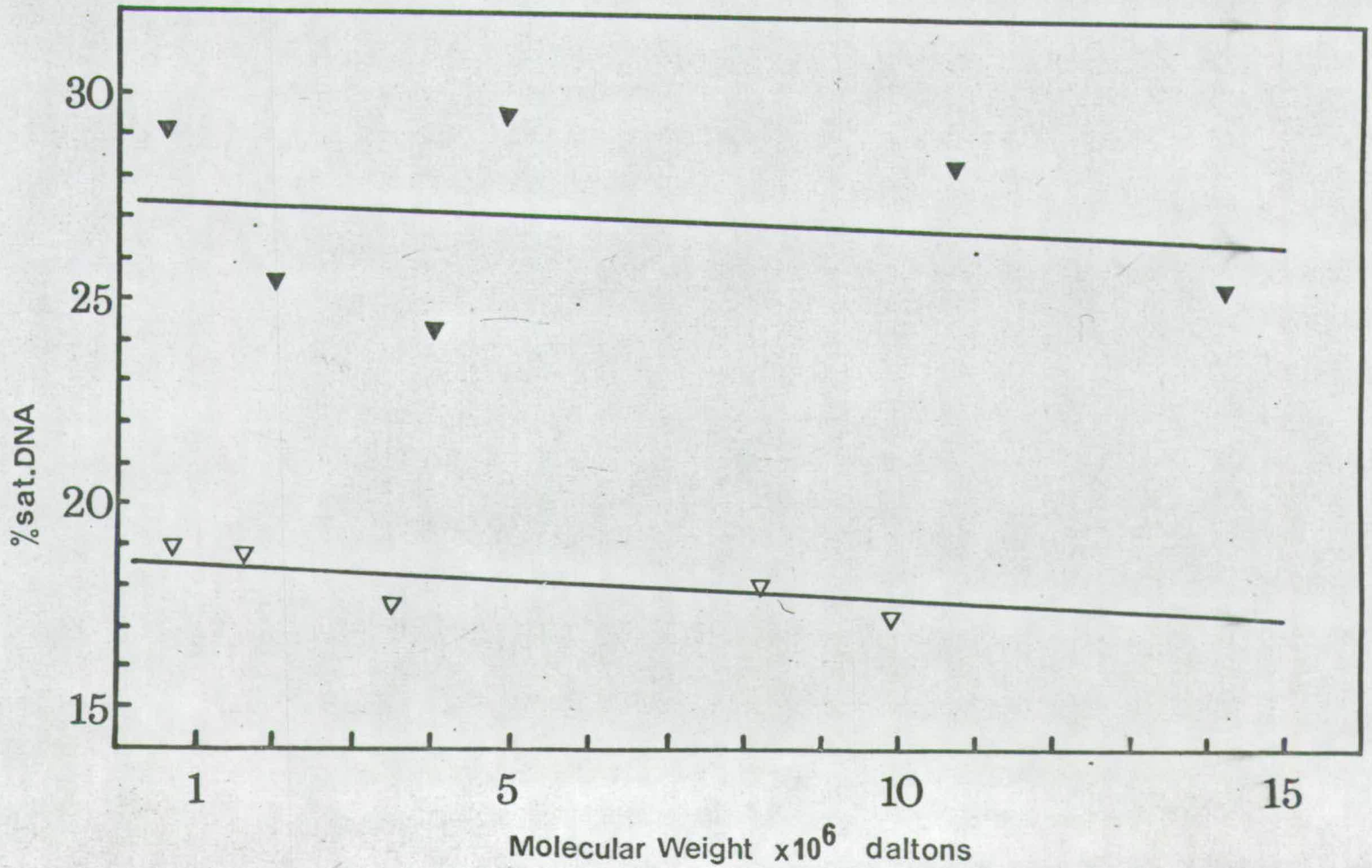
Melon	Sequential extraction No.	% Satellite		Buoyant-density ($g.cm.^{-3}$)			
				Mainband		Satellite	
		Seed	Fruit	Seed	Fruit	Seed	Fruit
Honeydew f	1	24.2	18.2	1.6923	1.6942	1.7057	1.7086
	2	23.9	18.0	1.6923	1.6941	1.7058	1.7086
	3	-	19.8	-	1.6939	-	1.7085

Fruit and seed material were homogenized in detergent medium. The aqueous layer was removed and treated with chloroform followed by nucleic acid precipitation by the addition of 2 vols. ethanol. The chloroform layer and interface were re-extracted with more detergent and the above process repeated. All the DNA was quantitatively removed from seeds by 2 extractions and from fruit by 3 extractions.

FIGURE 18

Effect of DNA size on the percentage satellite DNA.

Total DNA was prepared from fruit and seeds of a melon. The average molecular weight of each preparation was determined by the spill-over method of Studier (1965), and then each was successively sheared, initially by forcing the solution through a syringe and finally by sonication. The size at each stage was measured, and the quantitative distribution of satellite DNA determined by Model E centrifugation. —▼—▼— seed; —▽—▽— fruit.


30
25
20
15
%sat.DNA
1
5
10
15
Molecular Weight x10^6 daltons

Consequently, it seemed that the differences between fruit and seed DNAs were not artefacts of differential extraction.

(c) Size of DNA

Differences in the amount and buoyant-density of sat.DNA could possibly result from the DNA preparations of fruit and seed tissue having different molecular weights. DNA with as high a molecular weight as possible was, therefore, prepared from the seeds and fruit of a Honeydew melon and the percentage of sat.DNA in each determined. The DNA was then progressively sheared, initially by hand-shearing through a syringe, and then by sonication. At each stage, the average molecular weight and the percentage sat.DNA were determined (fig.18). Although the percentage satellite in both seed and fruit increased slightly as the molecular weight decreased, this increase was attributed to the lack of resolution of satellite and mainband as DNA size decreased. There was no tendency for the percentage of sat.DNA in the fruit to approach that of the seed. Thus, it was concluded that the difference between fruit and seed was not due to the size of the DNA.

(ii) Variation in satellite DNA during development

On the assumption that all meristematic cells contained a high percentage of sat.DNA, the development of tissues such as fruit, leaves, and cotyledons should show a decrease in percentage and a change in buoyant-density of the sat.DNA during the change from meristematic to mature tissue.

(a) Fruit development

It was not possible to study the development of melon fruits

since growth conditions in the greenhouse were not favourable. However, one sample of Ogen melons at different stages of development were obtained from Israel and a preliminary analysis made.

The percentage of sat.DNA in the fruit decreased with size (similar to ageing) from 22% in the smallest size fruit examined to 18% in the largest fruits. Furthermore, the density of the sat.DNA suggested an increase from 1.7084 to 1.7089 $g.cm.^{-3}$ (Table 10).

Table 10

Distribution and buoyant-densities of mainband and satellite DNAs during development of the Ogen melon.

Melon	Satellite DNA (% of total)		Buoyant-density ($g.cm.^{-3}$) Mainband		Satellite	
	Seed	Fruit	Seed	Fruit	Seed	Fruit
a	22.0	22.5	1.6950	1.6951	1.7085	1.7084
b	28.0	20.5	1.6949	1.6948	1.7083	1.7087
c	25.3	18.1	1.6947	1.6949	1.7081	1.7089
d	27.7	19.0	1.6947	1.6953	1.7080	1.7089

The melons a-d are in progressive order of increase in size (from 68 x 38 mm. to 155 x 116 mm. in diameter) and, therefore, presumably in order of increasing age.

In the seeds from the same melons, the percentage of sat.DNA increased from 22% in the seeds of the smallest melon to 28% in the seeds of the largest melon, whilst the sat.DNA density decreased from 1.7085 to 1.7080 $g.cm.^{-3}$ (Table 10).

Thus, in the youngest melon, the density of seed and fruit sat.DNAs were very similar (1.7085 and 1.7084 $g.cm.^{-3}$ respectively) and the percentage of sat.DNA was the same in both cases (22%), whereas in the older melons, there was a difference in buoyant-density (1.7080 and 1.7089 $g.cm.^{-3}$ in seed and fruit respectively) and in the percentage of sat.DNA (18% and 28% in fruit and seed respectively).

More extensive studies were made on the development of cucumber fruits which were readily produced under greenhouse conditions. It was assumed that a reasonable measure of cucumber maturation was the volume, as this was an easily measured parameter. On account of the great range of cucumber volume, the $\log_{10}$ cucumber volume (log.vol.) was used when the volume was measured in mm^3.

In cucumber fruit (var. Kariha), the percentages of the 2 sat.DNAs were constant at 25% and 16% (for the less-dense and more-dense satellites respectively, fig.19d) when the log.vol. was between 2.0 and 4.1, i.e. for young fruits up to ca.120 mm. long. Throughout this range, the densities of the 2 sat.DNAs were also constant (1.7012 and 1.7060 $g.cm.^{-3}$, fig.19b,c). Older, mature fruits (log.vol. 5.1 - 6.0, ca.250-260 mm. in length) showed a slight but significant ($p < 5\%$) decrease in percentage of each of the

FIGURE 19

Effect of maturation on the density of cucumber DNA and the percentage of satellite DNA.

Cucumber volume was used as a parameter for measuring maturation. a, mainband density; b, density of satellite 2; c, density of satellite 1; d, satellite DNA as a percentage of the total DNA (—□—□— satellite 2; —○—○— satellite 1).

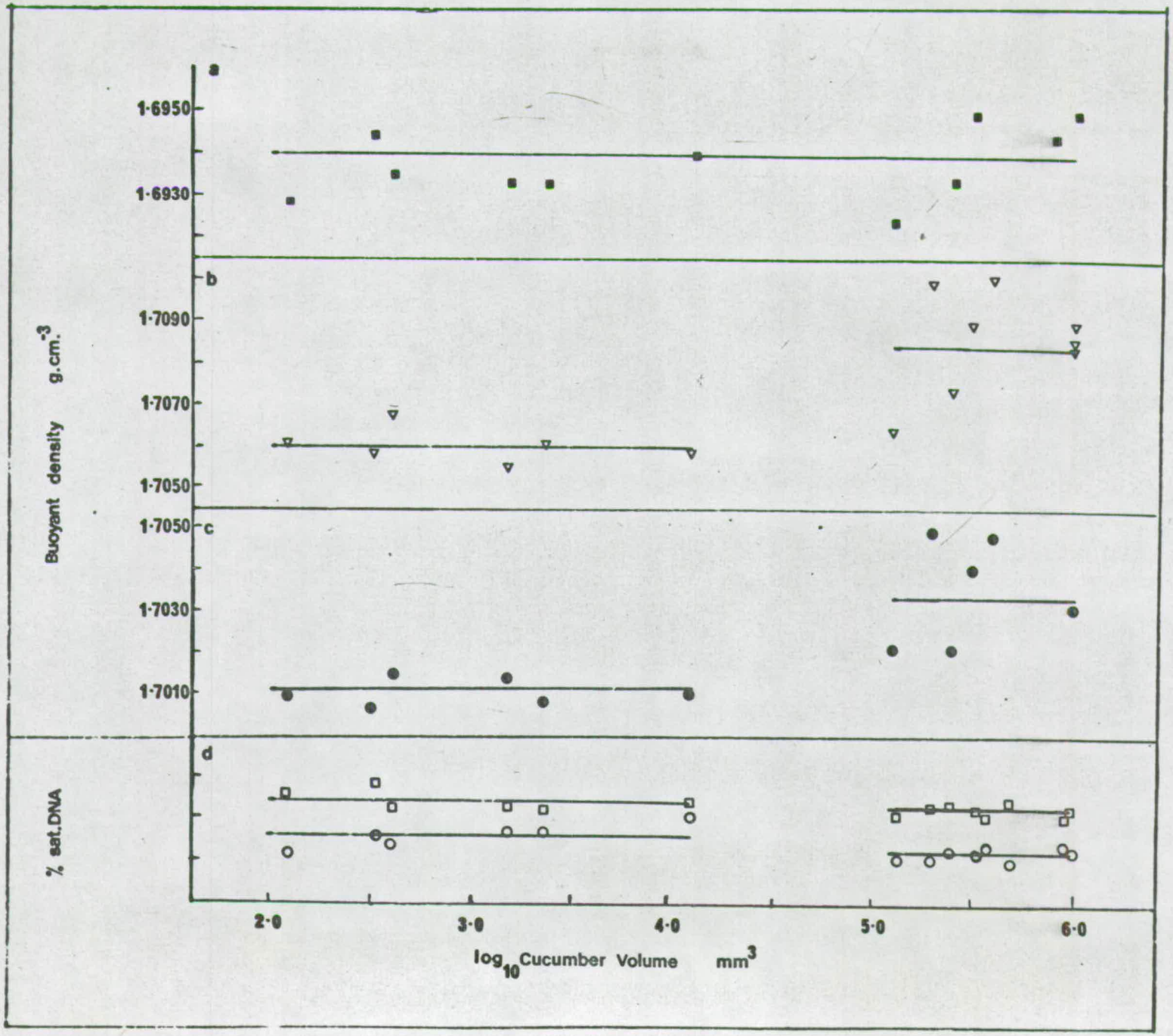
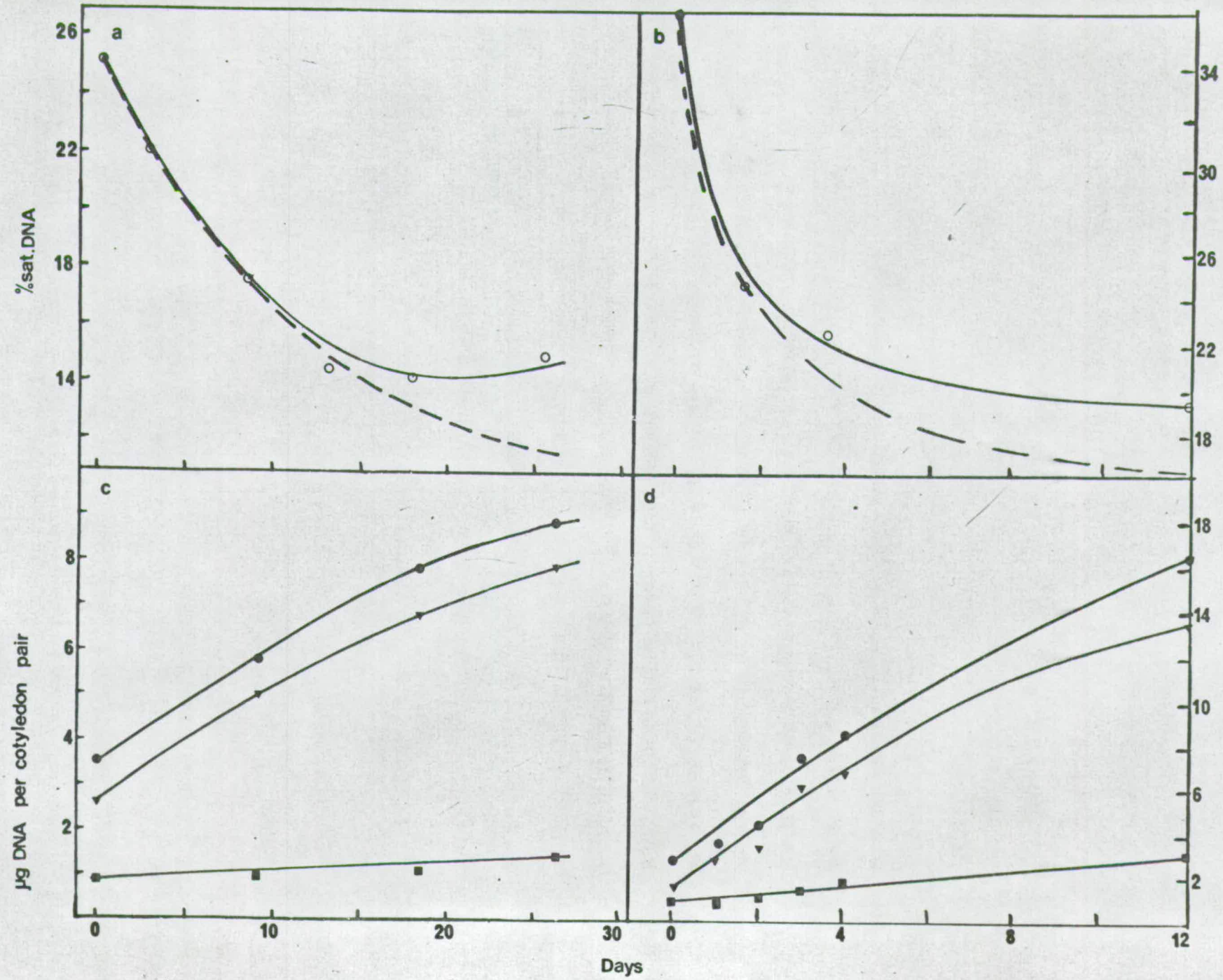

a
1·6950
1·6930
b
1·7090
1·7070
1·7050
1·7050
c
1·7030
1·7010
Buoyant density g.cm.-3
d
% sat.DNA
2·0
3·0
4·0
5·0
6·0
log10 Cucumber Volume mm3

FIGURE 20

Changes in satellite DNA during growth of cotyledons. Melon seeds were germinated and the DNA was prepared from the cotyledons at various stages during development. Decrease in the percentage of satellite with time in cotyledons of: a, Spanish Winter melon h; b, Spanish Winter melon k. In a, b the dotted line shows a theoretical dilution curve whereas the solid line shows actual values. Increase in the amount of DNA in melon cotyledons following germination in: c, Spanish Winter melon h; d, Spanish Winter melon k. In c, d —●—●— total DNA, —▼—▼— mainband DNA, —■—■— satellite DNA.


a
b
c
d
%sat.DNA
µg DNA per cotyledon pair
Days
26
22
18
14
34
30
26
22
18
8
6
4
2
18
14
10
6
2
0
10
20
30
0
4
8
12

sat.DNAs (23% and 12%, fig.19d) as well as a highly significant ($p < 0.1\%$) increase in the density of each satellite (1.7034 and 1.7084 $g.cm.^{-3}$, fig.19b,c). Unlike melon, the density of the mainband DNA did not significantly change with ageing but remained constant at 1.6940 $g.cm.^{-3}$ (fig.19a).

(b) Cotyledon development

Following the germination of melon seeds, the percentage of sat.DNA in the cotyledons decreased continually with time (fig.20a,b). For the first 10 days, the rate of increase in DNA per cotyledon pair was linear, but subsequently the rate decreased and tended towards zero (fig.20c,d). Both mainband and satellite DNAs increased at a linear rate for ~12 - 15 days, and from this initial rate, the accumulation rate of sat.DNA (i.e. µg. sat.DNA per day/µg. total DNA per day) represented 6.3% and 17.0% of the total DNA accumulation in Spanish Winter melons h and k respectively. Sat.DNA, therefore, appeared to be accumulated at a constant percentage during cotyledon development, but this percentage differed markedly from that in the seed cotyledons.

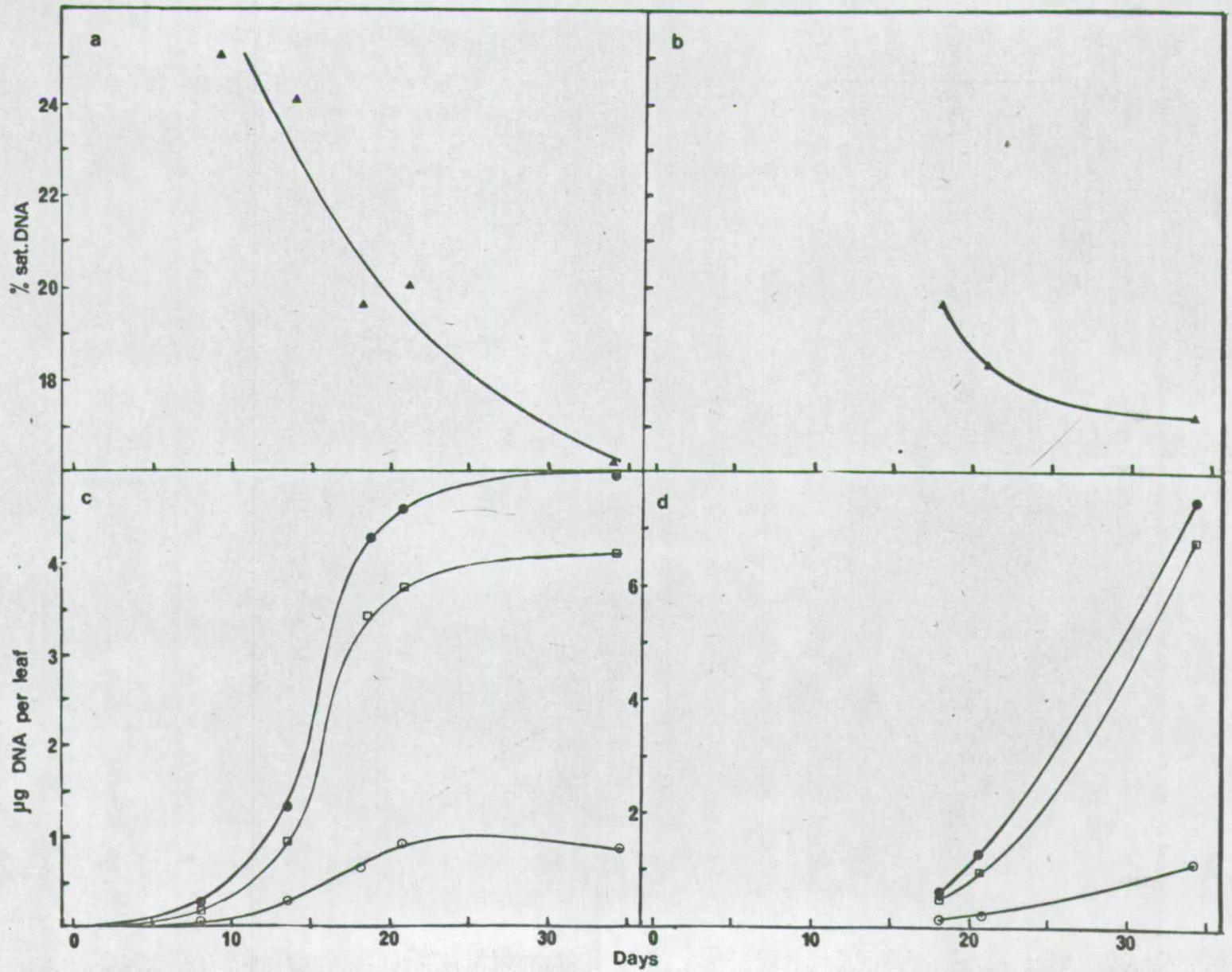
(c) Leaf development

As in cotyledons, there was a distinct drop in the percentage of sat.DNA during the development of melon leaves. This was true of both the first and second leaves to appear after germination (fig.21a,b). In this case, the change in the percentage of sat.DNA could not be due to dilution of existing DNA but only to changes in the

FIGURE 21

Changes in satellite DNA during growth of leaves.

Melon seeds were germinated and the DNA was prepared from the first and second leaves to appear in Spanish Winter melon h. Decrease in the percentage of satellite DNA with time in: a, first leaf; b, second leaf. Increase in the amount of DNA in melon leaves following germination: c, first leaf; d, second leaf. —●—●— total DNA, —□—□— mainband DNA, —○—○— satellite DNA.


a
b
c
d
% sat.DNA
µg DNA per leaf
Days
24
22
20
18
4
3
2
1
6
4
2
0
10
20
30

relative rates of accumulation of satellite and mainband. The absolute amounts of the various DNAs present per leaf underwent a sigmoidal increase with time (fig.21c,d). Although the second leaf commenced growth later, both leaves showed a decrease in the sat.DNA percentage. The first leaf of a Spanish Winter melon showed a 21-fold increase in total DNA in an 8-day period (from 8 to 16 days after planting, fig.21c), during which time the sat.DNA percentage fell from 25% to 20% on account of there having been only a 15-fold increase in sat.DNA in the leaf (fig.21a). Similarly, the second leaf showed an 11-fold increase in total DNA in a 16-day period (from 18 to 34 days after planting, fig.21d), during which time the sat.DNA percentage fell from 19% to 17% on account of there having been only a 9-fold increase in sat.DNA in the leaf (fig.21b).

Results of ^{32}P-orthophosphate labelling of melon seedlings

Although the accumulation of satellite and mainband DNA was equivalent to synthesis if DNA was completely stable, the different proportions of sat.DNA might have arisen not at the level of replication, but of turnover. Preliminary experiments on the synthesis of DNA suggested that turnover of sat.DNA might be important.

13 - 14-day old Spanish Winter melon seedlings (melons h and k) were labelled for 24 hr. with ^{32}P-orthophosphate by intake through the roots. Total DNA preparation from the leaves was followed by fractionation on preparative CsCl gradients. The percentage of newly-synthesized sat.DNA (counts) was 31.4% (k) and 33.7% (h) compared with 22.7% (k) and 20.9% (h) in the total leaf DNA (OD_{260})

FIGURE 22

Rate of synthesis of satellite DNA in leaves of Spanish Winter melons.

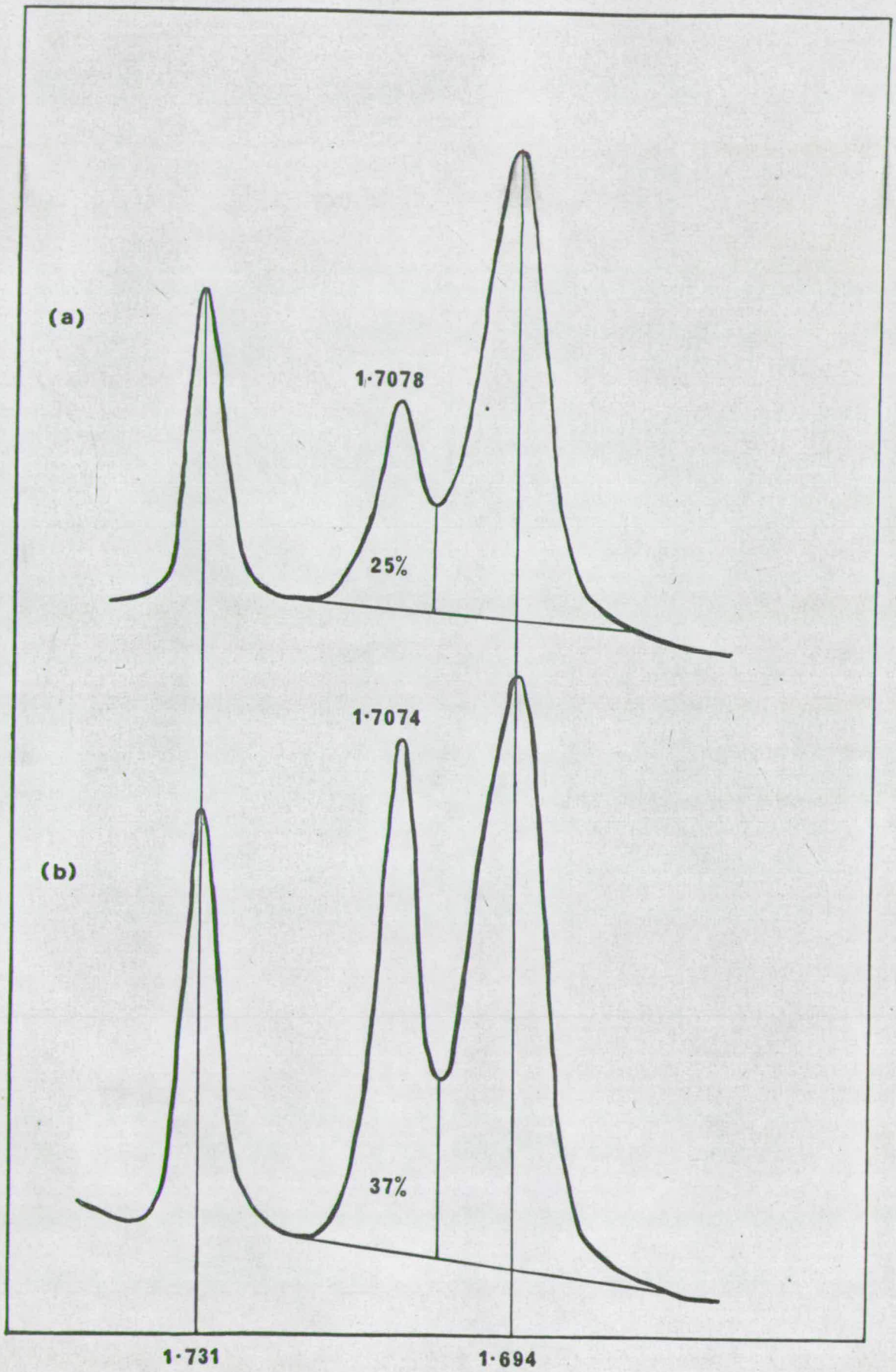
Spanish Winter melon seedlings were labelled with ^{32}P-orthophosphate by uptake through the roots. DNA prepared from the leaves of these seedlings was fractionated on a preparative CsCl gradient. The gradient was pumped from the tube and the optical density at 254nm. was continuously recorded (continuous line). Each fraction was counted on a filter in Toluene-Butyl PBD scintillation fluid by a scintillation counter (histogram). a, Spanish Winter melon h: 14-day old seedlings. O.D. 21% satellite DNA; radioactivity 34% satellite DNA; b, Spanish Winter melon k: 13-day old seedlings. O.D. 23% satellite DNA; radioactivity 31% satellite DNA.

Optical Density 254nm
Fraction
Counts
a
b

FIGURE 23

Effect of NaCl on satellite DNA distribution.

Spanish Winter melon e fruit was homogenized and a crude nuclear preparation made by centrifugation at 2,500xg for 10 min. at 0°C (a) and then resuspended in 2.5M NaCl for 5 min. at room temperature. The insoluble material was recovered by centrifugation at 10,000xg for 20 min. at 0°C and DNA prepared (b). DNA was prepared and then analysed by Model E centrifugation.


(a)
1·7078
25%
1·7074
(b)
37%
1·731
1·694

(fig.22). This implied that the sat.DNA was less stable than the mainband DNA and had a greater turnover rate.

5. Functions of Satellite DNA

A. Chromosome structure and organization

Certain properties of sat.DNA, such as the centromeric location of mouse sat.DNA, suggested that it might be involved in chromosome structure and organization of the nucleus. Such a rôle would be consistent with the observation that euchromatin was more soluble in NaCl than heterochromatin (Maio and Schildkraut, 1969). It has also been reported that such salt treatment might reveal sat.DNA components undetected in total DNA preparation (Huguet and Jouanin, 1972). Experiments were designed to confirm the results of Huguet and Jouanin (1972) with plants containing sat.DNA. A crude nuclear fraction was obtained by centrifugation of a homogenate in Honda medium from fruit of a Spanish Winter melon (melon e) at 2,500 xg for 10 min. at 0°C. DNA was prepared from half of the pellet by the normal detergent treatment for total DNA preparation. The other half was resuspended in 2.5M NaCl for 5 min. at room temperature. The insoluble material was recovered by centrifugation at 10,000 xg for 20 min. at 0°C and DNA prepared. The NaCl-insoluble fraction contained only 26% of the DNA of the untreated pellet but the percentage of the sat.DNA was enriched from 25% to 37% in the NaCl-insoluble pellet (fig.23).

Nuclear material (Spanish Winter melon e) was treated with various concentrations of NaCl and DNA was prepared from the

FIGURE 24

Effect of NaCl concentration on satellite DNA distribution. Spanish Winter melon e fruit was treated as described in fig.23, except that the concentration of NaCl was varied from 1M (b) to 2.5M (c) to 5M (d). The control preparation with no salt treatment is shown in (a).

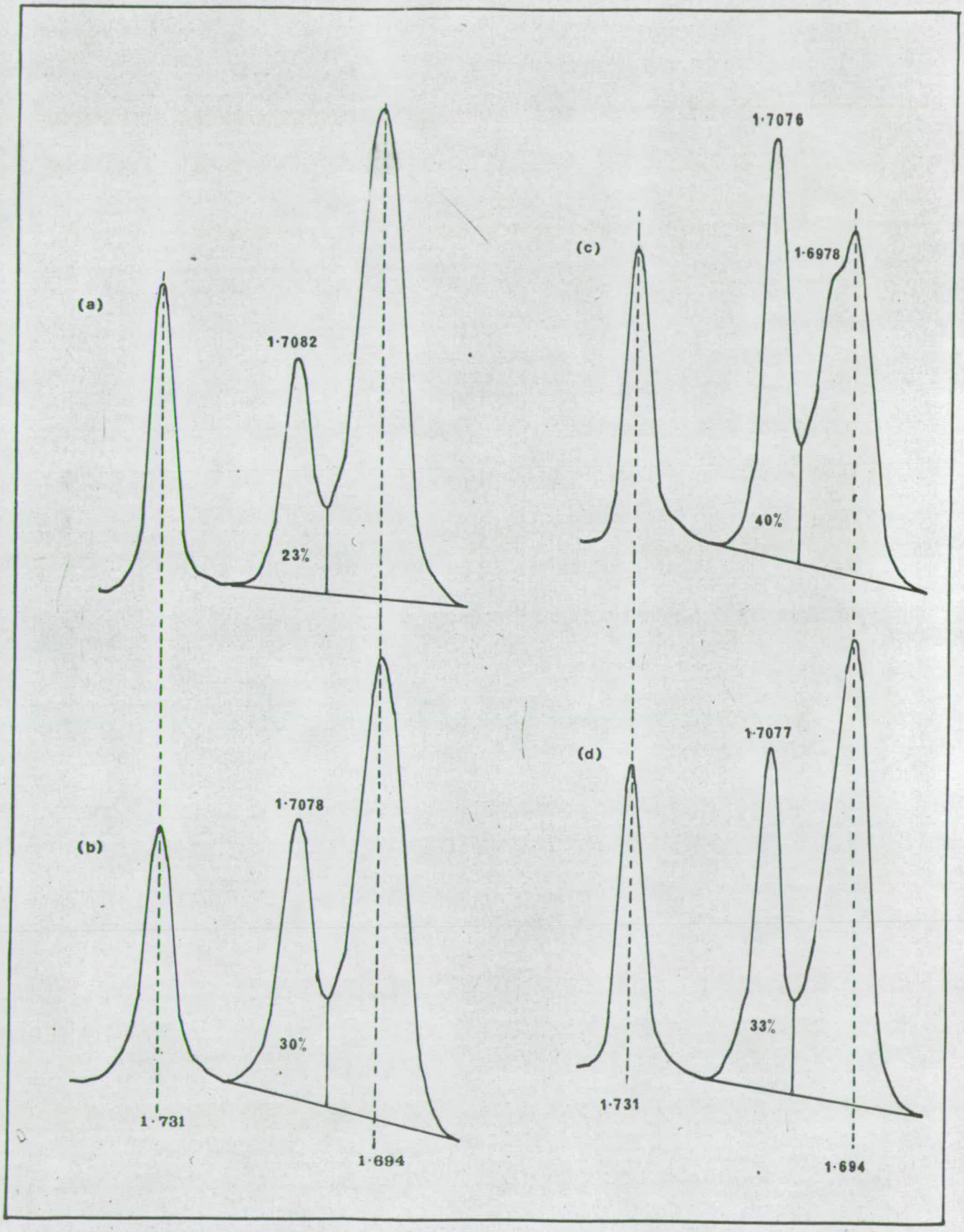
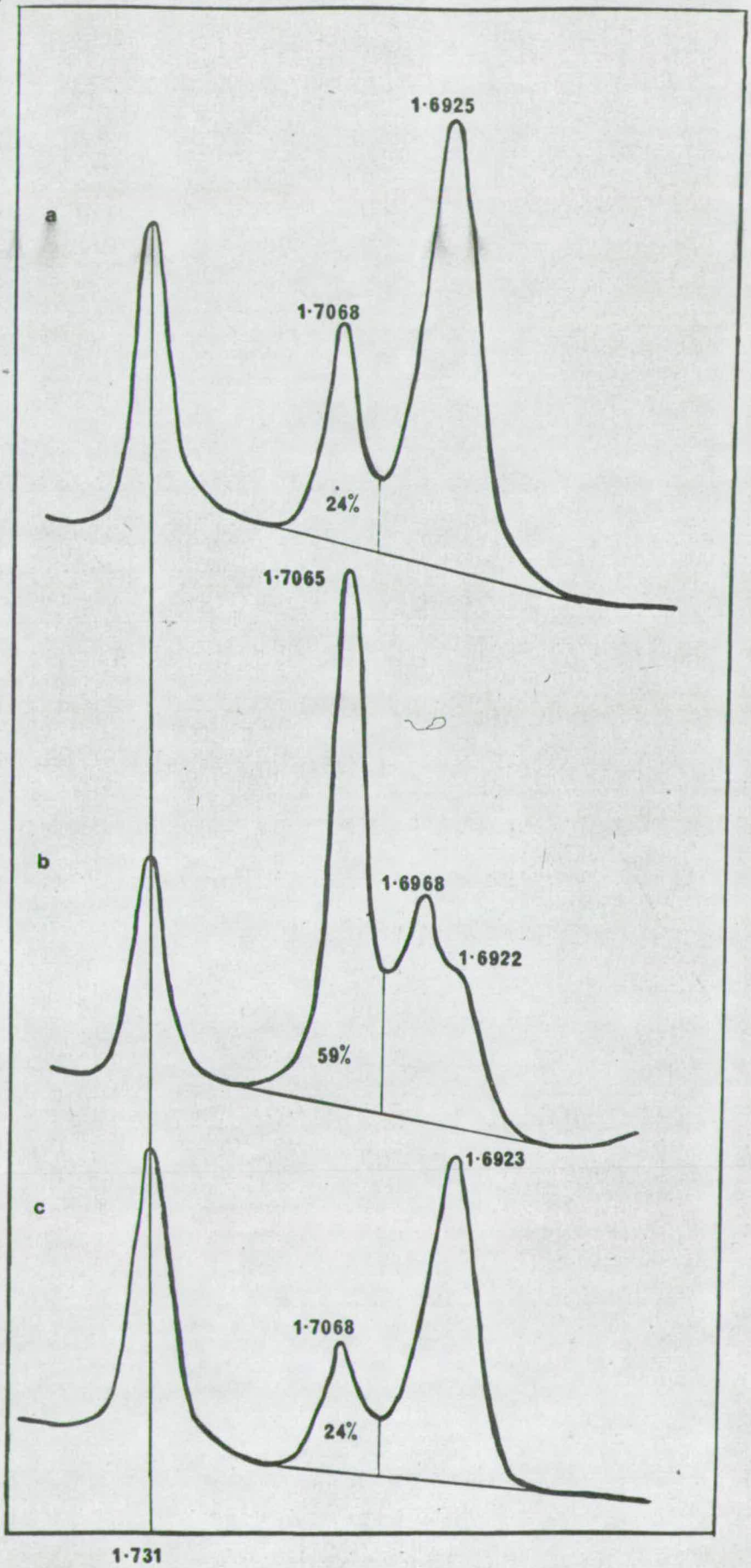

(a)
1·7082
23%
(b)
1·7078
30%
1·731
1·694
(c)
1·7076
1·6978
40%
(d)
1·7077
33%
1·731
1·694

FIGURE 25

Effect of NaCl treatment on DNA of Spanish Winter melon f. A crude nuclear preparation was made from fruit of Spanish Winter melon f by centrifugation at 1,500xg. a, DNA prepared from the pellet from the 1,500xg centrifugation. The pellet was resuspended in 2.5M NaCl for 20 min. at room temperature. The insoluble material was pelleted by centrifugation at 10,000xg for 40 min. at 0°C (b). The NaCl-soluble DNA material was recovered and analysed (c) and was essentially similar to the total-cellular material.


1·6925
a
1·7068
24%
1·7065
b
1·6968
1·6922
59%
1·6923
c
1·7068
24%
1·731

insoluble material. The NaCl-insoluble fractions were all enriched for sat.DNA, the 2.5M NaCl sample having the greatest enrichment (40%) as well as having a shoulder of density 1.6978 $g.cm.^{-3}$ (fig.24). Consequently, 2.5M NaCl was regarded as being the optimum concentration.

The crude nuclear pellet, which itself showed a slight increase in percentage of sat.DNA relative to the total-cellular DNA (Spanish Winter melon e: total cellular DNA = 17% satellite, crude nuclear pellet = 23% satellite), represented a recovery of only 2% of the total DNA. The 2.5M NaCl-insoluble fraction was, in turn, only 36% of the crude nuclear material. Therefore, although the NaCl-insoluble material contained 40% sat.DNA, this represented only 0.3% of the DNA from a total preparation, or 1.8% of the total sat.DNA.

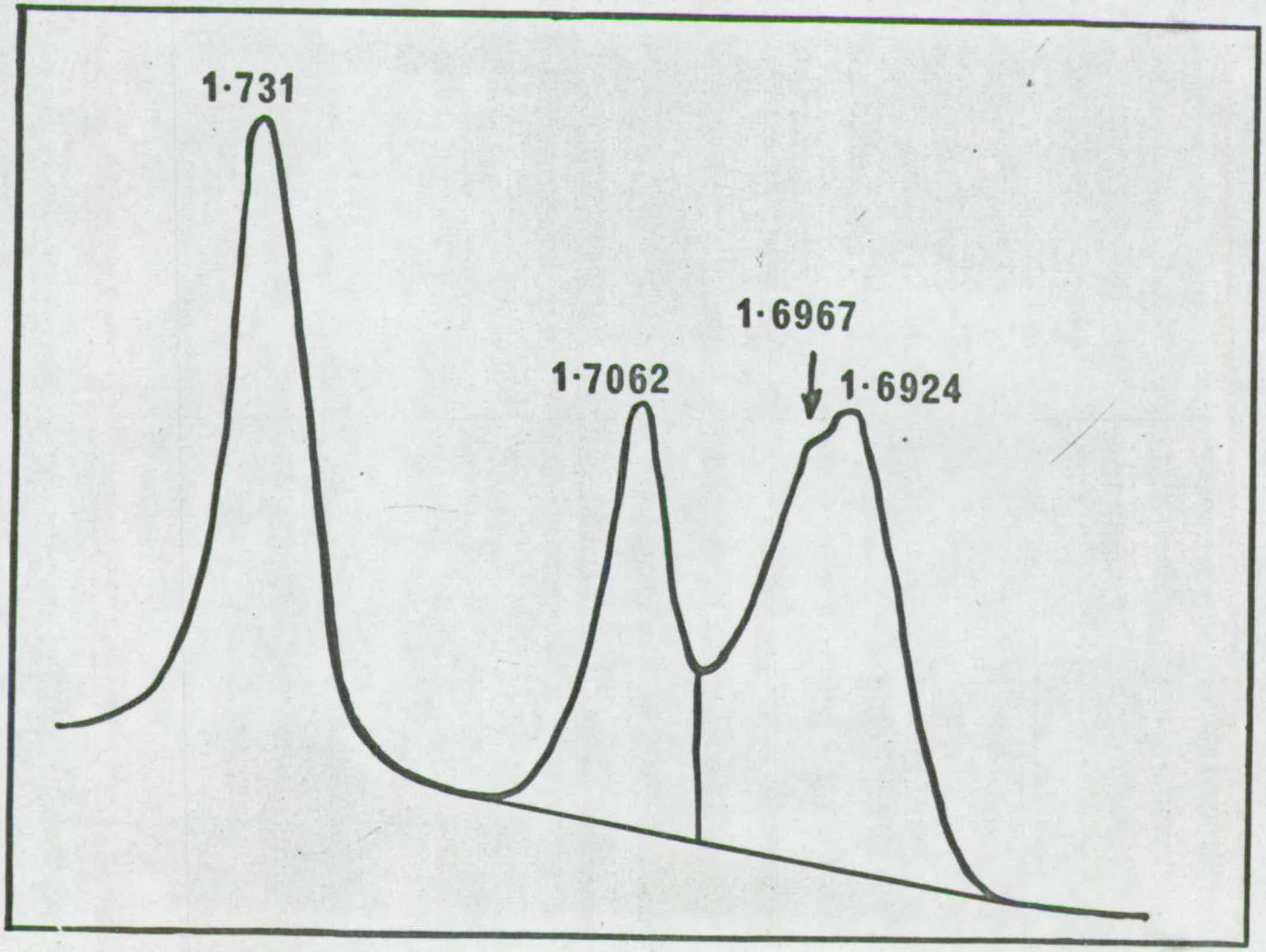
Experiments with another Spanish Winter melon fruit (melon f) yielded, in the 20% of the nuclear material insoluble in 2.5M NaCl in 20 min., 59% of sat.DNA, a second peak of density 1.6968 $g.cm.^{-3}$, and a shoulder of density 1.6922 $g.cm.^{-3}$ Again, the percentage of sat.DNA in the "nuclear" fraction (24%) was greater than the value from the total-cellular DNA preparation (18%). The NaCl-soluble fraction contained the remaining 80% of the "nuclear pellet" DNA which was essentially identical to the DNA from the crude-nuclear fraction (fig.25).

Since the absolute amount of sat.DNA present in the NaCl-insoluble preparations was so small, it was possible that the "satellite DNA" percentage might be an artefact caused by the enrichment of mitochondrial DNA (density $\sim$ 1.706 $g.cm.^{-3}$;

FIGURE 26

DNA from a mitochondrial fraction.

A crude nuclear preparation was made from fruit of Spanish Winter melon f by centrifugation at 1,500xg. The supernatant was centrifuged at 10,000xg for 40 min. DNA was prepared from the pellet and analysed by Model E centrifugation.


1·731
1·7062
1·6967
1·6924

Wells and Ingle, 1970). It should be noted that the Triton X-100 treatment, normally included in preparations of nuclear material, was not used in these differential salt extractions. This hypothesis was supported by the presence of DNA of density 1.697 $g.cm.^{-3}$ which was characteristic of the density of chloroplast DNA (Wells and Ingle, 1970).

A fraction greatly enriched with plastids and mitochondria was obtained by centrifugation of the fruit homogenate at 10,000 xg for 40 min. after removal of the bulk of the nuclear material at 1,500 xg. The 10,000xg pellet contained DNAs of densities 1.6967 and 1.7062 $g.cm.^{-3}$, the denser DNA accounting for 33% of the DNA from the pellet (fig.26). These densities were taken as the values of chloroplast and mitochondrial DNAs respectively and were seen to be identical to the "shoulder" and "satellite" DNAs in the NaCl-insoluble fraction in figs.24 and 25. Microscopic analysis of the 1,500 xg pellet from a Spanish Winter melon (melon j) showed that all the nuclei were ruptured and suggested that their DNA had been largely solubilized by the NaCl solution within 20 min., whereas the unbroken mitochondria and plastids were unaffected and produced the post-NaCl pellet from which the DNA was prepared. The density of the DNA in the NaCl-insoluble fraction, 1.7065 $g.cm.^{-3}$, compared with 1.7068 $g.cm.^{-3}$ in the total-cellular DNA preparation, was taken to show the density difference between mitochondrial and true nuclear satellite DNAs in this fruit.

This conclusion was supported by further data from other melons and some marrow fruits (Table 11).

Table 11

Distribution and buoyant-densities of satellite DNAs from fruits of Cucurbitaceae.

Fruit		Fraction	Buoyant-density ($g.cm.^{-3}$)	% of total
Melon				
Spanish Winter	e	Total	1.7080	18.2
		NaCl-insoluble	1.6978) 1.7076)	40.4
	f	Total	1.7068	22.0
		NaCl-soluble	1.7068	23.6
		NaCl-insoluble	1.6968	24.8
			1.7065	58.9
Marrow				
Vegetable Spaghetti	a	Total	1.7056	18.6
		NaCl-soluble	1.7062	16.1
		NaCl-insoluble	1.7050	31.4
	b	Total	1.7064	16.6
		NaCl-soluble	1.7064	13.8
		NaCl-insoluble	1.7054	24.9

Total DNA was prepared as usual. The NaCl procedures were performed on a crude chromatin preparation obtained by homogenizing fruit material in ice-cold Honda medium, filtering through nylon gauze (pore-diameter 25μm) and centrifuging the filtrate at 1,500 xg for 10 min. 2.5M NaCl was added to the pellet followed by centrifugation at 10,000 xg for 10 min. The supernatant was the NaCl-soluble fraction and the pellet the NaCl-insoluble fraction.

FIGURE 27

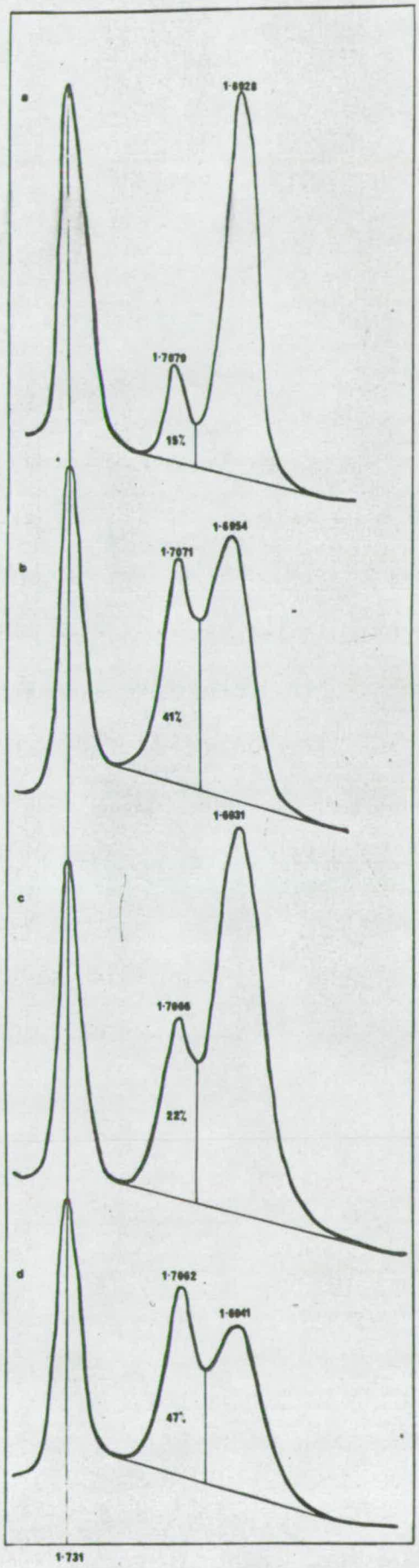
Effect of Triton on crude nuclear preparations of melon fruit. A 10,000xg pellet was prepared as described in fig.26. DNA was prepared from part of this whilst the remainder was treated with Triton X-100 at room temperature. The suspension was subsequently centrifuged and DNA extracted from both the supernatant (Triton-soluble fraction) and from the pellet (Triton-insoluble fraction).

a, Total cellular DNA of Spanish Winter melon g.

b, DNA prepared from the 10,000xg pellet.

c, DNA prepared from the Triton-insoluble fraction.

d, DNA prepared from the Triton-soluble fraction.


a
1·6928
1·7079
15%
b
1·6954
1·7071
41%
1·6931
c
1·7066
22%
d
1·7062
1·6941
47%
1·731

The NaCl-insoluble fraction always showed enrichment by mitochondrial and plastid DNAs. The NaCl-soluble fraction, however, was typical of a total-cellular DNA preparation. This was taken to show that nuclei were ruptured during preparation, that NaCl solubilized the DNA from these nuclei, and that plastids and mitochondria were not ruptured during preparation and consequently did not have their DNA solubilized by NaCl.

Addition of Triton X-100 to a suspension of the 1,500xg or 10,000xg pellet should solubilize the mitochondria and plastids, so that only nuclear material would be recovered by centrifugation at 10,000xg for 10 min. The 10,000xg pellet from Spanish Winter melon (melon g) contained 41% DNA of density 1.707 $g.cm.^{-3}$ (mainly mitochondrial), but when this pellet was treated with Triton, the subsequent DNA preparation contained less DNA of this density (22%) and was more typical of total-cellular DNA (15% satellite, density = 1.708 $g.cm.^{-3}$). The Triton-supernatant fraction contained 47% of DNA of density 1.7062 $g.cm.^{-3}$ from the solubilized mitochondria (fig.27).

The 1.7062 and 1.6967 $g.cm.^{-3}$ peaks were consistently decreased in DNA prepared from Triton-treated pellets. However, in practice, it was rare that the degree of solubilization attained 100% - presumably owing to clumping of organelles in the pellet. Furthermore, even if 100% solubilization had been attained, the nuclear pellet DNA might be expected to contain a higher percentage of sat.DNA, since in DNA from a total-cellular preparation, chloroplast DNA was masked by the mainband DNA and calculations to obtain the

FIGURE 28

Effect of NaCl extraction on Triton-treated nuclear material. A crude nuclear preparation (1,500xg pellet) was made from fruit of Honeydew melon e. Half of the material was treated with Triton X-100 and the Triton-insoluble fraction obtained by centrifugation. Both the control and Triton-treated pellets were resuspended in 2.5M NaCl pH6.0 for 20 min. at room temperature. The NaCl-insoluble material was pelleted by centrifugation at 10,000xg for 30 min. at 0°C and DNA was prepared from the pellets. a, NaCl-insoluble nuclear material which was not treated with Triton; b, NaCl-insoluble fraction of the Triton-insoluble pellet.

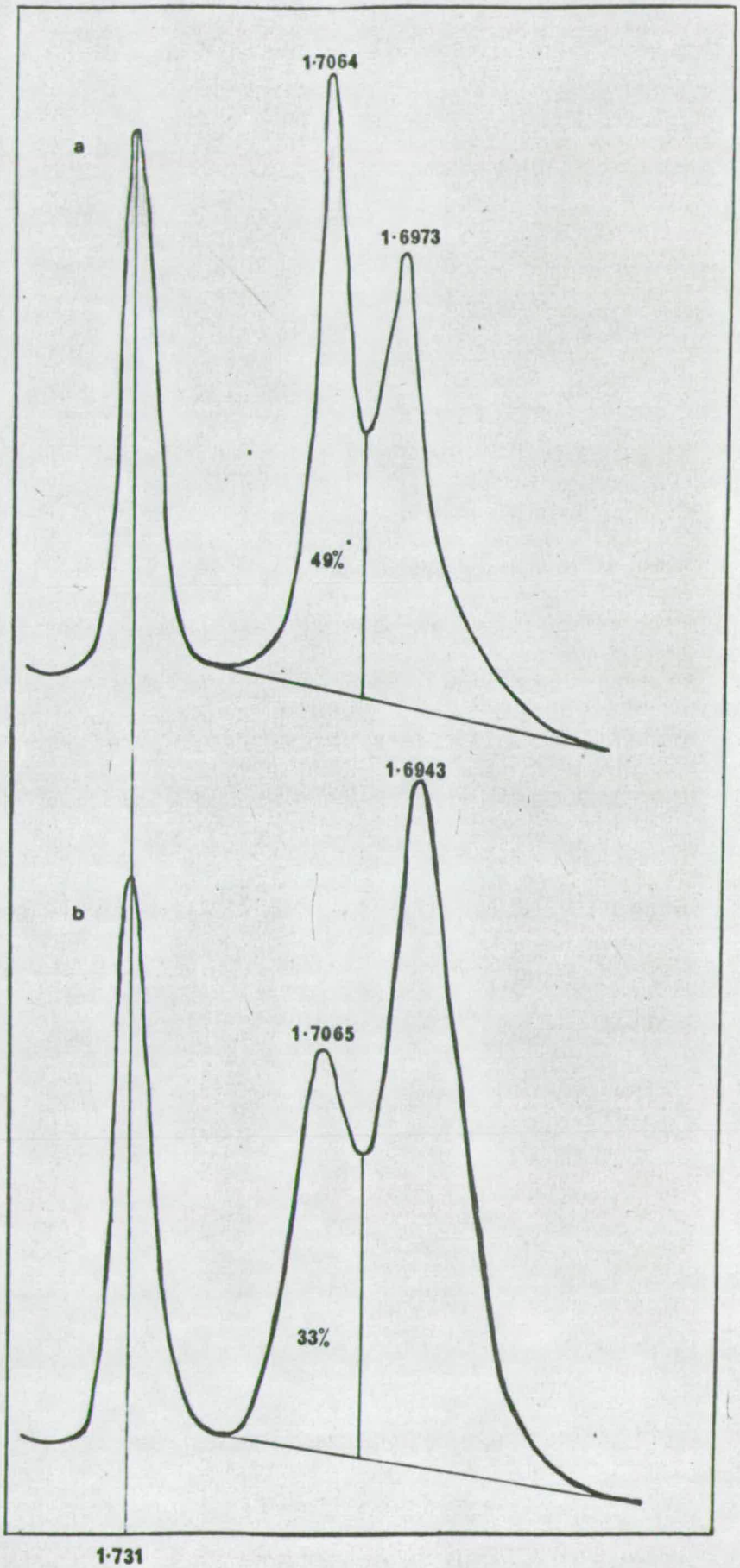

1·7064
a
1·6973
49%
1·6943
b
1·7065
33%
1·731

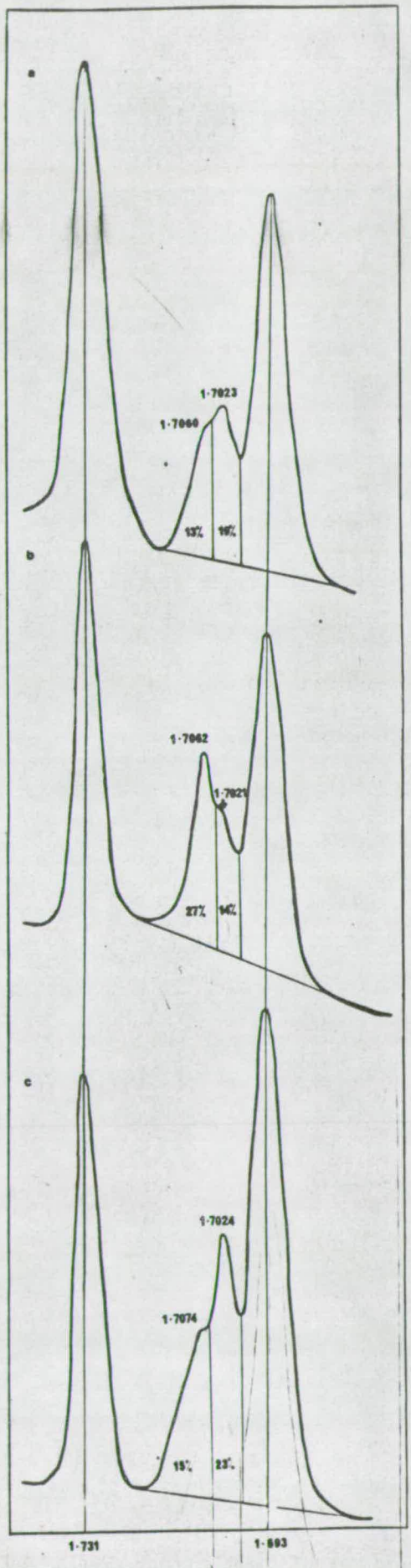
FIGURE 29

Effect of NaCl extraction on preparations of cucumber fruit. A crude nuclear preparation (1,500xg pellet) was resuspended in 2.5M NaCl pH8.4 for 20 min. at room temperature. DNA was prepared from both the NaCl-soluble and insoluble fractions, separated by centrifugation at 10,000xg for 30 min. at $0^{\circ}C$.

a, Total cellular fruit DNA.

b, NaCl-insoluble fraction.

c, NaCl-soluble fraction.


a
1·7023
1·7060
13%
19%
b
1·7062
1·7021
27%
14%
c
1·7024
1·7074
15%
23%
1·731
1·693

sat.DNA percentage would obtain a value which was an underestimate of the true value. For example, following Triton-treatment, the NaCl-insoluble pellet of a Honeydew melon (melon e) showed only a moderate decrease in sat.DNA percentage (33% satellite) compared with the non-Triton-treated pellet (49% satellite) (fig.28).

The enrichment of mitochondrial rather than satellite DNA by the salt treatment should be more obvious in tissues where the sat.DNA density differed significantly from that of the mitochondrial DNA. Cucumber contained 2 sat.DNAs (1.702 and 1.707 $g.cm.^{-3}$). Treatment of crude nuclear fractions (1,500xg) with 2.5M NaCl, pH8.4 for 20 min. resulted in an apparent relative increase in the denser sat.DNA (1.706 $g.cm.^{-3}$, mitochondrial or satellite 2) compared with the lighter satellite (1.702 $g.cm.^{-3}$, satellite 1). As there had been no Triton-treatment, this greatly supported the suggestion that the apparent enrichment of sat.DNA was caused by mitochondrial DNA, since in the event of true enrichment of satellite, one might have expected both sat.DNAs to have been enriched (fig.29).

The sat.DNA in Sweet orange (Citrus sinensis) at 1.712 $g.cm.^{-3}$ was quite distinct from mitochondrial DNA. The "crude nuclear" pellet (2,500xg) from leaves was treated with 2.5M NaCl, pH6.0 for 20 min. DNA prepared from the NaCl-insoluble material contained 2 peaks only: one at 1.6952 $g.cm.^{-3}$ - significantly different from that of nuclear mainband DNA (1.6941 $g.cm.^{-3}$) - which was probably chloroplast DNA, and the other peak at 1.7068 $g.cm.^{-3}$ - vastly different from that of the sat.DNA (1.7116 $g.cm.^{-3}$) - which was mitochondrial DNA (Wells and

FIGURE 30

Effect of NaCl extraction on nuclear preparations of orange leaf.

A crude nuclear preparation (2,500xg pellet) made from leaves of sweet orange was resuspended in 2.5M NaCl pH6.0 at room temperature for 20 min. DNA was prepared from both the NaCl-soluble and insoluble fractions, separated by centrifugation at 10,000xg for 10 min. at 0°C.

a, Total cellular leaf DNA.

b, NaCl-insoluble fraction.

c, NaCl-soluble fraction.

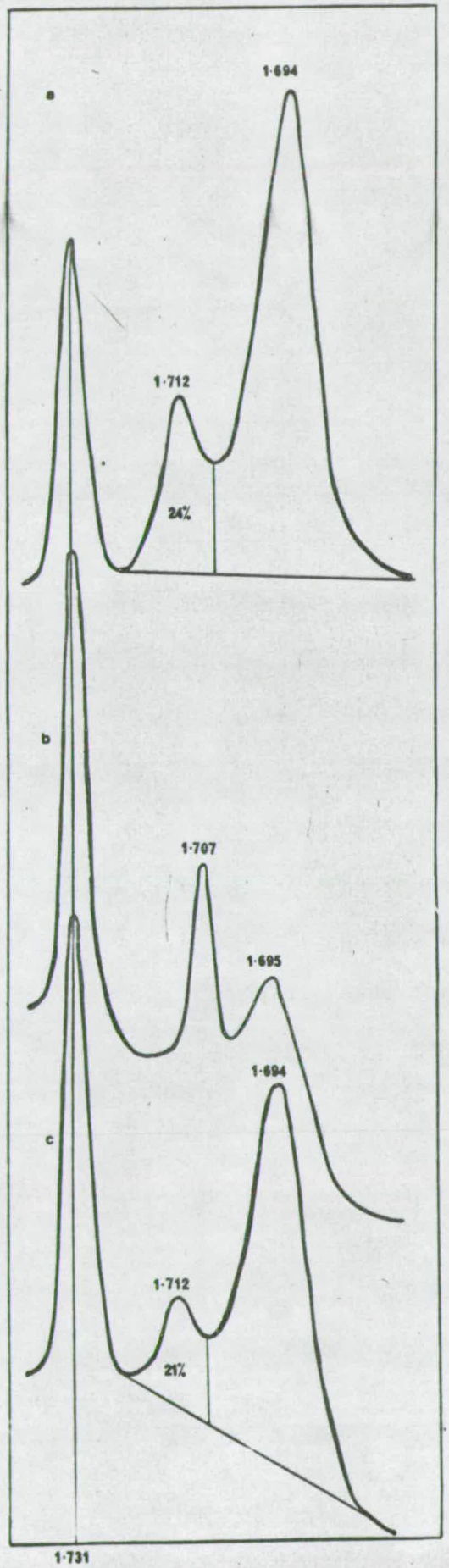

a
1·694
1·712
24%
b
1·707
1·695
c
1·694
1·712
21%
1·731

PLATE 1

Chromosomes of *Tradescantia virginiana*.

Anthers were squashed in lacto-propionic orcein and photographed.

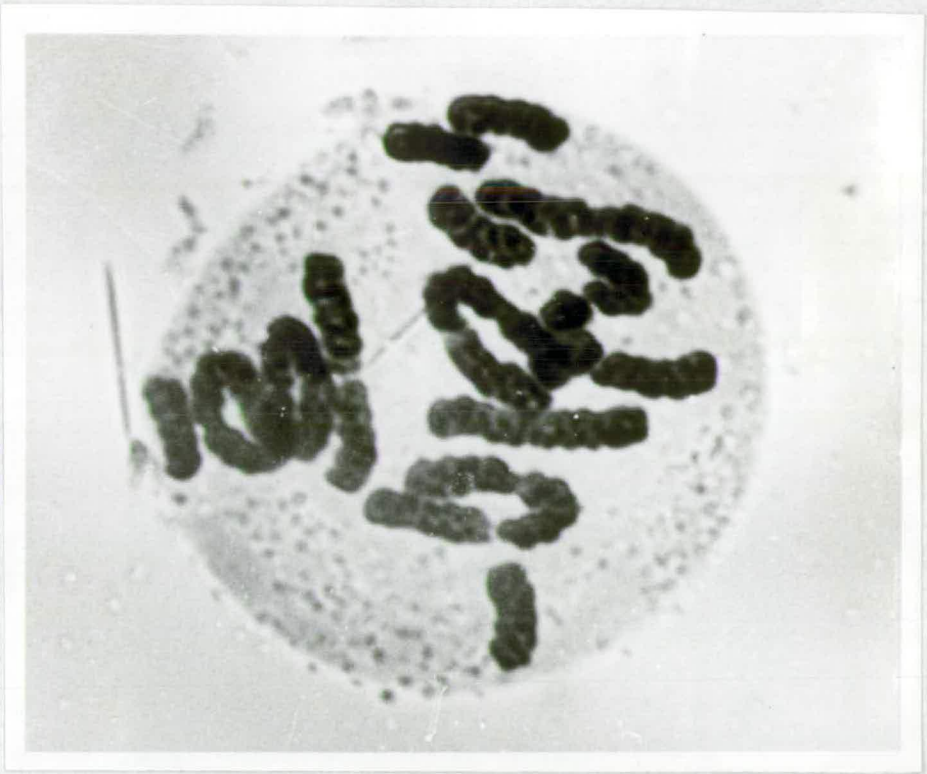
Tetraploid nucleus (24 chromosomes).

PLATE 2

Chromosomes of *T. virginiana*.

Anthers were squashed in lacto-propionic orcein and photographed.

Tetraploid nucleus + 3 B-chromosomes.





Ingle, 1970) (fig.30b). The NaCl-soluble fraction was similar in DNA composition to the total-cellular DNA (fig.30c). This was presumably because all the nuclei were ruptured during isolation, whereas a large number of mitochondria and chloroplasts were still intact.

Consequently, crude nuclear preparations from plant material contained very few intact nuclei. Treatment with salt did not appear to differentially solubilize the euchromatin with subsequent enrichment of heterochromatin and sat.DNA. The buoyant-density situation was confused by the stability of cell organelles which were present in nuclear preparations which had not been treated by Triton X-100.

B. Correlation of satellite DNA with the presence of B-chromosomes.

The presence of sat.DNA in populations of the grasshopper Myrmeleotettix maculatus was correlated with the presence of B-chromosomes (Gibson and Hewitt, 1970). Analyses were, therefore, made of plant species containing B-chromosomes. Clones of Tradescantia virginiana, containing either the normal tetraploid chromosome complement or tetraploid plus 3 B-chromosomes, were obtained from the garden (Plates 1 and 2). These B-chromosomes were euchromatic. DNA prepared from these 2 clones was identical in density (1.695 $g.cm.^{-3}$) and there was no evidence of a sat.DNA in either case. Since the B-chromosomes in T. virginiana were euchromatic, analyses of rye (B-chromosomes partially heterochromatic) and Puschkinia (B-chromosomes highly heterochromatic) DNAs were made but in neither case was there any sat.DNA.

It was concluded that in the plant species examined, there

FIGURE 31

Density of rRNA genes in cucumber DNA.

DNA prepared from cucumber var. Kariha fruit was fractionated on a preparative CsCl gradient in the MSE 10x10 angle rotor at 38,000 r.p.m., 25°C for 66 hr. The gradient was then dripped from the tube and the optical density at 260nm. recorded for each fraction (continuous line). The DNA in each fraction was alkaline denatured, neutralized, and fixed to a Millipore membrane (Gillespie et al., 1965). The membranes were incubated with ^{3}H-rRNA in 6xSSC at 70°C for 2 hr. and then washed once with 6xSSC, 3 times with 2xSSC, incubated with 10µg.cm.$^{-3}$ ribonuclease at 25°C for 15 min., dried, and counted (histogram).

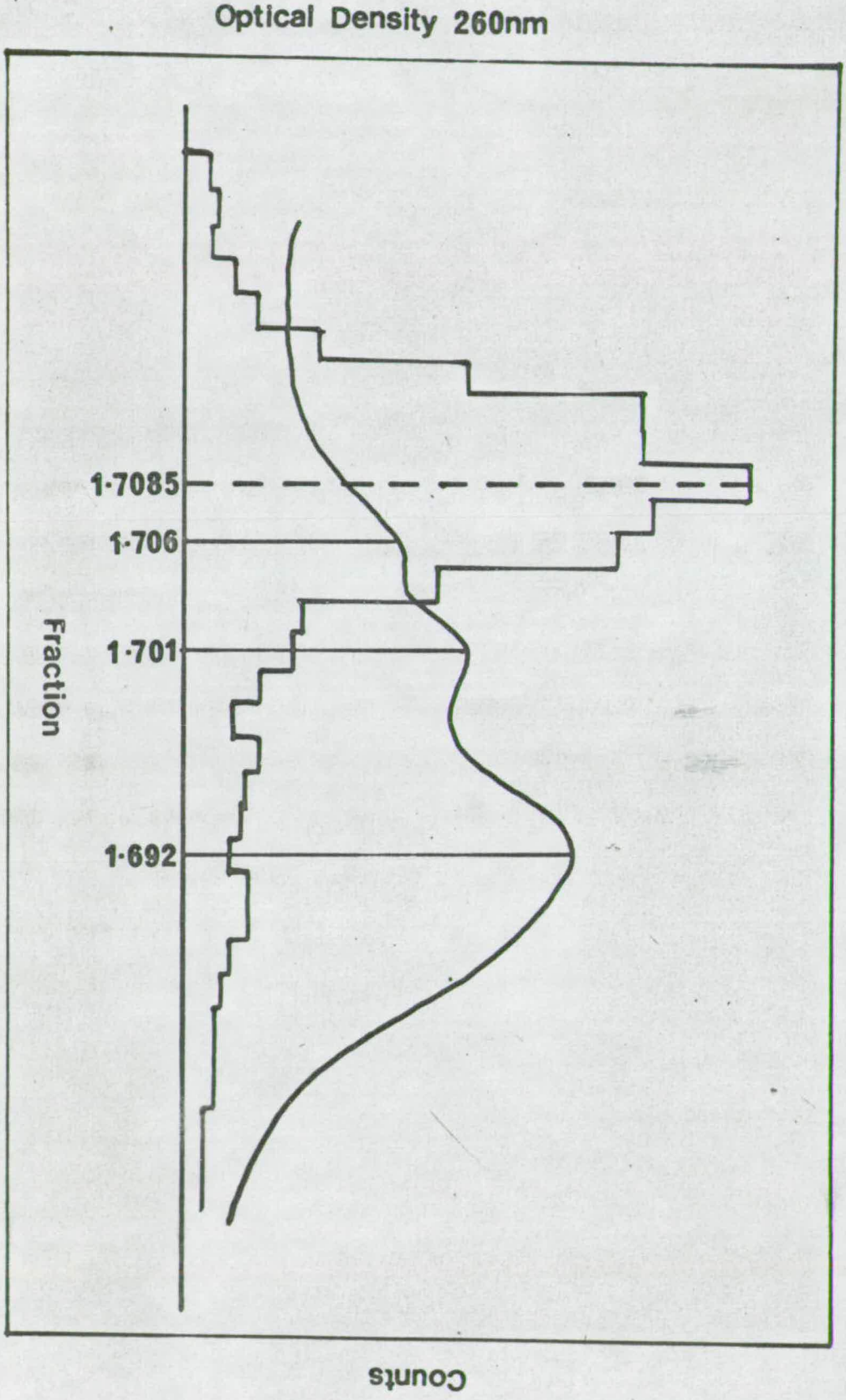

Optical Density 260nm
1·7085
1·706
1·701
1·692
Fraction
Counts

FIGURE 32

Density of rRNA genes in marrow fruit.

DNA prepared from marrow fruit was fractionated and hybridized with rRNA as described in fig.31.

OD_{260} (continuous line); counts (histogram).

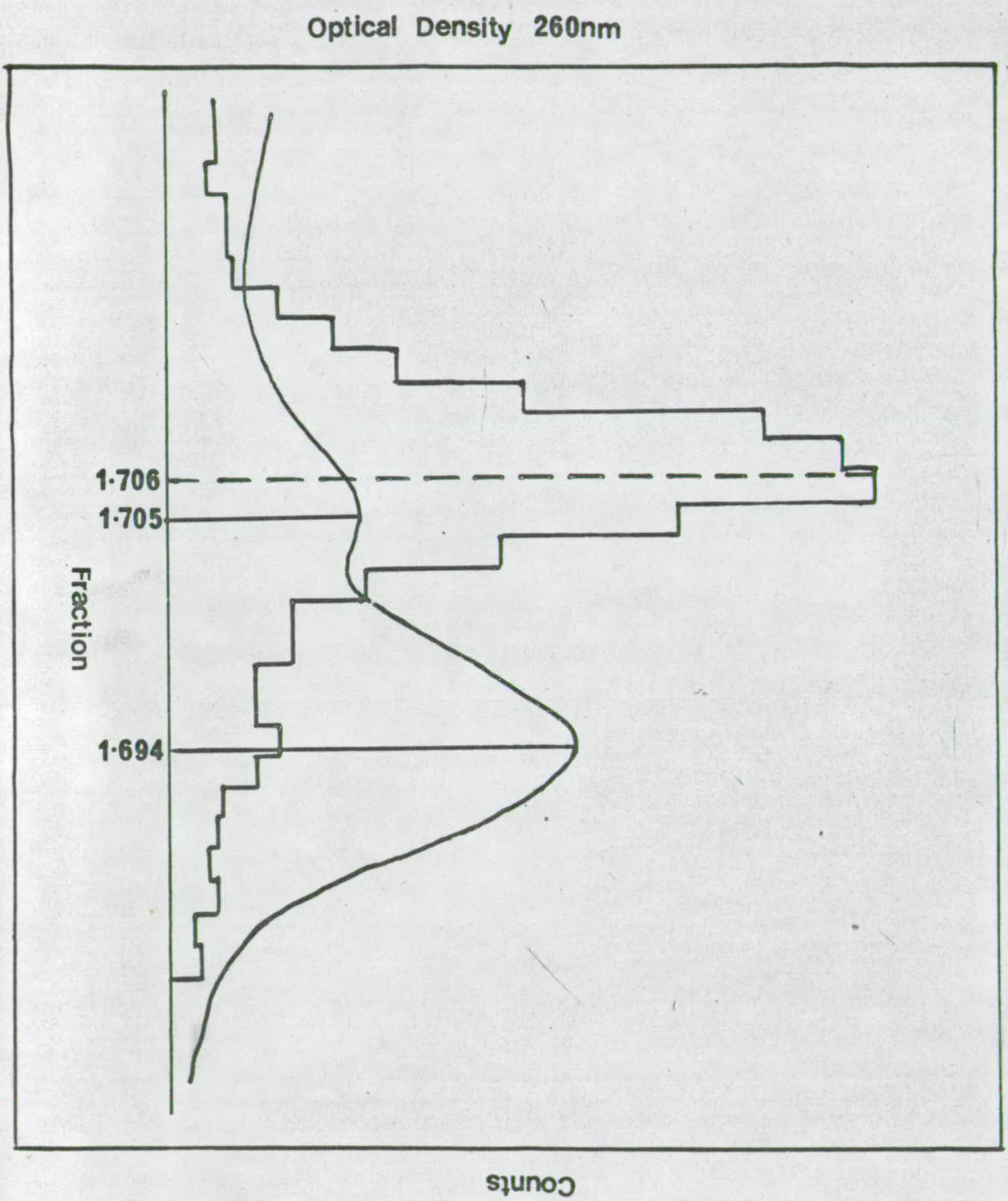
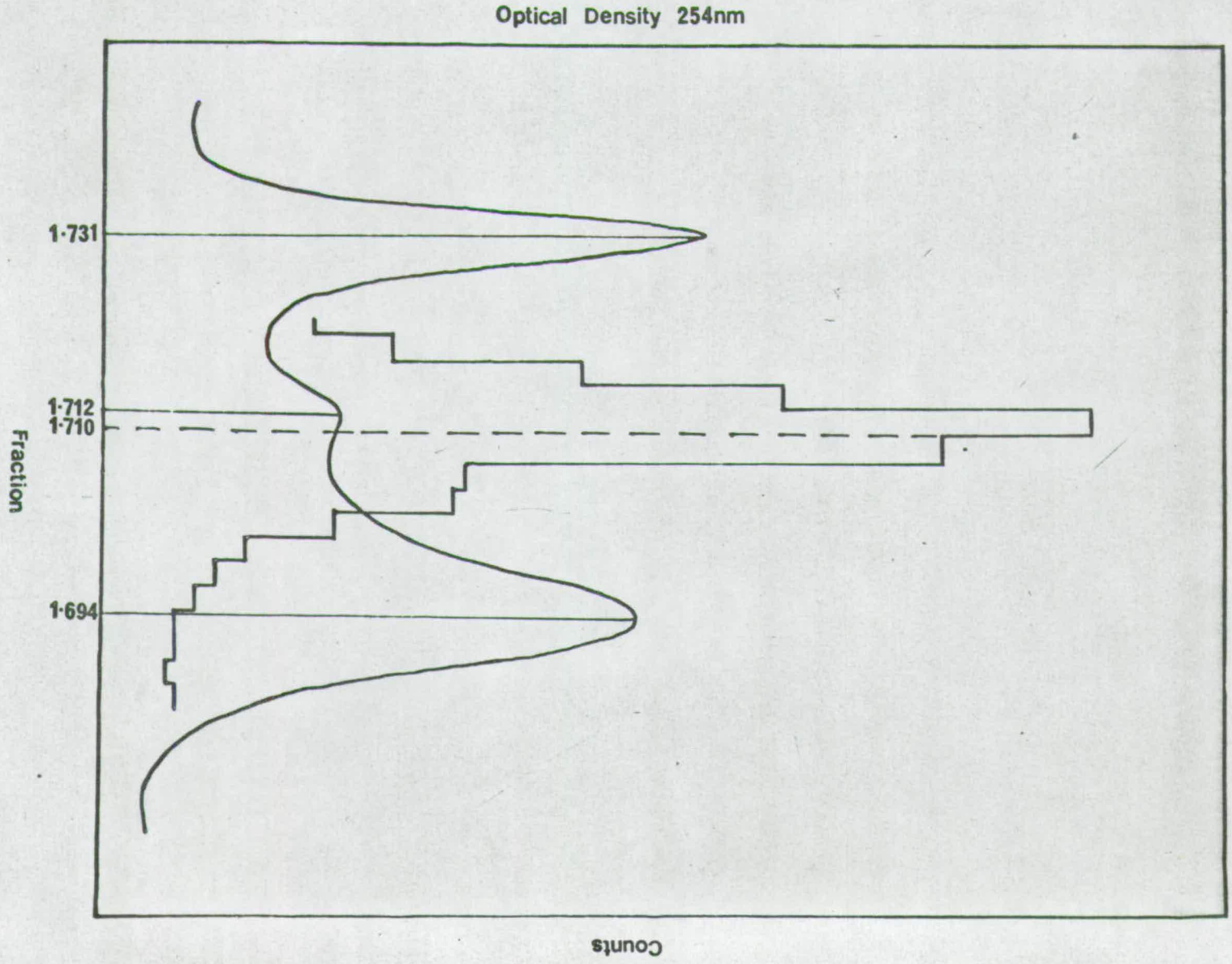

Optical Density 260nm
1·706
1·705
1·694
Fraction
Counts

FIGURE 33

Density of rRNA genes in orange leaves.

DNA prepared from the young leaves of sweet orange was fractionated and hybridized with rRNA as described in fig.31, except that the gradient was pumped from the tube and the optical density at 254nm. continuously recorded.

Continuous line (OD_{254}); Histogram (counts).


Optical Density 254nm
1·731
1·712
1·710
1·694
Fraction
Counts

was no correlation between B-chromosomes and sat.DNA.

C. Correlation of satellite DNA with rRNA genes

The higher hybridization with rRNA of sat.DNA from preparative CsCl gradients (compared with mainband DNA) and the higher percentage hybridization in plants containing satellite led Matsuda and Siegel (1967) to propose that sat.DNA was the same as ribosomal DNA (rDNA - DNA containing the genes for ribosomal RNA). This conclusion would be consistent with the observation that sat.DNA renatured rapidly. Quétier, Guillé and Vedel (1968) proposed that the stress-sat.DNA, also, represented a massive amplification of the rRNA genes. The relationship between sat.DNAs and rRNA genes was, therefore, studied.

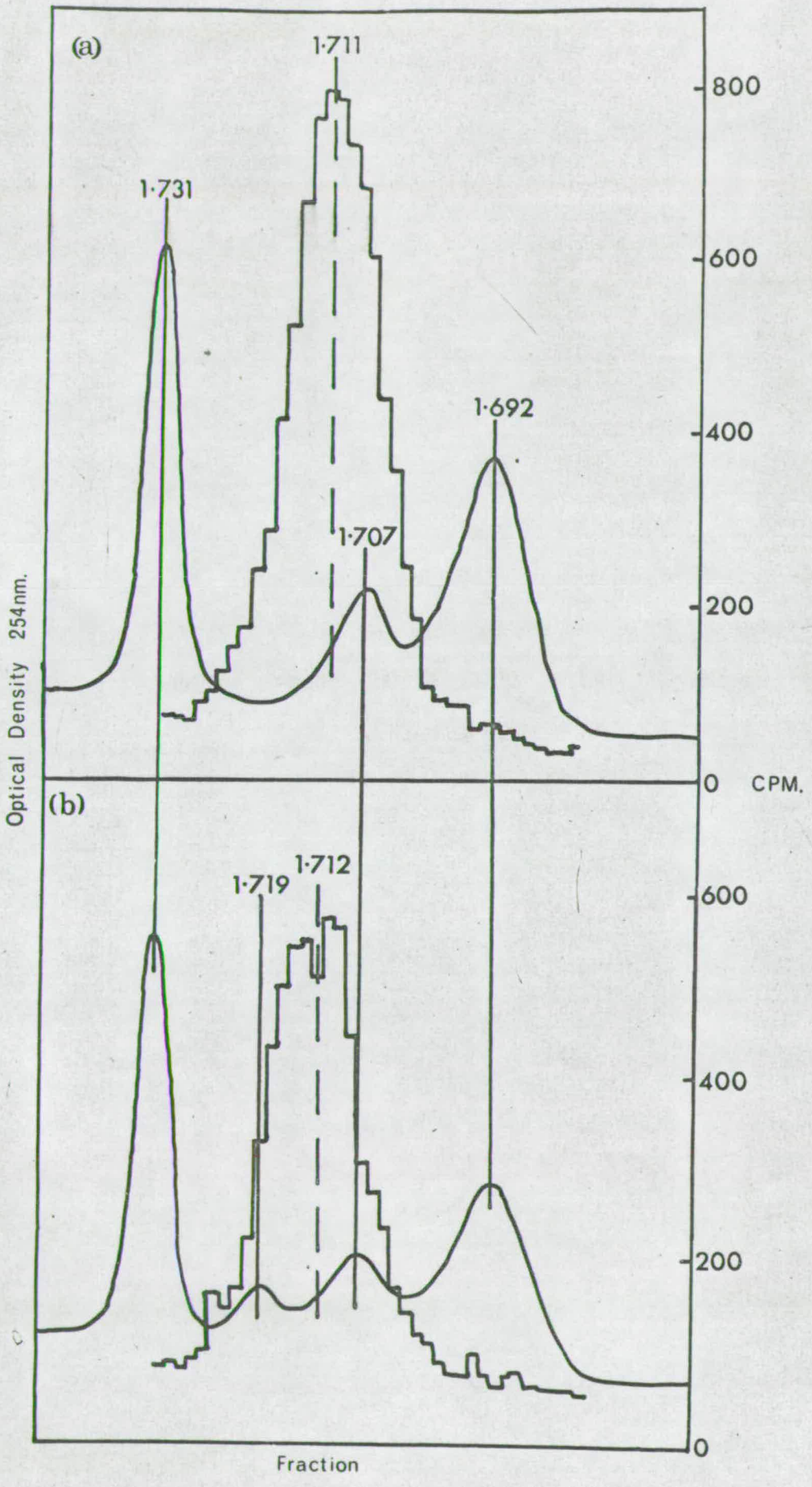
DNA was fractionated on a preparative CsCl gradient. The DNA in each fraction was denatured and fixed to a filter. rRNA was then hybridized to the DNA on the filters. Since it had been shown that hybridization was not dependent on the source of the rRNA (Matsuda and Siegel, 1967), ^{3}H-rRNA (Molecular weight 1.3×10^{6} daltons) from artichoke was used. The rRNA hybridized to cucumber DNA with a peak at a density of 1.708 - 1.709 g.cm.$^{-3}$ - distinct from that of both of the satellites (1.701 and 1.706 g.cm.$^{-3}$, fig.31). The hybridization peak with marrow DNA was at 1.706 g.cm.$^{-3}$ compared with the sat.DNA at 1.705 g.cm.$^{-3}$ (fig.32). The density of the hybridization peak in Sweet orange (1.710 g.cm.$^{-3}$) was slightly less than that of the sat.DNA (1.712 g.cm.$^{-3}$) (fig.33).

rRNA was hybridized to DNA prepared from melon seeds, both fresh and cold-stored (the latter containing a stress-sat.DNA).

FIGURE 34

Density of rRNA genes in melon DNA.

DNA prepared from Honeydew melon seeds was fractionated and hybridized with rRNA as described in fig.33. a, DNA from fresh seeds; b, DNA from seeds after 14 days cold-storage. Continuous line (OD_{254}); Histogram (counts).


(a)
1·711
1·731
1·692
1·707
800
600
400
200
0
CPM.
(b)
1·719
1·712
600
400
200
0
Optical Density 254nm.
Fraction

The peak of hybridization was at 1.711 - 1.712 $g.cm.^{-3}$ in both cases, i.e. denser than the normal sat.DNA (1.707 $g.cm.^{-3}$) but less-dense than the stress-sat.DNA (1.719 $g.cm.^{-3}$) (fig.34). These data, together with the fact that rRNA had a peak of hybridization at densities of 1.706 - 1.711 $g.cm.^{-3}$ with DNA containing no sat.DNA (Scott and Ingle, 1973) indicated that neither normal sat.DNA nor stress-sat.DNA (bacterial DNA) corresponded to the genes for rRNA.

Table 12

DNA content and percentage hybridization with rRNA

Plant	% DNA hybridized	DNA pg./2C nucleus	No. of copies of rRNA gene/2C nucleus
Wheat (Triticum aestivum)	0.092*	30	12,700
Jerusalem artichoke (Helianthus tuberosus)	0.022*	22.5	1,580
Swisschard (Beta vulgaris)	0.20 *	2.5	2,300
Pea (Pisum sativum)	0.17 *	10.0	7,800
Onion (Allium cepa)	0.09 *	31.6	13,300
Marrow var. Courgette (Cucurbita pepo)	0.6 +	2.6	7,200
Cucumber var. Improved Telegraph (Cucumis sativus)	0.8 +	1.8	6,600
Cucumber var. Kariha (Cucumis sativus)	0.8 +	1.9	7,000
Melon var. Hero of Lockinge (Cucumis melo)	0.18 +	2.0	1,700
Orange (Citrus sinensis)	0.27 +	1.8	2,200

* Ingle and Sinclair, Nature (1972) 235, 30-32.

\+ Plants containing a satellite DNA.

For hybridization, 1.3×10^6 daltons molecular weight rRNA was used. DNA prepared from the various plant species was hybridized with rRNA at 3 or 5$\mu g.cm.^{-3}$ for 2 hr. in 6xSSC at 70°C. The DNA of the nucleus was determined by comparative spectrophotometry on the basis that pea contained 10×10^{-12} g./2C nucleus.

The percentage of DNA which hybridized to rRNA varied from plant to plant (Table 12). Those plants containing sat.DNA certainly tended to have a higher percentage hybridization than those without a satellite. The percentage hybridization was not, however, a direct measure of the number of copies of the rRNA gene, since this depended on the DNA content per nucleus. From the percentage hybridization and the amount of DNA per nucleus, it was possible to calculate the no. of grams of DNA complementary to rRNA, i.e. rDNA. In the case of cucumber, this was 0.8% of 1.9×10^{-12}g. i.e. $\underline{1.5 \times 10^{-14}}$g. Avogadro's rule stated that 1.3×10^{6}g. (gram-molecular weight of the rRNA = gram-molecular weight of the rDNA) contained 6×10^{23} moles.

$\therefore 1.5 \times 10^{-14}$g. contained $\dfrac{6 \times 10^{23} \times 1.5 \times 10^{-14}}{1.3 \times 10^{6}}$ moles

= $\underline{7{,}000 \text{ moles, or copies of rRNA gene}}$.

These plants containing sat.DNA, and with a high percentage hybridization, did not necessarily contain a greater number of copies of the rRNA gene than did plants without a sat.DNA, e.g. onion and wheat each contained 13,000 copies whereas melon contained only 2,000 (Ingle and Sinclair, 1972).

From Table 12, with the exceptions of wheat, pea, onion and artichoke, all the other species contained approximately 2pg.DNA/2C nucleus. These species all contained sat.DNAs (except Swisschard) whereas those with higher amounts of DNA/2C nucleus had no sat.DNA. Swisschard (2.5pg.DNA/2C nucleus) had no sat.DNA although containing only as much DNA as marrow.

From these results, it was postulated that only plants with a small amount of DNA per nucleus had sat.DNAs, but not all plants with a small amount of DNA per nucleus contained satellite.

SECTION 4: DISCUSSION

The investigation into the distribution of sat.DNAs among plant species (Table 2) shows that no sat.DNAs are present in any of the monocotyledon species examined. All these species contain large amounts of DNA per nucleus (Table 12). No dicotyledon species examined with more than 2-3pg. DNA per 2C nucleus is found to contain a sat.DNA. It is postulated that a pre-requisite for a nucleus to possess a sat.DNA is that it should contain no more than this amount of DNA. Amongst the dicotyledons, sat.DNAs are present in orders ranging from the most primitive to the most advanced (Takhtajan, 1969). Furthermore, although one species in a genus might contain a sat.DNA, it does not necessarily follow that every species in that genus will have a satellite. An alternative classification of plants based on amino-acid substitution in cytochrome c was proposed by Boulter, Ramshaw, Thompson, Richardson and Brown (1972). Although only a few species were examined, the phylogenetic tree proposed for these species correlates better with the distribution of sat.DNAs than does Takhtajan's classification. The more-highly evolved families at the top of the tree - Cucurbitaceae, Cruciferae, and Leguminosae - all contain sat.DNAs in at least some of their species, whereas the families lower down the tree, with the exception of the Solanaceae, do not contain sat.DNAs in any of the species examined. However, the fact that the Solanaceae do not fit into this pattern is not a serious discrepancy. It would be unwise to assume that sat.DNAs have arisen only once in evolution and that all species containing sat.DNAs have arisen after this event. Sutton and McCallum (1971) suggested that even in species as closely related as mouse and guinea-pig, the "ancestral sequence" of the sat.DNA might be quite distinct. This suggests that sat.DNA

appeared after these species diverged. It would, therefore, appear to be injudicious to suggest that all plants with a sat.DNA have arisen from the same initial species. Certainly, Takhtajan believes that the Geraniales (including the Linaceae) have arisen directly from the Rutales (including the Rutaceae). The investigations described herein show that those members of the Rutaceae containing a sat.DNA (Citrus and Fortunella genera) have a sat.DNA of density 1.712 $g.cm.^{-3}$, whereas the sole member of the Linaceae investigated (i.e. flax) has a sat.DNA of density 1.689 $g.cm.^{-3}$ These two sat.DNAs are respectively the densest and the least-dense found. It is, indeed, difficult to envisage how these could have arisen from the same ancestral sequence. Experiments to indicate homology between sat.DNAs from different species, such as DNA/DNA reassociation or in situ hybridization with transcribed sat.DNA, were not undertaken, and so it is not possible to state how closely related are the sat.DNAs at the top of the phylogenetic tree of Boulter et al. Their densities range from 1.702 - 1.707 $g.cm.^{-3}$ - a fairly wide range, but this does not necessarily mean that they could not have the same ancestral sequence. However, the Solanaceae, which also contain sat.DNAs in this density range, are well removed in the classification of Boulter et al. from the other sat.DNA-containing families mentioned above. If his positioning of the Solanaceae is correct, therefore, it suggests that the Solanaceae at least must have individually developed sat.DNAs. If this procedure is possible in one family, then presumably it is possible in others.

One factor that is evident from Table 2 is that only species which have been highly cultivated contain a large amount (>10% or so) of sat.DNA. No other plants possess large amounts of sat.DNA.

Beridze (1972) noted that in the genus *Phaseolus*, the species that had a 'New World' origin contained about 30% of sat.DNA, whereas those from the 'Old World' had 15% or less of sat.DNA. He proposed that the age of cultivation of these various species might be an influencing factor. This does not appear to be so, since Purseglove (1968) concluded that all these 6 species have been cultivated for at least 3 millennia, and some for as many as 10 millennia. Furthermore, pea, which has been cultivated since the Greek era at least, does not contain a sat.DNA. There could, however, be some significance in Beridze's theory, since Purseglove stated that the *Cucurbitaceae* and the *Citrus* and *Fortunella* genera in the *Rutaceae* have been cultivated for many millennia. The *Solanaceae*, however, appear to have been cultivated for less than 1,000 years and these have less sat.DNA than the *Cucurbitaceae*, *Rutaceae*, or *Leguminosae* in general (Table 2), and the species, those of which contain a sat.DNA, have all been cultivated for much longer. Accepting that the history of cultivation may be changed in view of further archeological data, there does seem to be a correlation between the age of cultivation and the amount of sat.DNA. Whereas there are ancient-cultivated species with no sat.DNA, there does not appear to be any newly-cultivated species (such as *Skimmia japonica* in the *Rutaceae*) with vast amounts of sat.DNA, and certainly no uncultivated plant examined contains more than about 7% (e.g. *Bryonia dioica* in the *Cucurbitaceae*).

A possible modification of Beridze's theory is to substitute "breeding" for "cultivation". All the plants containing >10% satellite have been intensively bred for some characteristic (usually fruit quality). Artificial breeding can perform in a decade what would take a century or more of natural selection. Consequently,

"age of cultivation" may not be the most valid criterion in considering plants such as melon but rather the "intensity of breeding".

In the majority of plants examined which contain sat.DNA, the satellite buoyant-densities are greater than the mainband density (Table 2), and, of these, 54% have a sat.DNA of density = 1.705 - 1.707 $g.cm.^{-3}$ There does not appear to be any obvious reason for the high frequency of this type of satellite, especially as some species contain sat.DNA with densities as low as 1.689 $g.cm.^{-3}$ or as high as 1.712 $g.cm.^{-3}$ Furthermore, some plants contain 2 sat.DNAs e.g. cucumber (both denser than mainband) and Lobularia maritima (one denser and one less-dense than mainband). The question arises as to why these plants need 2 sat.DNAs. Clearly, the answers to these questions cannot be given until the function of sat.DNA is known. Walker (1971) suggested that the sat.DNA might confer an advantage on the individual chromosome possessing it and that this advantage might lie in its exerting some effect on the pairing of chromosomes at meiosis or in the attachment of the chromosomes to the mitotic spindle. If this kind of reason is correct, then the sat.DNA could have an important rôle in DNA-protein binding. It is reasonable to suppose that there is an optimum composition for the protein which is associated with sat.DNA function, and consequently one might expect this composition to be the most-frequently occurring. This could explain the predominance in the species examined of the 1.705 - 1.707 $g.cm.^{-3}$ satellite in Table 2. However, it must be borne in mind that examination of a wider range of species might show no concentration of sat.DNAs in this range.

The intra-cellular distribution of sat.DNA has been investigated and it has been shown to be nuclear. This has been demonstrated

directly, by isolating nuclei and extracting the DNA from them, and indirectly, by showing that the sat.DNA is not of mitochondrial, chloroplast, or bacterial origin.

The density of chloroplast DNA, which appears to be constant in a range of plant species at 1.697 $g.cm.^{-3}$ (Wells and Ingle, 1970), is very different from the majority of sat.DNAs. Although plastid DNA is not confused with sat.DNA buoyant-density, it is material in concealing the true percentage of satellite in DNA from a total-cellular preparation, since plastid DNA is present within the mainband peak. Consequently, the percentage of sat.DNA is calculated relative to nuclear plus plastid DNAs and is an underestimation proportional to the plastid DNA percentage (1 - 5%). DNA prepared from a crude nuclear pellet which has been treated with Triton X-100 to solubilize plastids and mitochondria gives a truer estimate of the sat.DNA percentage, and this is invariably greater than the estimate obtained from a total-cellular DNA preparation by a few percent.

Mitochondrial DNA, constant at 1.706 $g.cm.^{-3}$, also differs in density from certain sat.DNAs e.g. Citrus and flax. In species where the mitochondrial DNA and the sat.DNA have approximately the same density, then the sat.DNA is present in amounts too large to be accounted for by mitochondrial DNA, which is present as less than 1% of the total DNA.

Bacterial contamination of the plant material was estimated and the amount of DNA that could be contributed by the bacteria calculated. In vigorously growing plants, the bacterial contamination is of the order of 10^{-10} - 10^{-13}g. DNA per gram of material, compared with 10^{-6}g. sat.DNA, i.e. 10^{4} - 10^{7} times too small to be able to account for the sat.DNA being bacterial in origin. This is not, however, the

case for plant material placed under conditions of physiological stress, which result in the production of a stress-sat.DNA (Pearson and Ingle, 1972). This was shown to be bacterial DNA and will be discussed later.

The intranuclear distribution of sat.DNAs in animals has shown that they are associated with nucleoli (Schildkraut and Maio, 1968) and constitutive heterochromatin, in particular that in the centromere region of the chromosomes (Maio and Schildkraut, 1969; Jones, 1970; Jones and Corneo, 1971). The experiments of Maio and Schildkraut (1969) showed that there was an enrichment of sat.DNA in the NaCl-insoluble fraction of isolated mouse chromosomes, and Huguet and Jouanin (1972) reported a similar result when wheat nuclei were extracted with 2.5M NaCl. The results described in this thesis of similar experiments to those of Huguet and Jouanin show that the enrichment of "sat.DNA" is, in fact, an enrichment of mitochondrial DNA. It is probable that tissue homogenization results in a nuclear pellet containing damaged nuclei but intact chloroplasts and mitochondria. Subsequent NaCl treatment solubilizes the bulk of the chromatin, preferentially leaving DNA present in intact organelles. The supernatant containing the solubilized nuclear material gives a DNA analysis on Model E centrifugation which is identical to DNA from total-cellular or the initial nuclear material. The addition of Triton X-100 to the crude nuclear pellet before NaCl treatment should disrupt the chloroplast and mitochondrial membranes without any effect on nuclear membranes. NaCl-digestion of the Triton-treated pellet should, therefore, yield a pellet of pure nuclear material.

Experiments similar to those of Maio and Schildkraut (1969) using isolated chromosomes are not feasible using the plant species containing

sat.DNAs. This is because the chromosomes in the plants with sat.DNAs, such as cucumber, melon, and orange, are far too small (~1μm). This unfortunate limitation also means that *in situ* hybridization cannot be employed to localise the sat.DNA in the chromosomes, since the resolution of the silver grains on the autoradiograph is of the same order of magnitude as the size of the chromosomes.

Nevertheless, the observation that in all species containing a sat.DNA, the amount of DNA per nucleus is small (~2pg./2C nucleus) and the fact that the chromosomes themselves are very small might well be significant. No plant with a reasonably large amount of DNA per 2C nucleus or per chromosome has been found to contain a sat.DNA. It could, therefore, be that the small size of the chromosome favours sat.DNA, or *vice versa* that sat.DNA gives a selective advantage to chromosomes of small size. The possibility that the sat.DNA may be present as a fixed amount in all chromosomes (rather than a fixed percentage) and consequently only shows up in plants with small chromosomes appears to be ruled out by the observation that artichoke, containing 108 small chromosomes, contains no sat.DNA. The restriction of sat.DNA to small genomes may account for the absence of satellite from monocotyledons. All plant species examined have been shown to contain highly repetitious DNA (Britten and Kohne, 1967) similar in complexity to sat.DNA but not necessarily different in buoyant-density from the mainband (less-repetitious) DNA. Unless the densities of the two DNA classes are distinct they do not, of course, segregate on a neutral CsCl gradient.

The reports on animal sat.DNAs being associated with constitutive heterochromatin (Yasmineh and Yunis, 1970; Arrighi, Hsu, Saunders and Saunders, 1970) and with heterochromatic B-chromosomes in grasshoppers

(Gibson and Hewitt, 1970) instigated an investigation into the presence or absence of sat.DNAs in plant B-chromosomes. Tradescantia virginiana contains no sat.DNA in plants either with or without B-chromosomes (euchromatic B-chromosomes) and neither do rye or Puschkinia (heterochromatic B-chromosomes). Nevertheless, it must be borne in mind that these species are monocotyledons, none of which contain sat.DNA in any of the species examined, and, furthermore, that all these species contain a large amount of DNA per 2C nucleus and that no such nucleus has ever been shown to contain a sat.DNA.

In animals, human mainband DNA has been shown to be heterogeneous with regard to buoyant-density on a CsCl gradient (Corneo, Bianchi, Ginelli and Polli, 1966). The sat.DNAs in human tissues, however, are found to be homogeneous, always displaying the same density when different fractions of the peak are subsequently analysed (Corneo, Ginelli and Polli, 1968; 1970b; 1971b).

Of the plants examined, marrow leaf DNA is similar to the animal satellites in that the sat.DNA is very homogeneous (1.706 $g.cm.^{-3}$) when taken from fractions displaying original densities ranging from 1.702 - 1.714 $g.cm.^{-3}$ (Table 3) whereas the mainband shows heterogeneity from 1.692 - 1.697 $g.cm.^{-3}$ Thus in marrow, the sat.DNA is very homogeneous and the mainband slightly heterogeneous, but nowhere near as heterogeneous as human mainband DNA which ranges from 1.690 - 1.720 $g.cm.^{-3}$ (Corneo, Ginelli and Polli, 1970b).

In cucumber (var. Kariha) fruit and leaf DNAs, the situation is more complex. The present work finds that the mainband DNA is very similar to the mainband DNA in marrow, showing components from 1.688 - 1.694 $g.cm.^{-3}$ The sat.DNA re-runs to buoyant-densities close to the density of the fraction from which they are taken, rather than

re-running simply to the peak densities of the two satellites. No DNA re-runs to within the range 1.695 - 1.699 $g.cm.^{-3}$ Subsequent more detailed analysis of cucumber DNA showed greater homogeneity of the two satellite components (Ingle, Pearson and Sinclair, 1973). This difference may be explained by the different molecular weights of the DNA preparations. If the initial preparation had been more sheared, this could result in more apparent heterogeneity. The evidence therefore indicates that the sat.DNAs in cucumber and marrow are homogeneous, at least when of large molecular weight, whereas the mainband is slightly heterogeneous.

Perhaps the most striking property of animal sat.DNAs is the rapid rate at which the denatured, separated strands reassociate. Only collisions between complementary strands result in renaturation, and therefore the concentration of each sequence establishes the rate, and for fixed amounts of DNA, this concentration is determined by the number of different sequences present. The renaturation of sat.DNA from orange was studied. The increase in buoyant-density from 1.712 - 1.722 $g.cm.^{-3}$ on denaturation indicates that the satellite is double-stranded. Upon being subjected to renaturing conditions, the DNA is completely renatured within 5 min. at a concentration of $3\mu g.cm.^{-3}$, which shows the orange DNA to have a complexity of less than $4x10^6$ daltons compared with a complexity of $140x10^6$ daltons for T_4 DNA. This sequence is, therefore, reiterated about $5x10^4$ times in the orange genome. These values are very similar to those obtained for melon sat.DNA (Ingle, Pearson and Sinclair, 1973) where there is a unit of $2.5x10^6$ daltons, reiterated about 10^5 times. These values indicate that the repeated sequence in these plant sat.DNAs is about 100x shorter than the T_4 phage genome and about 1,000x shorter than the E. Coli genome.

Mouse sat.DNA contains ca.1.5x10^6 copies of a sequence 2x10^5 daltons in length from renaturation data (Waring and Britten, 1966). However, the T_m of renatured sat.DNA is 5°C lower than the T_m of native sat.DNA. The difference in T_m is a measure of the degree of mismatching in the duplex, and in mouse this corresponds to about 8%, presumably reflecting an average divergence of about 4% from the common ancestor of the sequences. Using this data, Sutton and McCallum (1971) showed that the ancestral sequence complexity could be as small as 8x10^4 daltons. In melon and orange, there is less than 1% mismatching, showing that there has been little divergence from the ancestral sequence in the plant satellites. The plant sat.DNAs, therefore, appear to be rather more complex than mouse satellite, and the ancestral sequence would appear to be about the same as the present sequence - $\sim$ 2x10^6 daltons. Southern (1970) postulated, however, that the sat.DNAs in mouse and guinea-pig might have been reiterated from sequences as short as 6 - 13 base-pairs long and that errors or mutations had occurred during this replication. Consequently, the apparent sequence length is now much greater than it originally was. To produce the guinea-pig α-sat.DNA by the saltatory process would require 10^7 copies of the basic sequence to be provided at one time. More than 10^6 mutations would then be introduced by subsequent divergence. In this case, non-random frequencies of the mutations might be taken to indicate that selection had favoured some mutations over others. An alternative mechanism for development of sat.DNA would be to increase the sequence slowly by unequal crossing over. In this case, too, the distribution of mutations should be random in the absence of selection.

If, however, copies of the sequence were produced by a multiplication process and the periods between multiplication steps

were long enough to allow mutations to be introduced, the amount of any mutation in the final sequence would be determined by the point in the multiplication process at which it occurred: a mutation occuring early, when there were only a few copies of the sequence, would be multiplied many times and be present at a higher frequency in the final sequence than mutations occurring late, which would be multiplied less frequently. It can be shown that the number of mutations in the sequence is compatible with accepted values for the mutation rate and the age of the sat.DNA. Assuming that the mutation rate is the same for the sat.DNA as for the rest of the genome, and ignoring effects of selection, it is evident that a repeated sequence produced by saltation or by unequal crossing over will receive the same fraction of mutations as the rest of the DNA over the same period of time. This would also be true for a repeated sequence produced by slow multiplication. The mutation rate has been calculated as around 4.2×10^{-9} changes per base-pair per year. At this rate, ca.50×10^{6} years would be needed to change 20% of the bases in sat.DNA (Southern, 1970) and 20% would have had to be altered to give the present sequence in guinea-pig α-sat.DNA from the proposed 6 base-pair sequence. Conventional zoological data suggests that the divergence point of guinea-pig from the other rodents could have occurred as long ago as 50×10^{6} years (Simpson, 1959).

However, as the plants show much less mismatching than the animals in their sat.DNAs, it might be reasonable to assume that the plant sat.DNAs are phylogenetically younger than the animal sat.DNAs and that consequently they have not been replicated as many times, thereby lessening the number of opportunities for mutations to occur. If this were so, then it would mean that the plant sat.DNAs had an

ancestral sequence about the same length as their present sequence - $\sim 2 \times 10^6$ daltons - which is much greater than the proposed 6 - 13 base-pair ancestral sequence in the animals (10^3 - 10^4 daltons).

Although *in situ* hybridization experiments with the sat.DNA-containing plants have not been possible, it is possible to deduce that the repeated sequences must be clustered in, at least, 5 - 6 copies. This is indicated from experiments in which DNA was prepared as gently as possible, thereby maintaining as high a molecular weight as possible. The DNA was then sheared in consecutive steps, and at each stage, the molecular weight and the percentage of sat.DNA were determined. Decreasing the molecular weight from $> 10 \times 10^6$ daltons to ca.0.6×10^6 daltons had no significant effect upon the percentage of sat.DNA, or upon the buoyant-density of the DNA. The length of the repeating unit in melon sat.DNA is about 2×10^6 daltons. If such units occurred singly and were generally distributed throughout the genome, then, since the DNA separating repeated units from each other would be of lower %GC (mainband DNA), fragments of satellite-containing DNA of size $> 2 \times 10^6$ daltons would have a lower buoyant-density than sat.DNA fragments of size $< 2 \times 10^6$ daltons, which would contain pure sat.DNA. Since neither the density nor the percentage of satellite does alter significantly with decreasing molecular weight, it can be concluded that DNA fragments up to at least 10×10^6 daltons contain nothing but the satellite sequence. This shows that there must be at least 5 - 6 tandem copies of the sequence, and there are probably many more, as in animal sat.DNAs (Waring and Britten, 1966).

It has been suggested (Matsuda and Siegel, 1967) that the sat.DNA consists of multiple copies of the rDNA. This is known to be the situation in *Xenopus laevis* (Birnstiel, 1967; Brown and Dawid, 1968).

Matsuda and Siegel separated satellite from mainband DNA fractionated on a preparative CsCl gradient and hybridized each with rRNA. They found that the percentage hybridization was much higher with the sat.DNA fraction than with the mainband DNA fraction. In Xenopus, somatic tissues contain 0.3% rDNA whereas oöcytes contain 30% of this DNA. The situation is similar in Dytiscid water beetles (Gall, Macgregor and Kidston, 1969). In Dytiscus marginalis and Colymbetes fuscus, a high density sat.DNA is found in somatic cells and in sperm. Hybridization experiments show that all the rDNA is located in this satellite, although quantitatively making up only a small fraction of the satellite. In both species, the DNA isolated from ovariole tips is enriched with respect to the sat.DNA. A parallel enrichment of the rDNA has been shown in ovariole tips of Colymbetes. It was proposed that in somatic cells and sperm, the rDNA is part of an extensive region of high density DNA in one or more chromosomes, but that in oögonia and oöcytes, the entire high density region is replicated extrachromosomally and appears as Giardina's body.

The experiments described in this thesis show that the density of the rDNA may be similar, but not identical, to the density of the sat.DNA. In other tissues examined, the sat.DNA buoyant-density is very different from that of the rDNA, this difference being particularly marked in flax (Timmis and Ingle, 1973) with a sat.DNA at 1.689 $g.cm.^{-3}$ and rDNA at 1.709 $g.cm.^{-3}$ Furthermore, in a whole range of species containing no sat.DNA, the density of rDNA ranges from 1.703 to 1.712 $g.cm.^{-3}$ (Scott and Ingle, 1973). The density of rDNA in cucumber, melon and marrow is greater than the sat.DNA buoyant-density, but in orange, the rDNA is slightly less-dense than the sat.DNA. There is, therefore, no strict correlation in the plants examined

between the rDNA and the sat.DNA, any similarity between their buoyant-densities probably being fortuitous. Furthermore, Matsuda and Siegel (1967) showed that of the plants they examined, the percentage hybridization with rRNA was greater in those species containing a sat.DNA. The present work, together with that of Ingle and Sinclair (1972) shows that this is not necessarily so. Orange and melon DNAs, containing a sat.DNA, are very similar to the 0.2% hybridization of swisschard DNA, which does not have a satellite (Table 12). It is more valid to consider the number of copies of the rDNA sequence per telophase nucleus than to consider the percentage hybridization. Melon and orange, both with approximately 20% of sat.DNA, contain roughly the same number of copies of the rDNA sequence per telophase nucleus as swisschard. Cucumber and marrow, however, contain more copies, but not as many as the monocotyledons wheat and onion which contain no sat.DNAs. In fact, the highest percentage hybridization is not correlated with the largest number of genes. The largest number of genes is found in the low percentage hybridization species. The higher percentage hybridization in satellite-containing plants is probably a result of the small genomes of these plants.

Differential replication of DNA has been indicated during the development of a range of eucaryotic somatic tissues by a variety of experimental techniques. Non-doubling of the DNA during the development of polyploid cells was shown in locusts (Fox, 1970) and testis wall in beetles (Fox, 1971). In the latter case, it was shown that the heterochromatin:euchromatin ratio in the polyploid cells differs from the ratio in the diploid cells. The differential replication of heterochromatin has also been described in cultured

orchid protocorms (Nagl, 1972), which contain two populations of nuclei, one the result of regular endopolyploidy and the other containing a much larger content of heterochromatin. Lorick (1970) demonstrated that cells in the testis sheath of mealy bugs, containing one set of 5 heterochromatic and one set of 5 euchromatic chromosomes, undergo endomitosis resulting in a polyploid number of euchromatic chromosomes but only the haploid set of heterochromatic chromosomes, owing to the lack of replication of this latter material. Polytene chromosomes in the salivary glands of Drosophila have been shown to fall below multiples of the doubling DNA series owing to reduction in the amount of chromocentric heterochromatin (Rudkin, 1965), whereas polytene chromosomes in the suspensor cells of Phaseolus indicate regular DNA doubling up to 8192C (Brady, 1973). Drosophila nuclei from polytene cells contain only 5% of the fast-renaturing, repetitious DNA, whilst the non-polytene cells contain 20% of this DNA (Dickson, Boyd and Laird, 1971). This heterochromatic, repetitious DNA is, at least in part, analagous to sat.DNA since sat.DNA is greatly under-represented in polytene chromosomes (Gall, Cohen and Polan, 1971). However, repetitious or sat.DNAs are not uniformly under-replicated during polytenization since Drosophila virilis DNA shows normal replication of one sat.DNA fraction whilst two other satellite components are under-replicated (Blumenfeld and Forrest, 1972). Hennig (1972) showed differential DNA replication during polytenization in Drosophila hydei, and during this development, rDNA is under-replicated, there being only 40 copies of the rRNA gene compared with 280 per haploid genome in a diploid cell (Hennig and Meer, 1971).

Blumenfeld and Forrest (1972) showed that the percentage of sat.DNA varies during the development of D. virilis, dropping from

40% in the embryo to 28% in the adult fly. It is not clear whether this variation is due simply to the proportion of under-replicated polytene cells present in the various stages, or whether differential replication in other polyploid cells contributes towards the total change.

In a large number of individual melon plants, the percentage of sat.DNA varies with the plant tissue (Table 7). It is clearly seen that the seeds and the roots contain the highest percentages of sat.DNA, whereas the hypocotyls and flowers contain less, and the fruit, mature leaves and mature cotyledons contain least of all. Furthermore, as shown in Table 8, the densities of the 2 components of the seed DNA (mainband and satellite) are both less than the densities for the 2 corresponding components of the fruit DNA.

In cucumber, there is certainly a difference in density between the sat.DNAs of the fruit and the seed, although the mainband DNA density appears to be the same. Furthermore, as the fruit matures, so the percentages of the 2 sat.DNAs decrease and the densities of the fruit sat.DNAs increase (fig.19).

The result of one experiment on developing melon fruits and seeds suggests that, in the very young fruits and seeds, the percentage of sat.DNA is identical, as are the densities of the mainband and satellite DNAs. In the older fruits and seeds, however, the percentage of sat.DNA in the fruit decreases whereas in the seeds it increases. Furthermore, the densities of the mainband and satellite DNAs decrease in the seeds and increase in the fruits. These values, then, show that the DNA in the very young fruits and seeds is identical, whereas in older fruits, the values diverge to give the typical difference between melon fruit and seed (Table 10).

It was possible that the differences in percentage satellite in fruit and seed DNA might be artefacts introduced by preferential DNA extraction. In order to test this hypothesis, different DNA extraction methods were used and the DNAs compared. The usual method of extraction (homogenization in Kirby medium) was compared with a method involving homogenization in SSC/SDS followed by a pronase digestion. The results of the two methods are identical in showing that melon seed contains a higher percentage of sat.DNA than melon fruit (Table 8). In addition, repeated extractions using Kirby medium were employed in an attempt to extract all the DNA from samples of seed and fruit. This appeared to be successful in that the 3rd extraction from seeds and the 4th extraction from fruit contained no DNA. All the fruit extractions contained about 18% sat.DNA, and both the seed extractions contained about 24% sat.DNA (Table 9). Thus, it appears that the method does not preferentially extract sat.DNA and that the differences between seed and fruit DNAs are real. The difference in percentage satellite is not due to the size of the DNA analysed or to the organization of satellite sequences within the genome, since the percentage satellite is essentially constant with decrease in the size of the DNA fragment.

Sat.DNAs in these plants, therefore, are in a state of flux during differentiation. Both densities and sat.DNA percentages alter. The variation in the amount of sat.DNA is readily explained in terms of a differential replication of satellite relative to mainband. The change in buoyant-density suggests either that the satellite sequence changes during differentiation, or that the satellite is composed of a range of different sequences which themselves undergo preferential replication. The latter explanation

is more likely and is supported by the observation that melon satellite does contain at least 2 components (Ingle, Pearson and Sinclair, 1973) and also by the fact that the density of the mainband changes. The change in mainband density suggests that massive differential replication must occur. Alternatively, it is possible that the young fruits and seeds contain a certain percentage of the cytosine bases present as 5-methyl-cytosine and as the fruits develop and the DNA is replicated, the degree of methylation is reduced. This would give rise to an increase in the buoyant-density of the DNA, since 5-methyl-cytosine is a larger molecule than cytosine and consequently displaces more CsCl, with the result that it exhibits a lower buoyant-density than unmethylated cytosine.

In cucumbers and melons, it has been shown that whereas the roots contain 2C and 4C nuclei, the fruit cells exhibit varying levels of ploidy up to 64C (Pearson, Timmis and Ingle, 1974). Furthermore, there are in both fruits many ploidy values which do not fit into a doubling DNA series. It has also been shown that in the fruits of cucumber, there are 2 populations of cells - a minor proportion with a large amount of heterochromatic material, and the majority with very little. No quantitative results were obtained for the amount of heterochromatin in melon 2C and polyploid nuclei owing to its diffuse nature, but subjective analysis suggests that there is less heterochromatin in the polyploid nuclei. There is, therefore, a correlation between reduced sat.DNA and less heterochromatin in fruit tissue. The correlation in cucumber is, however, not quantitative, since a 7% decrease in sat.DNA between seed and fruit is associated with a 20% decrease in heterochromatin. This decrease in percentage satellite in the polyploid fruit nuclei is consistent with the proposal

that sat.DNA might play some rôle in cell division.

Cucumber fruits contain some nuclei with much heterochromatin and some with very little. One might expect the nuclei with a lot of heterochromatin to contain correspondingly more sat.DNA than those with very little. However, DNA extracted from fruit only reflects the balance between the two classes of cells. The fact that the percentage satellite decreases as the fruit matures suggests that the proportion of cells with high heterochromatin content decreases with development, but no data are available on this point.

In melon at least, the fruits and seeds are not the only tissues that show sat.DNA changes during development. The percentage sat.DNA in cotyledons decreases during germination, although the buoyant-densities of the mainband and satellite DNAs remain constant. Likewise, developing leaves exhibit a similar decrease in percentage sat.DNA. The absolute amount of DNA in the cotyledons (initially equal to the amount of DNA in the seed) increases linearly for a while and then the rate decreases until there is no further increment with time (fig.20). From the initially linear section of this plot, the percentage rate of accumulation of sat.DNA can be calculated, which for Spanish Winter melon (k) is 17%. Consequently, although the percentage sat.DNA in the seed is 36% for this melon, given the above percentage rate for sat.DNA accumulation, and given an infinite length of time, the cotyledons would contain 17% of sat.DNA. The results confirm that after 12 days, the percentage has dropped to 19% in a curve which corresponds well with the predicted curve assuming this percentage rate of sat.DNA accumulation. The decrease in percentage sat.DNA in cotyledons is not, therefore, due to changes in the differential rates of synthesis of satellite and mainband.

In the leaves, the situation is basically the same, although in this case the amount of DNA per leaf is initially zero. Results for Spanish Winter melon (h) leaves (fig.21) show that at 8 days after germination, the first leaf contains 25% of sat.DNA and that the percentage decreases with time to 16% in the next 26 days. The change in percentage sat.DNA in leaf must be due to differential synthesis or accumulation rates of satellite and mainband DNAs. Measurement of the amounts of DNA per leaf can, of course, only supply information on the rate of percentage sat.DNA accumulation, not on the rate of synthesis. This was determined by labelling seedlings through the roots with ^{32}P-orthophosphate for 24 hr. A preliminary experiment was performed in duplicate melons to measure the percentage rate of sat.DNA synthesis in leaves 14 days after germination. From the 2 preparations, the mean percentage sat.DNA in the first leaf was 22%, whereas the radio-activity profile indicated 34% of the satellite component. Thus, the newly-synthesized DNA contained much more sat.DNA than did the total DNA. Therefore, although the leaves were accumulating sat.DNA at the rate of ca.16%, they were synthesizing it at the rate of 32%. This suggests that the sat.DNA is less stable than the mainband DNA and has a much greater rate of turnover. Consequently, pre-existing sat.DNA is constantly being replaced, whilst the leaf is increasing in its total DNA content but decreasing in its percentage of sat.DNA. It would be interesting to speculate as to whether this rapid turnover of sat.DNA exists in other plant tissues, e.g. the fruit, where the percentage of sat.DNA also decreases with time.

Very dramatic changes in the percentage of sat.DNA during plant development have been reported by Guillé, Quétier and Huguet (1968)

and Quétier, Guillé and Vedel (1968). They observed that under conditions of physiological stress, plant material developed a new dense sat.DNA termed the stress-sat.DNA. This satellite appeared when melon seeds were stored for 15 days at $4^{o}C$, or when tomato or cucumber seedlings were grown in the dark on distilled water for 21 days. A variation on this latter procedure was to decapitate or wound the seedlings, and this caused an increase in the rate of stress-sat.DNA synthesis.

This DNA was considered to be nuclear in origin since it was recovered in the pellet fraction of a nuclear preparation after treatment with Triton X-100. The Model E scan showed new sat.DNA as a narrow and symmetrical peak of density 1.722 $g.cm.^{-3}$ with a molecular weight of at least $9x10^{6}$ daltons which was resistant to RNase but digested by DNase. Hybridization of the stress-sat.DNA with 25S rRNA indicated that there was 100x more hybridization than with the mainband DNA. Upon denaturation, the density increased by 7 $mg.cm.^{-3}$ It was proposed that this small density increase upon denaturation might be because the DNA was undergoing transcription.

The present work (Pearson and Ingle, 1972) shows that cold storage of Cucurbitaceae seeds does produce a stress-sat.DNA similar to that described, but also that this DNA is bacterial in origin, resulting from massive bacterial contamination. Furthermore, the small increase in density observed upon denaturation is quite normal for DNA of this buoyant-density, since M. lysodeikticus DNA of native buoyant-density 1.731 $g.cm.^{-3}$ increases to 1.737 $g.cm.^{-3}$ on denaturation. Hybridization studies with melon DNA and rRNA show that the density of the rDNA (1.711 $g.cm.^{-3}$) is between the values of the densities of the normal satellite (1.706 $g.cm.^{-3}$) and the stress-satellite (1.718 $g.cm.^{-3}$) DNAs

(fig.34). It is possible, therefore, that the stress-satellite fraction collected by Quétier et al. (1968) contained the normal rDNA.

The stress-sat.DNA observed during the growth of seedlings in the dark might also be bacterial. Certainly in the present work, only a small trace of stress-sat.DNA was found in tomato seedlings so grown and none at all in the cucumber seedlings. It is quite possible that these seedlings were more sterile than those of Quétier et al. (1968) and so consequently did not contain sufficient bacteria for their DNA to be detectable in a total-cellular DNA extraction.

The sat.DNA arising as a result of the wounding of seedlings may similarly be explained in terms of bacterial contamination. Certainly, the resistance to infection of a wounded plant would be much less than that of a healthy, actively-growing plant and one might, therefore, expect to be able to detect bacterial DNA in total-cellular extractions from damaged plants but not from their healthy counterparts.

In conclusion, sat.DNA is present in many plant species, where it differs in buoyant-density from the rest of the genome. It appears to undergo differential replication during various stages of plant development. There is some indication that, compared with mainband DNA, it has a high rate of turnover. Since plant material is not usually sterile, precautions and checks must be taken to ensure that all DNA components present are of true plant origin.

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THE ORIGIN OF STRESS-INDUCED SATELLITE DNA IN PLANT TISSUES

G.G. PEARSON and J. INGLE

Botany Department, King's Buildings, Edinburgh University, Edinburgh, Scotland

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The stress-satellite DNA which is produced in seeds of Cucurbitaceae in response to cold storage is bacterial in origin. The appearance of this stress-satellite cannot therefore be taken as evidence for the amplification of the ribosomal-RNA genes under this condition of physiological stress.

Certain plants contain a nuclear satellite DNA, which differs from the normal nuclear DNA in its buoyant density in a CsCl gradient (Matsuda et al., 1967). Such satellite DNA's are normally denser than the mainband, for example in *Cucurbita pepo* the satellite is 1.706 $g.cm^{-3}$ compared with the mainband of 1.696 $g.cm^{-3}$. The satellite DNA has, therefore, a higher guanine plus cytosine (G+C) content (Sueoka et al., 1959) than the mainband DNA and, from the rate of renaturation, it appears to be considerably less complex than the bulk of the DNA (Matsuda et al., 1967). It has also been suggested, from hybridisation experiments, that this satellite DNA contains the genes for the ribosomal RNA (rRNA) (Matsuda et al., 1967).

More recently, an additional satellite has been described, with a much higher buoyant density of 1.722 $g.cm^{-3}$. This satellite DNA appears when plant tissues are exposed to certain physiological conditions of stress, such as growing seedlings on water in the dark for long periods, wounding dark-grown seedlings by bruising, slitting or decapitation, or storing seeds for many days at 4°C (Quétier et al., 1968; Guillé et al., 1968). The similarity of the type of growth resulting from such conditions to that stimulated by hormones, such as auxin and gibberellic acid, led to the suggestion that hormones may be directly involved with the appearance of this DNA. Hybridisation experiments indicated that this satellite DNA contained rRNA genes. Consequently the change in the amount of this DNA, from nothing in the young seedling or fresh seed to a large percentage of the total DNA in the dark-grown seedling or cold-stored seed, suggested a massive amplification of rRNA genes under these conditions of stress (Quétier et al., 1968; Guillé et al., 1968).

Since it had been concluded that there was no gross change in the number of copies of the rRNA gene during several phases of normal plant development (Ingle et al., 1971), it was of great interest to determine the origin of this stress-satellite DNA (designated as such to distinguish this very dense satellite (1.722 g.cm^{-3}) from the normal nuclear satellite (1.706 g.cm^{-3}), which appears to remain constant under stress conditions).

MATERIALS AND METHODS

Seedlings of tomato (*Lycopersicon esculentum* var. Ailsa Craig) and cucumber (*Cucumis sativus* var. Improved Telegraph) were grown in vermiculite moistened with water, in the dark at 22.5°C. Melon (*Cucumis melo*), cucumber (*Cucumis sativus*) and marrow fruits (*Cucurbita pepo*) were obtained commercially or grown in the greenhouse. The seeds were removed from the fruit, washed and stored in aluminium foil packets at 0–4°C. Total DNA was prepared by homogenisation of the tissue in a detergent medium, followed by chloroform and phenol deproteinisation, ethanol precipitation and purification of the crude DNA preparation by ribonuclease and pronase digestion. The DNA was pelleted by centrifugation (100,000 *g* for 16 hr) and analysed by CsCl equilibrium centrifugation in the Model E analytical centrifuge (Wells et al., 1970). Bacterial contamination of the plant material was determined by plating out samples of the tissue homogenised in water. The plates (nutrient agar 28 g/l) were scored for viable colonies after 2 or 3 days growth at 25°C.

RESULTS AND DISCUSSION

In preliminary experiments designed to confirm the observations of Quétier et al. (1968), tomato and cucumber seedlings were germinated in the dark for 20 days, and seeds from two melons were cold stored for 15 days. Analysis of DNA prepared from the hypocotyl tissue of 7 and 20 day old cucumber seedlings showed only the normal nuclear DNA components, the mainband at 1.693 and the double satellite peaks at 1.702 and 1.707 g.cm^{-3}. After 14 days growth the tomato hypocotyl contained a trace of the stress-satellite DNA, 1.720 g.cm^{-3}, in addition to the normal mainband (1.694 g.cm^{-3}) and satellite (1.705 g.cm^{-3}) DNA's. The cold-stored melon seeds, however, contained large amounts of the stress-satellite DNA's which were not present in the fresh seeds (fig. 1a). Honeydew melon seeds contained one stress-satellite DNA at 1.718 g.cm^{-3}, in addition to the normal components at 1.693 and 1.707 g.cm^{-3} (fig. 1b) whereas the Hero of Lockinge seeds contained stress-satellites at 1.698, 1.715, 1.719 and 1.726 g.cm^{-3} (fig. 1c). Further experiments were therefore directed towards the appearance of these stress-satellites during the cold storage of melon and other Cucurbitaceae seeds.

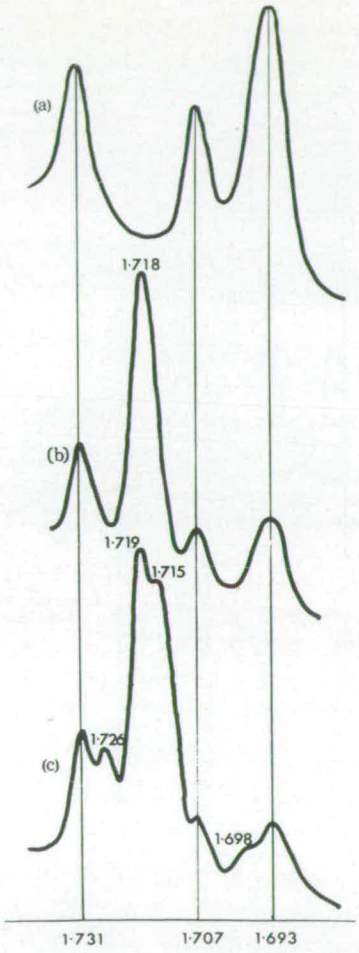


Fig.1. Microdensitometer tracings of ultraviolet photographs of melon seed DNA's. The DNA samples (2 to 3 μg) were adjusted to a density of 1.720 g.cm^{-3} with CsCl and centrifuged at 44,000 rpm for 20 hr at 25°C. The marker DNA from *Micrococcus lysodeikticus* has a density of 1.731 g.cm^{-3}. (a) DNA from fresh seed of Honeydew melon. Mainband and satellite at 1.693 and 1.707 g.cm^{-3}, respectively. (b) DNA from 15-day cold-stored seed of Honeydew melon. Mainband and satellite at 1.693 and 1.707 g.cm^{-3}, respectively, with stress-satellite at 1.718 g.cm^{-3}. (c) DNA from 15-day cold-stored seed of Hero of Lockinge melon. Mainband and satellite at 1.693 and 1.707 g.cm^{-3}, respectively, with stress-satellites at 1.698, 1.715, 1.719 and 1.726 g.cm^{-3}.

Several properties of the stress-satellite DNA prepared from stored seeds of Honeydew melon were determined. All of the components in the CsCl gradient were completely sensitive to deoxyribonuclease. Analysis of the CsCl gradient with

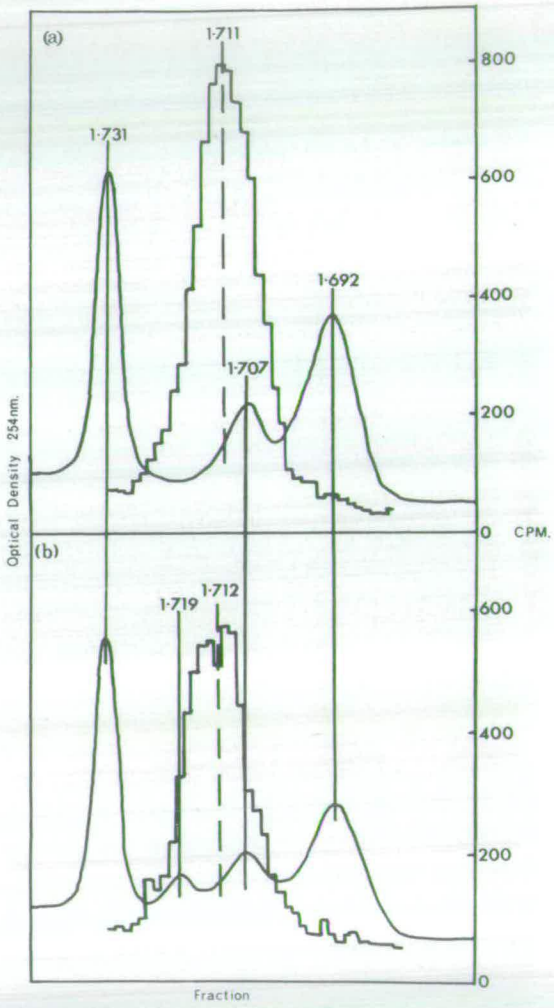


Fig.2. Density of rRNA genes in melon DNA. DNA prepared from Honeydew melon seeds was fractioned on a preparative CsCl gradient in the MSE 10×10 angle rotor at 38,000 rpm, 25°C for 66 hr. The gradient was pumped from the tube and the optical density at 254 nm was continuously recorded. The DNA in each fraction was alkaline denatured, neutralized, and fixed to a millipore membrane (Gillespie et al., 1965). The membranes were incubated with ^{32}P-rRNA in 6×SSC at 70°C for 2 hr and then washed once with 6×SSC, 3 times with 2×SSC, incubated with 10 μg/ml ribonuclease at 25°C for 15 min, dried and counted. (a) DNA prepared from fresh seeds; (b) DNA prepared from seeds after 14 days cold-storage.

Schlieren optics showed no evidence of polysaccharide contamination of the DNA peaks (Edelman et al., 1967). The stress-satellite DNA, isolated from preparative CsCl gradients, showed normal thermal melting with a T_m corresponding to 60%

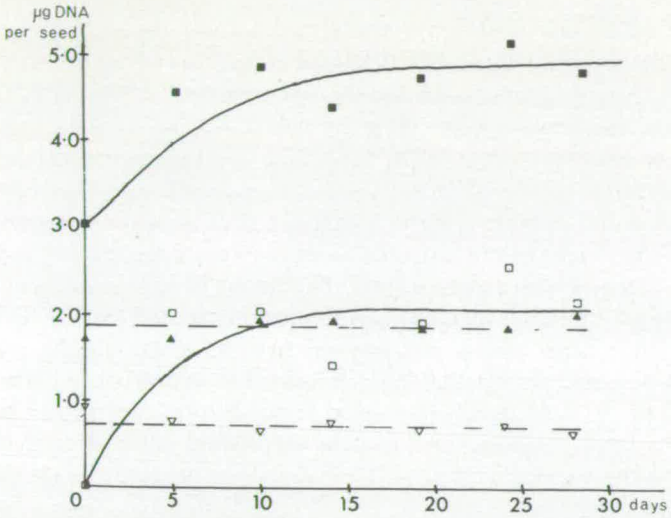


Fig.3. Appearance of stress-satellite DNA during cold storage. Seeds of a Musk melon were stored at 0–4°C. The total DNA content of the seed was calculated from the purified DNA preparation. The amounts of the individual DNA components were determined from the Model E fractionations. —■—■— Total DNA; —□—□— stress-satellite DNA (1.711 g.cm^{-3}); —▲—▲ mainband DNA (1.693 g.cm^{-3}); —▽—▽ normal-satellite DNA (1.707 g.cm^{-3}).

(G+C). On denaturation the density of the DNA increased by 0.008 g.cm^{-3}. It was suggested by Quétier et al. (1968) that this small increase in buoyant density on denaturation may be the result of the DNA being in process of transcription, but it is probably due to the high (G+C) content of the DNA; *Micrococcus lysodeikticus* DNA increases from 1.731 to only 1.737 g.cm^{-3} on denaturation. The stress-satellite DNA showed 50% renaturation after approximately 150 min, compared to 90 min and 1 min for 50% renaturation of *Escherichia coli* and melon satellite (1.707 g.cm^{-3}) DNA's, respectively, under identical conditions. The fractions of DNA from a preparative CsCl gradient were hybridised with rRNA to locate the position of the rRNA genes on the gradient (fig.2) (Ingle et al., 1970). The density of the genes (1.711–1.712 g.cm^{-3}) was not directly correlated with either the normal satellite DNA (1.707 g.cm^{-3}) or with the stress-induced satellite at 1.719 g.cm^{-3}.

These results confirmed that the stress-satellite was indeed DNA, but its complexity, 150-fold greater than the normal satellite, suggested that it was not largely composed of reiterated rRNA genes, a conclusion supported by the localisation of these genes at a density of 1.711 g.cm^{-3} on the gradient.

The appearance of the stress-satellite DNA was quantitated during the cold storage of seeds from a musk melon (fig.3). The total DNA content of the seed was calculated from the purified DNA preparation, and the individual amounts of the mainband, satellite and stress-satellite DNA's determined from the Model E fractions. The amount of the mainband and normal satellite DNA's remained constant

at 1.8 and 0.7 μg per seed, respectively, throughout the experiment, while the amount of the stress-satellite DNA increased to just over 2 μg per seed during the first 5 days, thereby almost doubling the DNA content of the seed. These results indicated that the stress-satellite arose by new synthesis of DNA rather than by conversion of existing nuclear DNA. Such time course experiments were repeated with seeds from a variety of melons, and from cucumber and marrow seeds. The results, summarised in table 1, showed that the density of the stress-satellite varied between different species and different varieties of the same species, and also varied to a limited extent within a given variety. The timing of appearance and the amount of the stress-satellite also varied considerably, and in certain cases, with one Honeydew melon, one Ogen melon and one marrow, no stress-satellite was produced (table 1). It was also shown that no stress-satellite was produced when the seeds were stored in the cold within the melon fruit, in toto, and further there was no change in the DNA fractionation when the seeds were removed from the fruit but maintained in the mucilaginous placental material for the cold storage period.

Table 1
The buoyant-densities of the stress-satellite DNA's produced during cold storage of seeds.

Seed*		Buoyant-densities of stress-satellite DNA's† ($g.cm^{-3}$)			
Melon					
Hero of Lockinge		1.698,	1.716,	1.719,	1.726
Honeydew	a			1.718	
	b		1.713,	1.718	
	c				1.718
	d	None			
Spanish Winter	a			1.717	
	b			1.718	
	c			1,718	
Ogen	a		1.711		
	b	None			
Musk			1.711		
Cucumber					
Kariha	a		1.711,	1.719	
	b			1.718	
Marrow					
Courgette	a	1.695,	1.714,	1.720	
	b	None			

* Melons a, b, c, d, etc., indicate seeds from 4 different fruits.
† The major components are underlined.

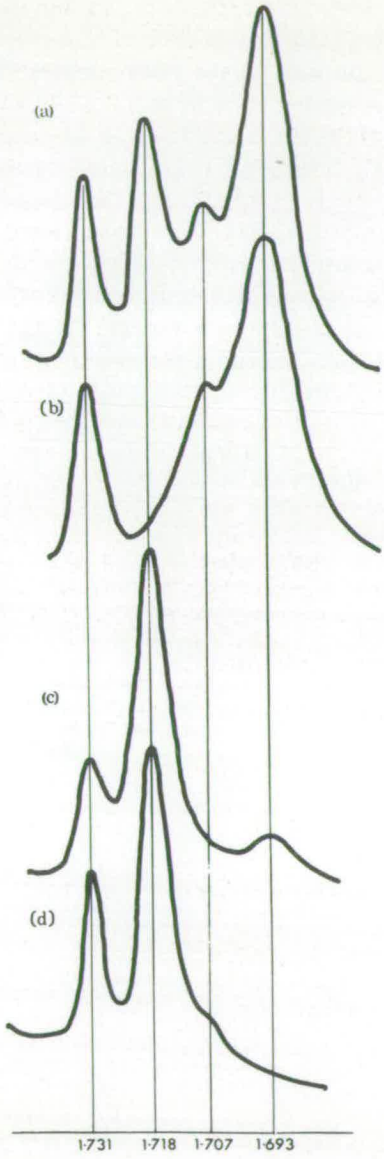


Fig.4. Localization of the stress-satellite DNA within the seed. Seeds of Honeydew melon, cold stored for 34 days, were separated into cotyledon and seed-coat tissue. DNA was prepared from these tissues, and from the complete seed, and analysed by Model E centrifugation (see fig.1). (a) DNA from the complete seed; (b) DNA from the cotyledon tissue; (c) DNA from the seed-coat tissue; (d) DNA prepared by washing the intact seeds in the detergent extraction medium.

It was observed in preliminary experiments that the yield of the stress-satellite, expressed as a percentage of the total seed DNA, varied considerably depending on the homogenisation method used in the DNA preparation. Homogenisation in a pestle and mortar, which resulted in a very low yield of total DNA, produced a much higher percentage of the stress-satellite DNA than after a very thorough tissue disintegration in the VirTis homogeniser. Following this indication that the stress-satellite DNA was more readily extracted than the normal DNA, cold-stored seeds were dissected and DNA was prepared from the separated cotyledon and seed coat tissue. Although the whole seed contained a large proportion of stress-satellite DNA ($1.718\ g.cm^{-3}$, fig. 4a), the cotyledons contained only the mainband ($1.693\ g.cm^{-3}$) and normal satellite ($1.707\ g.cm^{-3}$) DNA's (fig.4b). Essentially all of the $1.718\ g.cm^{-3}$ DNA was present in the seed coat tissue, together with a trace of mainband DNA (fig.4c, table 2).

Table 2

The location of the stress-satellite DNA in the seed and its correlation with bacterial contamination. The amount of stress-satellite DNA was calculated as described in fig. 3. The seed-coat DNA was the total amount of DNA prepared from the isolated seed-coats. The detergent wash DNA was the total amount of DNA extracted by washing the intact seeds in the detergent medium. The bacterial DNA was calculated from the number of bacterial contaminants per seed on the basis of 10^{-14} g per bacterium.

Seeds	No. days cold-storage	μg DNA/seed				No. bacteria per seed
		Stress satellite	Seed coat	Detergent wash	Bacterial	
Honeydew melon (c)	0	0	0	0	–	–
	20	1.5	1.4	–	–	–
	22	–	–	1.8	2.0	2×10^8
	24	1.7	1.8	–	–	–
Honey melon (d)	15	0	–	–	~0	40
	28	0	–	–	~0	200
Courgette marrow (a)	0	0	–	–	~0	20
	22	4.5	3.8	–	–	–
	46	8.8	6.8	6.1	>2	$>2\times10^8$

The restriction of stress-satellite DNA accumulation to the seed coat, which is composed largely of dead cells, indicated that this DNA was probably not of plant origin, but was the result of bacterial contamination on the outside of the seed coat. This was confirmed by 'washing' cold-stored seeds in the detergent extraction medium, without any homogenisation of the seed material. The DNA recovered

from the washing was identical in buoyant density (1.718 $g.cm^{-3}$, fig. 4d) and in yield to the stress-satellite component of total seed DNA (table 2). The presence and amount of the stress-satellite was also well correlated with the extent of the bacterial contamination, and the amount of bacterial DNA per seed, calculated on the basis of 10^{-14} g of DNA per bacterium, was similar to the stress-satellite component of the total DNA, to the DNA content of the seed coat and to the DNA prepared by detergent washing of the seed (table 2). Those seeds which produced no stress-satellite (see table 1) had very little bacterial contamination, even after long storage periods (table 2). Isolates of bacteria from different seeds were cultured. The DNA's prepared from such cultures were identical in buoyant density to the stress-satellite DNA's identified from the Model E fractions.

These results show that the stress-satellite DNA which is produced in seeds of the Cucurbitaceae in response to cold storage is bacterial in origin. This interpretation accounts for the variation, both quantitative and qualitative of this stress-satellite DNA, and is also in agreement with the complexity of the DNA, as measured by the rate of renaturation. It is possible, therefore, that other dense satellite DNA's observed in plant tissues, particularly those produced in response to stress conditions (Coudray et al., 1970), are similarly bacterial in nature. There is therefore no evidence for the gross amplification of the rRNA genes during normal plant development (Ingle et al., 1971) nor during conditions of physiological stress.

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Species Distribution and Properties of Nuclear Satellite DNA in Higher Plants

J. INGLE, G. G. PEARSON & J. SINCLAIR
Botany Department, King's Buildings, University of Edinburgh

Among dicots, satellite DNA was most abundant in the Cucurbitaceae and the Rutaceae. No satellite resolvable on CsCl gradients was found in monocots. The size range, homogeneity and complexity of satellite DNA were also studied.

SINCE the demonstration that nuclear DNA from certain eukaryotes may be fractionated into mainband and satellite components by neutral CsCl equilibrium centrifugation[3,4], the properties, functions and origins of satellite DNA have been extensively studied and discussed[5–7]. Satellite DNA of mouse differed from the mainband not only in buoyant density in CsCl, but also in the rate at which the separated satellite strands renatured. The rate of renaturation was approximately 1,000 times that of phage T4 DNA, indicating a sequence of approximately 300 nucleotide pairs, or 2×10^5 daltons[5]. As 10% of the cellular DNA is present as satellite, this represents several million copies of such a sequence.

Subsequent studies have shown that these copies are not identical[8], and indicate approximately 5×10^6 very similar sequences of about 130 nucleotide pairs, which appear to have evolved from a simple sequence of 8 to 13 nucleotides[9]. The two strands of the satellite DNA are so different in base composition that they separate completely in an alkaline CsCl gradient[10], a property which greatly facilitated the sequencing studies[9]. The satellite component in mouse, which is preferentially associated with the condensed fraction of interphase chromatin[11] and with nucleoli preparations[12], has now been localized at the centromeres of all the metaphase chromosomes by *in situ* hybridization[13,14].

Other rodents contain satellites which differ from that of mouse in amount, buoyant density and complexity, indicating species-specificity of this fraction[15]. Satellite DNA is very widespread as indicated by the presence of a distinct satellite in 48 out of 93 species of mammal[16]. Certain animals, for example *Drosophila pseudoneohydei*, contain more than one satellite component, and these satellites have different locations within the chromosomes[17].

Although the properties and location of satellite DNAs are now well described, their function and origin are still under discussion. Satellite DNA, which is generally associated with constitutive heterochromatin[18], does not appear to be transcribed[6], and its properties suggest a structural or organizational role, probably in relation to chromosome movement and pairing[6,7].

A few higher plants have been reported to contain satellite DNA, which renaturated much faster than the mainband DNA[19]. A relationship between the satellite DNA and the genes for rRNA was also suggested from this study, as satellite DNA isolated from a preparative CsCl gradient hybridized a greater amount of rRNA than did the mainband, and the DNA from three plants containing a satellite component hybridized to a greater extent with rRNA than did DNA from two plants without satellite[19]. An additional very dense (1.720 g cm^{-3}) satellite, which appeared in response to various stress conditions, and which was interpreted as representing a massive amplification of rRNA genes, has also been described[20]. The appearance of a similar satellite during cold storage of melon seeds, which has been explained in terms of bacterial contamination on the seed surface[21], throws doubt on the plant origin of any of the dense, stress-satellite DNAs.

The picture is further complicated by the fact that chloroplast and mitochondrial DNAs, which represent only a small percentage of the total DNA, are often greatly enriched in "nuclear" preparations. We present the results of an analysis of DNA from a large range of angiosperm species and describe the properties of the various satellite DNAs in terms of buoyant density, contribution as a percentage of the total genome, homogeneity and kinetic complexity. It should be stressed that in this context satellite DNA is defined as a minor component resolved by neutral CsCl centrifugation, and does not include fractions which appear as satellites after treatment with heavy metals[22]. All satellite DNAs so far examined are fast renaturing, or repetitious fractions, although not all repetitious DNAs appear as satellite components.

Species Distribution of Satellite DNAs

The plant species analysed (Table 1) were spread broadly across the taxonomic orders. One striking conclusion is the absence of satellite DNA amongst the 11 monocotyledon species examined, which ranged from the primitive Liliaceae to the highly evolved Gramineae, whereas 27 out of 59 dicotyledons contained satellite. This does not mean that repetitious DNA is absent from the monocotyledons, indeed rye contains a fraction of DNA with a renaturation rate comparable to that of the satellite component of dicotyledons. Such repetitious DNA is not, however, resolved as a discrete component on CsCl gradient analysis. It is perhaps pertinent to consider this difference in relation to the observations of Evans and Rees[23]. They confirmed the positive correlation between nuclear DNA content and duration of mitotic cell cycle and further concluded that, although the rate of increase in cycle time per unit of DNA was of the same order for both monocotyledons and dicotyledons, the complete cell cycle of the latter was, on average, 4 h longer. This difference was accounted for by variation in G1, that part of interphase preceding DNA synthesis. It was suggested that the very short G1 present in monocotyledons may be associated with the degree of chromosome coiling during division, as the DNA density of metaphase chromosomes from monocotyledons was only half that of dicot chromosomes. Such a difference in chromosome organization could well involve satellite DNA.

Satellite DNA was not preferentially associated with either the primitive or more advanced dicot families, being present in some of the most primitive (*Drimys*) and advanced (*Calendula*) species. It was most abundant in two particular families,

the Cucurbitaceae and the Rutaceae. Species containing no satellite DNA were, however, present in both families, *Momordica* in the Cucurbitaceae and *Choisya* and *Skimmia* in the Rutaceae. *Passiflora*, closely related to the Cucurbitaceae, contained no satellite. Within the Leguminosae, satellite DNA was present in the genera *Phaseolus*, but not in *Vicia* or *Pisum*. There was a suggestion from the Cucurbitaceae and Rutaceae that the presence and amount of satellite DNA may be related to intensive selection for, in these cases, fruit quality.

It was proposed from a comparison of satellite DNA in three American and three Asian species of *Phaseolus* that the latter, which contained less satellite, may be more ancient than the American species[24]. This conclusion is not particularly supported by the archaeological data[25]. In general the species distribution of satellite DNA makes little sense with either the generally accepted evolution of the flowering plant or with taxonomic classification. It is, however, interesting to note that mungbean, pumpkin and cauliflower, members of three of the families containing satellite DNA, are closely related in a phylogenetic tree based on the sequence of cytochrome *c*[26]. The number of species common to both the cytochrome *c* and satellite DNA analyses is too small to allow much discussion, although it would be intriguing to know the position of *Citrus* in the phylogenetic tree.

The Range of Satellite DNAs

The largest amount of satellite DNA was observed in the cucumber, where the two satellite components represented 44% of the nuclear DNA. The maximum percentage observed for a single satellite was 25% in melon, runner bean and the citrus species. The lower limit of detection is dependent on the density difference between the satellite and mainband, the size of the DNA being fractionated and the sensitivity of the analytical method. Consequently satellite components less than 5% of the total have not been included unless they are well resolved from the mainband. The 3% satellite of watermelon, which was separated by 15 mg cm^{-3} from the mainband, was a discrete component whereas the 5% satellite of white bryony (1.706 g cm^{-3}) was only a small shoulder on the mainband (1.696 g cm^{-3}).

Although the satellite in most species examined was denser than the mainband, two plants, flax and *Lobularia*, contained satellite DNA of lower buoyant density (Fig. 1). The analysis of melon, bean and two cucumber satellite DNAs by alkaline CsCl centrifugation showed only a single band, indicating no extreme differences in the guanine and thymine content between the two strands. Mouse satellite DNA was clearly resolved into two peaks under these conditions.

The analyses presented in Table 1 were on total DNA prepared from leaf tissue. Species with a satellite component in leaf DNA showed, however, a comparable satellite in DNA preparations from roots, cotyledons, hypocotyls, seeds or fruits. The presence of satellite DNA was not dependent on the method of preparation of the DNA. Although the best yields of DNA were consistently obtained by detergent extraction in the presence of EDTA followed by chloroform deproteinization[1], omission of EDTA, use of phenol instead of chloroform, hot extraction, and the use of pronase digestion in the presence of sodium lauryl sulphate had little effect on the relative amounts of satellite and mainband DNAs. The analysis was also independent of size of the DNA from 15×10^6 daltons, the maximum size prepared, down to 1×10^6 daltons, below which the lack of resolution became limiting.

Although total cellular DNA was analysed in these studies, the observation of satellite DNA was not influenced by either chloroplast DNA, which at 1.697 g cm^{-3} is not resolved from the mainband[1], or mitochondrial DNA. The latter, although of 1.706 g cm^{-3} buoyant density[1], constitutes such a small percentage of the total (less than 1%) that it is not seen in analyses of total DNA from, for example, onion roots (1.691 g cm^{-3}) which presumably contain a normal level of mitochondria. The plant material was obtained from the greenhouse or garden, and it has been shown that under certain conditions bacterial contamination may be sufficiently high to contribute a significant amount of DNA to the total preparation[21], so it was necessary to eliminate bacteria as a possible source of the satellite DNA. The bacterial contamination of

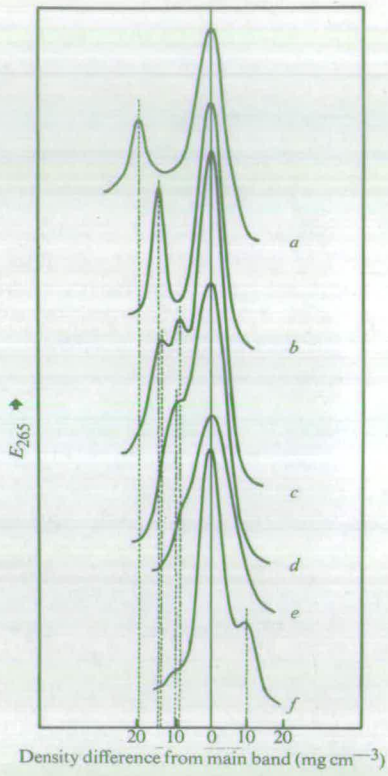


Fig. 1 The range of plant satellite DNAs. Total DNA prepared from leaf tissue was analysed by neutral CsCl equilibrium centrifugation at 44,000 r.p.m., 25° C for 20 h. The buoyant densities were calculated relative to the internal marker DNA of *Micrococcus lysodeikticus* (1.731 g cm^{-3})[1]. *a*, *Fortunella* DNA, 1.712 and 1.693 g cm^{-3}. *b*, Melon DNA, 1.706 and 1.692 g cm^{-3}. *c*, Cucumber DNA, 1.706, 1,702 and 1.694 g cm^{-3}. *d*, Runner bean DNA 1.702 and 1.693 g cm^{-3}. *e*, Turnip DNA, 1.704 and 1.696 g cm^{-3}. *f*, Flax DNA, 1.699 and 1.689 g cm^{-3}.

young leaves freshly harvested from the greenhouse varied from 10^4 to 2×10^6 bacteria per g of leaf. This would represent 10^{-4} to 2×10^{-2} μg of bacterial DNA (10^{-14} g DNA/bacterium) per 20 μg of leaf DNA, that is, 0.0005 to 0.1% of the total DNA, compared with 20% satellite DNA. The inside of melon and cucumber fruits, and the seeds from such fruits, were essentially sterile[21] and yet contained the full amount of satellite DNA. Analysis of DNA prepared from chromatin, which had been extensively washed with 4% 'Triton X-100' to solubilize the cell organelles, confirmed the nuclear origin of the satellite DNA.

Homogeneity of Satellite DNA

As DNA from eukaryotes has been shown to be heterogeneous with respect to base composition or buoyant density[27], we considered the possible heterogeneity of satellite DNA. DNA (50 to 100 μg) from melon, marrow and cucumber was fractionated by preparative CsCl centrifugation (Fig. 2) and samples throughout the mainband and satellite regions were subjected to Model E analysis. The buoyant density of the

Table 1 Species Distribution of Satellite DNAs

Order No.*	Family	Species	Common name	Buoyant density (g cm^{-3}) Main	Sat.	% Sat.	
A, Dicotyledons							
1	Magnoliaceae	*Magnolia soulangiana*		1.699	—	0	(1)
	Winteraceae	*Drimys piperita*		1.698	1.709	7	(1)
9	Ranunculaceae	*Ranunculus acris*	Meadow buttercup	1.699	—	0	(1)
		Ranunculus ficaria	Lesser celandine	1.696	—	0	(1)
		Helleborus niger	Christmas rose	1.694	—	0	(1)
		Anemone coronaria	de Caen	1.696	—	0	(1)
		Pulsatilla vulgaris	Pasque flower	1.699	—	0	(1)
		Clematis montana		1.700	—	0	(2)
		Trollius europaeus	Globe flower	1.698	—	0	(1)
		Aconitum napellus	Monkshood	1.697	—	0	(1)
16	Hamamelidaceae	*Hamamelis mollis*	Witch hazel	1.695	1.706	6	(1)
27	Caryophyllaceae	*Dianthus barbatus*	Sweet william	1.694	—	0	(1)
	Chenopodiaceae	*Beta vulgaris*	Swiss chard	1.693	—	0	(n)
		Spinacia oleracea	Spinach	1.695	—	0	(1)
32	Paeoniaceae	*Paeonia officinalis*	Paeony	1.692	—	0	(1)
35	Passifloraceae	*Passiflora antioquiensis*	Passionflower	1.700	—	0	(2)
36	Cucurbitaceae	*Cucumis melo*	Melon	1.692	1.706	25	(n)
		Cucumis sativus	Cucumber	1.694	1.702	28	(n)
					1.706	16	
		Cucurbita pepo	Marrow	1.696	1.706	18	(n)
			Pumpkin	1.695	1.707	16	(2)
			Squash	1.695	1.706	17	(4)
		Citrullus vulgaris	Watermelon	1.693	1,708	3	(2)
		Bryonia dioica	White bryony	1.696	1.706	5	(2)
		Momordica charantia	Balsam pear	1.695	—	0	(3)
		Lagenaria vulgaris	Bottle gourd	1.692	1.707	9	(2)
		Luffa cylindrica		1.696	1.707	6	(1)
38	Cruciferae	*Brassica rapa*	Turnip	1.696	1.704	21	(n)
		Brassica pekinensis	Chinese cabbage	1.695	1.703	17	(1)
		Raphanus sativus	Radish	1.700	—	0	(1)
		Lobularia maritima		1.695	1.688	23	(3)
					1.706	9	
40	Salicaceae	*Populus nigra*	Black poplar	1.693	—	0	(1)
50	Leguminosae	*Vicia faba*	Broad bean	1.694	—	0	(2)
		Vicia benghalensis	Purple vetch	1.694	—	0	(1)
		Pisum sativum	Pea	1.695	—	0	(n)
		Phaseolus coccineus	Runner bean	1.693	1.702	24	(n)
		Phaseolus vulgaris	French bean	1.693	1.703	19	(2)
		Phaseolus aureus	Mung bean	1.692	1.705	5	(1)
56	Rutaceae	*Citrus sinensis*	Sweet orange	1.694	1.712	23	(2)
		Citrus limonia	Lemon	1.694	1.711	23	(3)
		Citrus paradisi	Grapefruit	1.693	1.711	23	(2)
		Citrus nobilis. var. deliciosa	Tangerine	1.693	1.712	19	(1)
		Fortunella sp.	Kumquat	1.693	1.712	24	(1)
		Choisya ternata	Mexican orange	1.694	—	0	(1)
		Skimmia japonica		1.695	—	0	(1)
58	Linaceae	*Linum usitatissimum*	Flax	1.699	1.689	15	(8)
		Linum grandiflorum rubrum	Red flax	1.698	—	0	(1)
70	Solanaceae	*Solanum tuberosum*	Potato	1.695	1.707	4.0	(2)
		Solanum crispum		1.698	1.710	6	(2)
		Solanum capsicastrum	Christmas orange	1.693	—	0	(1)
		Lycopersicon esculentum	Tomato	1.694	1.705	8	(n)
		Atropa belladonna	Deadly nightshade	1.694	—	0	(1)
		Nicotiana tabacum	Tobacco	1.697	—	0	(1)
		Petunia hybrida	Garden petunia	1.696	—	0	(1)
71	Labiatae	*Perilla frutescens*		1.695	—	0	(1)
		Coleus blumei	Coleus	1.696	—	0	(2)
74	Compositae	*Tagetes erecta gigantea*	African marigold	1.692	—	0	(2)
		Calendula officinalis	Pot marigold	1.692	1.705	11	(4)
		Bellis perennis	Daisy	1.694	—	0	(1)
		Helianthus tuberosus	Jerusalem artichoke	1.695	—	0	(7)
		Hieracium aurantiacum	Orange hawkweed	1.696	—	0	(1)
B, Monocotyledons							
79	Liliaceae	*Lilium regale*	Regal lily	1.698	—	0	(1)
		Hyacinthus orientalis	Hyacinth	1.700	—	0	(n)
		Puschkinia libanotica		1.699	—	0	(3)
		Chlorophytum elatum variegatum	Spider plant	1.693	—	0	(1)
	Amaryllidaceae	*Allium cepa*	Onion	1.691	—	0	(6)
86	Commelinaceae	*Tradescantia virginiana*		1.695	—	0	(2)
89	Gramineae	*Secale cereale*	Rye	1.702	—	0	(9)
		Zea mays	Maize	1.701	—	0	(n)
		Triticum aestivum	Wheat	1.703	—	0	(7)
		Hordeum vulgare	Barley	1.701	—	0	(1)
92	Lemnaceae	*Lemna minor*	Duckweed	1.703	—	0	(1)

Total DNA was prepared from leaf tissue except in the case of orange, lemon and grapefruit, where seeds were used. The DNA was prepared and analysed by neutral CsCl equilibrium centrifugation as previously described[1]. The number of preparations analysed is shown in parentheses ($n > 10$).

* Order No. is taken from the classification of Takhtajan[2].

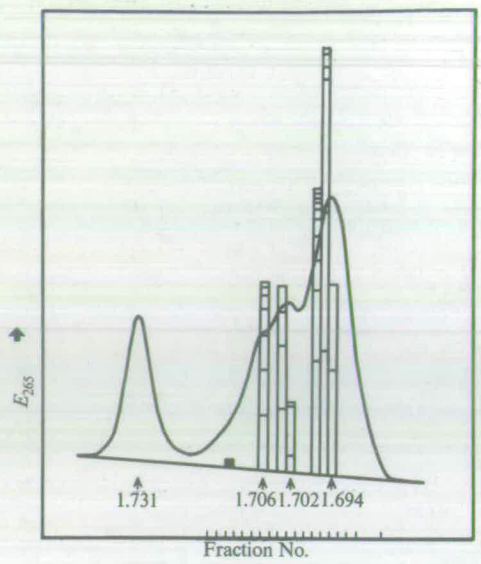


Fig. 2 Preparative fractionation and reconstitution of cucumber DNA. 110 μg of total cucumber fruit DNA was fractionated by preparative centrifugation in the 'MSE' 10×10 angle rotor at 35,000 r.p.m., 25° C for 66 h. *M. lysodeikticus* DNA (1.731 g cm^{-3}) was included as internal marker. The gradient was pumped from the tube and the optical density at 254 nm was continuously recorded (——). Each fraction from the mainband and satellite was analysed by Model E centrifugation, and the fractionation reconstituted (histogram) on the basis of the percentage distribution of components after analysis and the amount of DNA in each initial fraction.

rerun component(s) was compared with its buoyant density in the initial preparative gradient (Table 2). The mainband of cucumber DNA was heterogeneous from 1.692 to 1.696 g cm^{-3}, and a trace of 1.696 g cm^{-3} DNA was present in all the subsequent (denser) samples from the initial preparative gradient, up to a density of 1.717 g cm^{-3}. No components with density between 1.696 and 1.702 g cm^{-3} were observed, however, and with the exception of a single component at 1.704 g cm^{-3}, no DNA between 1.703 and 1.706 or between 1.707 and 1.712 g cm^{-3} was present.

From the percentage distribution of the various components resolved on the rerun, and the amount of DNA present in each initial fraction, it was possible to reconstitute the initial fractionation (Fig. 2). This showed the rather heterogeneous mainband, and three discrete satellite peaks at 1.702 to 1.703, 1.706 to 1.707 and 1.712 to 1.713 g cm^{-3}, with very little DNA of intermediate densities. Comparable analyses of melon and marrow DNAs again indicated heterogeneity of the mainband, but homogeneity of the buoyant density of the satellite DNA (Table 2).

Complexity of Satellite DNAs

The complexity of satellite DNAs from a range of species was determined from a comparison of their rate of renaturation relative to T4 DNA. The time required for 50% renaturation was estimated from Model E analyses of samples taken during the early stages of renaturation. With T4 DNA, at 20 μg $ml.^{-1}$ and hand-sheared to approximately 2×10^6 dalton double-stranded fragments, 50% renaturation occurred after about 8 min in SSC (0.15 M NaCl, 0.015 M Na citrate, *p*H 7.2) at

Table 2 Homogeneity of Mainband and Satellite DNAs

Melon				Marrow				Cucumber				
Initial density	Rerun densities			Initial density	Rerun densities			Initial density	Rerun densities			
1.686	1.691							1.686	1.692			
1.688	1.692							1.689	1.693			
1.690	1.693			1.692	1.693			1.691	1.694			
1.693 M	1.694	1.708 t		1.694 M	1.695			1.693 M	1.694			
1.696	1.695	1.707 t		1.697 M	1.696			1.694 M	1.695			
1.698	1.696	1.708 t		1.699	1.697	1.708 t		1.696	1.696			
1.701	1.697	1.706 s		1.702	1.698	1.708 t		1.698	1.696	1.702 s		
1.704	1.697	1.706		1.704	1.698	1.706 s		1.700	1.696	1.702		
1.706 S	1.697	1.706		1.707 S	1.697	1.707		1.701 S	1.696	1.702		
1.709	1.696	1.707		1.709 S	1.697	1.707		1.703 S	1.695	1.703		
1.712	1.697	1.707		1.712	1.696	1.708		1.705	1.694	1.704		
1.714	1.697	1.707		1.714	1.696	1.708		1.706 S	1.695 s	1.706		
								1.708 S	1.696 s	1.707		
								1.710	1.696 s	1.707		
								1.712	1.696	1.707		
								1.713	1.696	1.707	1.713 s	
								1.715	1.695	1.706	1.712	
								1.717	1.695	1.706	1.712	

DNA (50 to 100 μg) was fractionated by preparative CsCl equilibrium centrifugation (Fig. 2) and average buoyant density of each fraction estimated from the known densities of the mainband and *M. lysodeikticus* DNAs. Approximately 2 μg of DNA from each fraction was analysed by Model E CsCl centrifugation.

The positions of the mainband and satellite peaks in the preparative fractionation are indicated by M or S respectively. Where two or more components were resolved on the rerun the major component is underlined. Components present as shoulders or traces are indicated by s or t respectively.

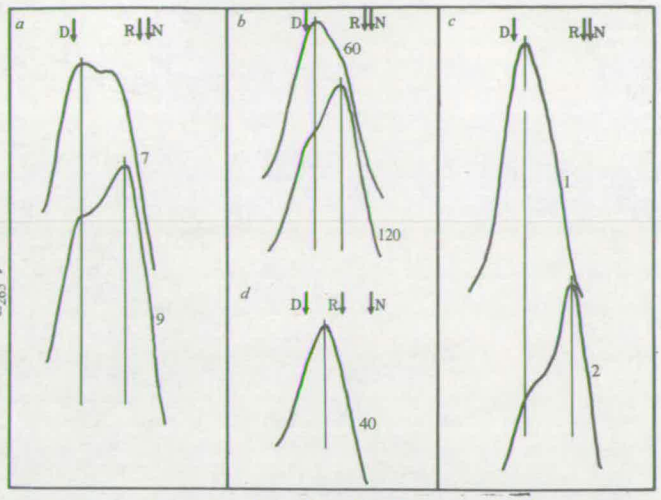


Fig. 3 Rate of renaturation of satellite DNAs. DNA was denatured in 0.1 × SSC by heating at 100° C for 5 min, and then quickly cooled. A sample of the denatured DNA was analysed by Model E CsCl centrifugation. The salt concentration was then increased to 1 × SSC, and the temperature raised to T_m − 25° C. Samples were analysed in the Model E after various time intervals. The arrows marked D, R, and N indicate the buoyant densities of the denatured, renatured for 22 h and native DNAs respectively. *a*, T4 DNA, 20 μg ml.$^{-1}$, renatured for 7 and 9 min. *b*, *E. coli* DNA, 20 μg ml.$^{-1}$, renatured for 60 and 120 min. *c*, Melon satellite DNA, 2 μg ml.$^{-1}$, renatured for 1 and 2 min. *d*, Runner bean satellite DNA, 2 μg ml.$^{-1}$, renatured for 40 min.

T_m − 25° C (Fig. 3, Table 3). At 2 μg ml.$^{-1}$ 50% renaturation required approximately 80 min. The renaturation of *E. coli* DNA took eleven times longer than T4, which is consistent with the relative complexities of these two DNAs, and mouse satellite DNA, which on this basis should require less than 0.1 min for 50% renaturation, was completely renatured after 1 min. The different plant satellite DNAs varied at least 30-fold in renaturation rate. Melon satellite DNA was 50% renatured after 1.5 min (Fig. 3, Table 3) indicating a complexity of 2.5×10^6 daltons, or 4,000 nucleotide pairs, relative to T4 of 1.4×10^8 daltons. The Model E analyses also indicated the presence of a second component in the melon satellite, renaturing at least 100 times slower than the 2.5×10^6 daltons fraction.

Satellite DNAs from orange, flax and cucumber all renatured at rates comparable to that of melon, whereas those from marrow, and particularly runner bean and turnip, were slower, taking about 40 min for 50% renaturation. This indicated a complexity of 70×10^6 daltons. Under comparable conditions the mainband DNAs showed essentially no renaturation (Table 3). The plant satellites appear therefore to be 10 to 100 times more complex than mouse satellite, and several of them contain two or more components with different rates of renaturation. These values are probably in excess of the true complexities due to the effect of nucleotide mismatching on the renaturation rate. The observed complexities of calf and *Apodemus* satellites were reduced from 9×10^6 and 12×10^6 to 3×10^5 and 2×10^5 daltons respectively when corrections were made for mismatching[8]. Preliminary experiments with melon satellite indicate, however, a minimum of mismatching, and the greater complexities of plant satellites would be consistent with the similarity in composition of the two strands, as indicated from the alkaline CsCl fractionations. On the basis of a complexity of 2.5×10^6 daltons, the melon genome would contain approximately 10^5 copies of the satellite sequence.

We thank Professor R. Brown for his support, and Mr R. McCluskey for his suggestions and supply of material.

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Table 3 Renaturation of Satellite DNAs

DNA	Time for 50% renaturation (min)	Extent of renaturation*	Complexity relative to T4
T4	80	13/15	1
E. coli	900	12/13	11
Mouse satellite	<0.3	13/17	<0.004
Melon satellite	1.5	12/13	0.02
Melon mainband	—	2/16	—
Orange satellite	<2.5	8/8	<0.03
Flax satellite	<2.5	6/12	<0.03
Flax mainband	—	1/14	—
Marrow satellite	10	—	0.1
Turnip satellite	40	7/13	0.5
Turnip mainband	—	1/14	—
Runner bean satellite	40	8/14	0.5
Runner bean mainband	—	1/14	—
Cucumber sat. I	<4	11/15	<0.05
Cucumber sat. II	<4	9/13	<0.05
Cucumber mainband	—	2/15	—

All DNA samples were hand-sheared to approximately 2×10^6 daltons double stranded fragment, and renaturation results have been corrected to a concentration of 2 μg ml.$^{-1}$. The time for 50% renaturation was estimated from Model E centrifugation analyses as shown in Fig. 3.

* Expressed as the decrease in buoyant density during 22 h renaturation relative to the increase in buoyant density observed on denaturation (mg cm^{-3}).

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The Differential Replication of DNA during Plant Development

G. G. Pearson, J. N. Timmis, and J. Ingle

Botany Department, King's Buildings, Edinburgh University

Abstract. The percentage of satellite DNA, as defined by neutral CsCl equilibrium centrifugation, varied in different tissues of a plant. Satellite DNA formed a higher percentage of the genome in meristematic tissues (seed, root tip) than in mature, differentiated tissues (fruit, leaf, cotyledon). The buoyant density of the satellite peak, and in some cases of the mainband, also varied significantly between seed and fruit tissue. This variation in the percentage of satellite DNA was correlated with a difference in the heterochromatin content of nuclei from cucumber root tips and fruit. The fruit contained at least two classes of polyploid nuclei, differing in their heterochromatin content.

Introduction

Differential replication of DNA has been indicated during the development of a range of eucaryote somatic tissues by a variety of experimental techniques. The determination of nuclear DNA content by Feulgen microspectrophotometry indicated a tissue specific, non doubling of DNA during the development of polyploid cells in locusts (Fox, 1969). Microspectrophotometry, coupled with heterochromatin-euchromatin estimation, showed that the testis wall in beetles contained a minor class of nuclei in which the heterochromatic DNA had undergone 2 or 3 fewer rounds of replication than the DNA located in euchromatin (Fox, 1971). The differential replication of heterochromatin has also been described in cultured orchid protocorms, which contained two populations of nuclei, one the result of regular endopolyploidy and the other containing a much larger content of heterochromatin (Nagl, 1972). Cells in the testis sheath of mealy bugs, containing one set of 5 heterochromatic and one set of 5 euchromatic chromosomes, undergo endomitosis resulting in a polyploid number of euchromatic chromosomes but only the haploid set of heterochromatic chromosomes, due to the lack of replication of this latter material (Lorick, 1970).

Spectrophotometric measurements of Feulgen stained nuclei also showed that during the development of polytene chromosomes in the salivary glands of *Drosophila* the total absorbance fell below multiples of that present in diploid nuclei. This appeared to be due to a reduction in the amount of chromocentric heterochromatin (Rudkin, 1965). A

comparable study of polytene chromosomes in the suspensor cells of *Phaseolus*, however, indicated a regular DNA doubling up to 8192C (Brady, 1973). The nature of the DNA which is under-replicated in *Drosophila* polytene nuclei has been studied by a variety of methods. A comparison of the renaturation kinetics of DNA prepared from polytene and non-polytene cells showed that the polytene genome contained only 5% of fast renaturing, or repetitious DNA, compared with 20% in non-polytene cells (Dickson, Boyd and Laird, 1971). This heterochromatic, repetitious DNA was at least in part analogous to satellite DNA, since Gall, Cohen and Polan (1971) showed, by isopycnic neutral CsCl centrifugation and by *in situ* hybridisation, that satellite DNA was greatly under-represented in the polytene chromosomes. However, repetitious or satellite DNAs are not uniformly under-replicated during polytenization. Isopycnic centrifugation of *Drosophila virilis* DNA showed normal replication of one satellite fraction whilst two other satellite components were under-replicated (Blumenfeld and Forrest, 1972). Various repetitious fractions of DNA have similarly been shown by hybridisation studies to be replicated to different levels during the development of the polytene chromosomes in *Drosophila hydei* (Hennig, 1972a). Ribosomal-DNA is under-replicated during this development, the number of copies of the ribosomal-RNA gene being only 40 compared with 280 per haploid genome present in a diploid cell (Hennig and Meer, 1971).

The percentage of satellite DNA, as measured by neutral CsCl equilibrium centrifugation, varies during the development of *Drosophila virilis*, decreasing from 40% in the embryo to 28% in the adult fly (Blumenfeld and Forrest, 1972). It is not clear whether this variation is due simply to the proportion of under-replicated polytene cells present in the various stages, or whether differential replication in other polyploid cells contributes towards the total change. In this paper we present data showing that satellite DNA in plant cells varies, both quantitatively and qualitatively, during the development of certain tissues involving the production of polyploid cells. The decrease in the percentage of satellite DNA is correlated at the cytological level with the under-replication of heterochromatin.

Materials and Methods

Preparation of DNA

Total DNA or nuclear DNA was prepared by homogenising total tissue or incubating a nuclear preparation (Scott and Ingle, 1973) in a detergent medium containing 1% (*w/v*) tri-isopropylnaphthalene sulphonate, 6% (*w/v*) p-aminosalicylate, 50 mM NaCl, 50 mM tris-HCl pH 8.0, 10 mM EDTA, pH 8.0 and 6%

(*v/v*) *n*-butanol at room temperature. The concentration of NaCl was increased to 0.5 M and the mixture shaken vigorously with 1 to 2 volumes of chloroform: 3 methyl butanol-l (24:1, *v/v*). After centrifugation at 2500 g for 10 min., the aqueous phase was removed and further deproteinzied by shaking with a phenol mixture (phenol containing 10% (*v/v*) m-cresol and 0.5% (*w/v*) 8-hydroxyquinoline, saturated with 50 mM tris-HCl, pH 8.0). Total nucleic acid was then precipitated from the aqueous phase by the addition of 2 volumes of ethanol and storage at 0°C overnight. The crude nucleic acid, collected by centrifugation (2500 g, 10 min) was washed with 80% ethanol containing 0.2% sodium dodecyl sulphate (SDS) and dissolved in 0.1 × SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.2). The salt concentration was increased to 1 × SSC, and the preparation was digested with DNase-free RNAse (50 μg/ml) for 1 hour at 37°C, followed by self-digested (15 min., 20°C) pronase at 400 μg/ml for 2 to 4 hours at 37°C. The DNA was precipitated with 2 volumes of ethanol at 0° for 1 hour, washed with 80% ethanol containing 0.2% SDS and dissolved in 0.1 × SSC. After increasing the salt concentration to 1 × SSC the DNA was recovered by centrifugation at 120000 g for 16 hours at 4°C. This procedure routinely yielded DNA preparations with a good U.V. spectrum and with better than 75% of the 260 nm absorption being due to DNA, as analysed by the diphenylamine assay (Burton, 1956) and Model E analysis.

Model E Analysis

Two to three μg of DNA were dissolved in CsCl solution (initial density of 1.707 g cm^{-3}) and centrifuged to equilibrium (20 hour) at 44000 rpm at 25°C in a Beckman Model E analytical centrifuge. *Micrococcus lysodeikticus* DNA (1 μg, buoyant density of 1.731 g cm^{-3}) was included with each sample to facilitate the quantitative estimation of the DNA in each preparation and the calculation of the buoyant density of the sample DNA. The centrifugation had previously been calibrated by determining the resolution of two standard DNAs (1.700 and 1.731 g cm^{-3}) when centrifuged in different initial CsCl densities and assuming a linear gradient between these two values (Wells and Ingle, 1970). The percentage of satellite component was estimated from the areas of the respective peaks of a microdensitometer (Joyce Loebl) tracing of the U.V. film, exposed to be within the linear range of the film. Where resolution of the two peaks was incomplete curves were fitted assuming symmetry of the mainband.

A measure of the errors involved in the determination of buoyant density and the relative distribution of mainband and satellite peaks was assessed by analysing one DNA preparation from melon seeds by 6 different centrifugations over a period of 2 weeks. The buoyant densities of mainband and satellite were 1.6921 ± 0.0002 and 1.7056 ± 0.0002 g cm^{-3} respectively (Table 1). The satellite component, expressed as a percentage of the total DNA, varied from 23.1 to 25.6, with a mean of 24.4 ± 1.0%. The reproducibility of DNA preparation was also checked by preparation and Model E analysis of 6 replicate samples of melon seed DNA (Table 1). The variability in DNA preparation was similar in magnitude to that of the analysis, both in terms of density and percentage of satellite DNA.

Estimation of Nuclear DNA Content and the Distribution of Heterochromatin

Nuclei were extracted from root and fruit material of melon (*Cucumis melo*, var. Spanish Winter) and cucumber (*Cucumis sativus*, var. Kariha) by a modification of the method of McLeish (1963). Young radicles were fixed in 2% (*w/v*) formaldehyde in 33 mM phosphate pH 7.0 for 20 min. at 0°C. The root tip was

Table 1. The accuracy of buoyant density and percentage satellite determinations.

Six preparations of total DNA were made from samples of 20 seeds obtained from one melon (S1 to S6). Each sample was analysed by Model E centrifugation, and analysis of S1 DNA was repeated at 6 different centrifugations.

Sample	Buoyant Density ($g \cdot cm^{-3}$)		Satellite (% of total)
	Mainband	Satellite	
S1	1.6919[a]	1.7055	24.9
	1.6924	1.7059	24.6
	1.6922	1.7057	23.2
	1.6921	1.7056	23.1
	1.6921	1.7054	25.2
	1.6919	1.7055	25.6
S1 mean	1.6921 ± 0.0002	1.7056 ± 0.0002	24.4 ± 1.0
S2	1.6918	1.7055	27.0
S3	1.6924	1.7057	26.6
S4	1.6922	1.7057	23.3
S5	1.6923	1.7057	24.2
S6	1.6923	1.7058	23.9
S1–6 mean	1.6922 ± 0.0002	1.7057 ± 0.0002	25.0 ± 1.5

[a] Value used for the $S_{1\text{-}6}$ analysis.

macerated on a slide and the large debris removed. The extracted nuclei were precipitated onto the slide with ethanol and dried in diethyl-ether vapour. Small pieces of the fruit tissue were fixed in 4% formaldehyde (66 mM phosphate, pH 7.0) for 1 hour at 0°C. The pieces were then squashed and the homogenate filtered through nylon mesh (25 μ). The nuclei were recovered by centrifugation at 200 g for 2 min., and dried onto slides. After a thorough washing the slide preparations were hydrolysed, stained in Feulgen reagent, washed in SO_2 water and mounted in glycerol. The amount of stain, and by inference the amount of DNA, per nucleus was measured at 560 nm using a Barr and Stroud integrating microdensitometer (Type GN2).

For an estimation of heterochromatin-euchromatin distribution some of the Feulgen stained preparations were dehydrated, mounted in Euparal, and allowed to dry under slight pressure. Photographic negatives, taken with a Zeiss photomicroscope, were scanned on a recording microdensitometer (Joyce Loebl) to estimate the relative densities of heterochromatic and euchromatic regions of the nucleus. From a number of scans of different cucumber fruit nuclei the heterochromatin staining was on average 2.5 times as dense as that of the euchromatin. The areas of heterochromatin and euchromatin were measured from tracings of the negatives (all of a constant magnification), and these areas were multiplied by 2.5 and 1.0 respectively to give relative DNA contents in arbitrary units. Since the area measurement was the major component in this calculation, the inaccuracy inherent in the determination of average density difference was of minor importance in the estimation of heterochromatin-euchromatin distribution.

Table 2. Variation in the amount of satellite DNA present in melon tissues

Variety	Satellite (% total DNA)						
	Seed	Root tip	Flower	Hypocotyl	Leaf	Cotyledon	Fruit
Hero of Lockinge	26.1	25.8	21.5	19.7	15.6	14.4	17.1
Honeydew	29.4	—	—	22.4	—	17.2	—
Musk (b)	29.5	—	—	—	18.9	17.6	17.1
Spanish Winter (c)	27.7	—	—	21.9	—	17.9	18.5
Spanish Winter (e)	24.3	—	—	—	16.2	19.2	16.7
Spanish Winter (g)	30.3	29.7	—	21.9	—	20.4	15.2
Spanish Winter (h)	24.8	—		—	16.3	14.1	20.9
Spanish Winter (j)	34.1	—	—	—	—	20.9	23.9
Spanish Winter (k)	36.3	—	—	—	15.1	19.3	—

The letter in parenthesis identifies an individual melon.

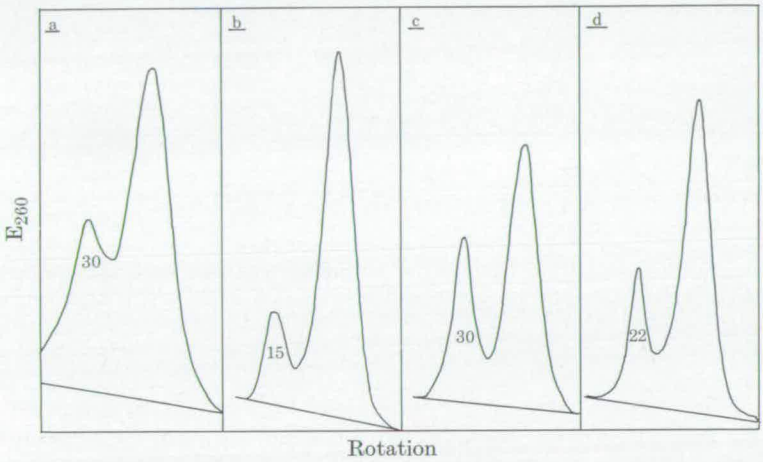


Fig. 1. Analyses of DNA prepared from melon tissues. Total DNA was prepared from the seeds (a) and the fruit (b) of Spanish Winter melon (g, Table 2). Seeds of this melon were germinated and DNA was prepared from root tip (c) and hypocotyl tissue (d). The DNAs were analysed by Model E centrifugation. The value indicates the amount of satellite as a percentage of the total DNA

Results

Quantitative and Qualitative Variation of Satellite DNA in Different Tissues

The amount of satellite DNA, expressed as a percentage of the total, varied in the different tissues of melon (Table 2, Fig. 1). The values fell into two classes, for example with Hero of Lockinge the seed and root tip contained 26% satellite compared to 16% in the leaf, cotyledon and

Table 3. Distribution and buoyant densities of mainband and satellite DNAs from seed and fruit

Variety	Satellite DNA (% of total)		Buoyant density ($g \cdot cm^3$)			
			Mainband		Satellite	
	Seed	Fruit	Seed	Fruit	Seed	Fruit
Melon						
Hero of Lockinge	26.1	17.1	1.6924	1.6938	1.7058	1.7085
Honeydew (e)	29.2	21.8	1.6919	1.6933	1.7062	1.7076
(f)	24.7	18.4	1.6922	1.6936	1.7056	1.7079
(g)	23.4	18.1	1.6922	1.6926	1.7056	1.7066
(h)	25.5	17.3	1.6928	1.6939	1.7063	1.7082
Ogen (a)	30.6	18.2	1.6927	1.6937	1.7056	1.7088
(c)	24.9	19.1	1.6929	1.6930	1.7063	1.7067
Musk (a)	24.2	16.3	1.6923	1.6927	1.7057	1.7070
(b)	29.5	17.1	1.6926	1.6940	1.7056	1.7071
Spanish Winter (c)	27.7	18.5	1.6917	1.6931	1.7063	1.7075
(e)	24.3	16.7	1.6931	1.6932	1.7068	1.7076
(g)	30.3	15.2	1.6923	1.6928	1.7064	1.7079
(h)	24.8	20.9	1.6918	1.6928	1.7057	1.7070
(j)	34.1	23.9	1.6921	1.6925	1.7056	1.7069
Cucumber						
Kariha[a] sat 1	41	34	1.6939	1.6943	1.7011	1.7034
sat 2					1.7060	1.7084

[a] Data are the means from 7 samples of cucumber seed and fruit. Due to lack of resolution of the 2 satellites in cucumber the combined satellites are expressed as a percentage of total DNA.

fruit. Some tissues, such as the flower and hypocotyl had intermediate values. This pattern was common to all the melons analysed. The difference in percentage satellite, 26 to 16%, was far greater than the experimental error involved in the preparation and analysis, which was of the order of a 3% spread (Table 1). The reason for the variation in the percentage satellite in different Spanish Winter melons is not clear. It is probably due to inadequate identification and genetic variation. Classification of melons is very confused since it is a highly polymorphic species and hybridisation between all the forms occurs readily. The varietal names, with the exception of Hero of Lockinge, refer to types rather than to individual cultivars. The values in Table 1 show that seeds taken from an individual fruit are very uniform.

The fruit and seeds of an individual melon showed the largest difference in the percentage satellite DNA, and since commercially obtained melons were a convenient source of material further investigations

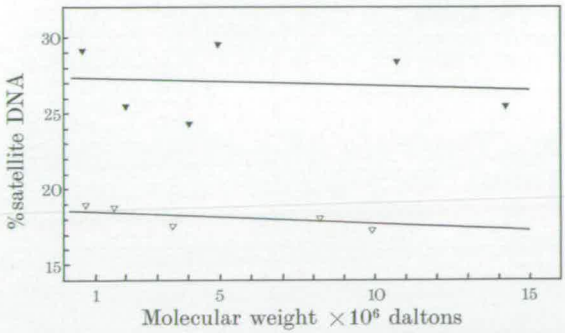


Fig. 2. Effect of DNA size on the percentage satellite DNA. Total DNA was prepared from fruit (-▿-) and seeds (-▾-) of a melon. The average molecular weight of each preparation was determined by the spill-over method of Studier (1965), and then each was successively sheared, initially by forcing the solution through a syringe and finally by sonication. The size at each stage was measured, and the quantitative distribution of satellite DNA determined by Model E centrifugation

concentrated on these tissues. With all of the melons examined the fruit tissue contained a smaller percentage of satellite DNA than the corresponding seeds (Table 3), and with the range of material analysed the difference between fruit and seed was highly significant ($p < 0.1\%$). However, the difference was not limited to the relative distribution of mainband and satellite DNAs, the buoyant density of both mainband and satellite peaks was greater in the fruit than in the seed. The difference was again highly significant ($p < 0.1\%$). The situation in cucumber was similar in that the percentage of satellite DNA was lower in the fruit than in the seed, and the density of both satellite components was significantly ($p < 0.1\%$) greater in the fruit. There was, however, no significant difference in the density of the mainband DNA.

The release of satellite DNA sequences from the rest of the genome will depend on the organisation of such sequences within the genome and on the size of the DNA fragments analysed. Within the range of 14 to 0.6×10^6 daltons the size of the DNA had no significant effect on the percentage of satellite DNA present in either seed or fruit tissue (Fig. 2). As the size of the DNA was decreased the resolution between the components was reduced, and the error of estimation of percentage satellite increased until, below 0.6×10^6 daltons, there was insufficient resolution to allow measurements to be made.

The observations that the highest percentage satellite DNA was present in meristematic tissues (seed and root tip, Table 2) and the lowest in mature tissues (fruit, leaf and expanded cotyledon) suggested that a decrease in percentage satellite should accompany the development of particular tissues. This should be particularly so for the cotyledons,

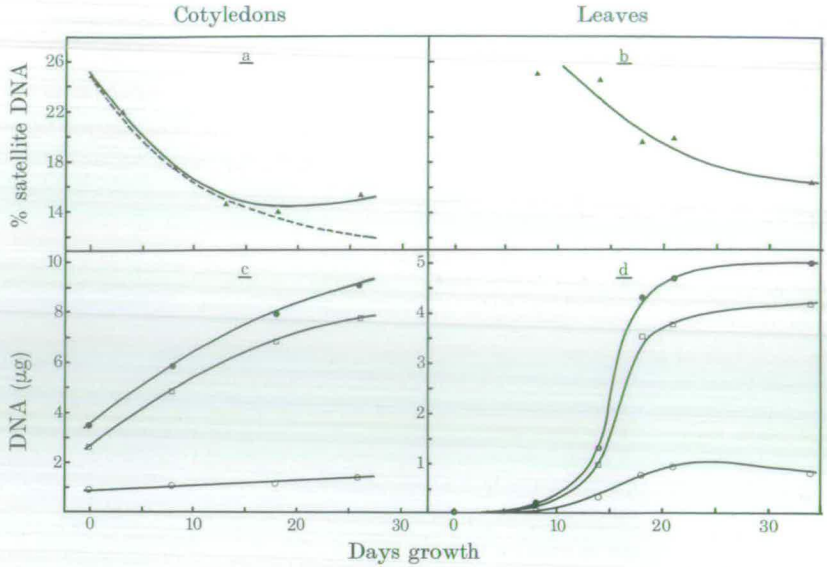


Fig. 3. Variation in the percentage of satellite DNA during the development of cotyledons and leaves. Seeds from a Spanish Winter melon (h, Table 2) were germinated and total DNA was prepared from the cotyledons and first leaves at intervals over a 35 day growth period. The percentage satellite DNA in the cotyledons (a) and first leaf (b) was estimated from Model E centrifugation, and the amount of mainband and satellite DNA in cotyledons (c) and first leaf (d) was calculated from the percentage satellite and the amount of total DNA extracted. In (c) and (d), -○- satellite, -□- mainband, -●- total. The dashed line in (a) represents a dilution curve for satellite DNA calculated from the observed linear accumulate rates of satellite and mainband DNAs shown in (c)

which constitute the majority of the seed tissue, and therefore change from a high percentage value to the lower value measured in the mature green cotyledons. Analysis of the distribution of satellite and mainband DNAs during cotyledon development showed that the satellite decreased from 25 to 14% of the total over a period of 15 days (Fig. 3). During this period the accumulation of both mainband and satellite components was essentially linear, and the reason for the relative decrease in satellite DNA was due to the slow accumulation rate of the satellite (0.022 μg DNA/day) compared with 0.255 μg DNA/day of mainband (Fig. 3c). The rate of satellite DNA accumulation was therefore only 8% of the total during this period, compared to 25% in the seed cotyledons. The decrease in the percentage satellite DNA during this 15 day period fits a dilution curve calculated from the accumulation rates. The constancy of the percentage of satellite at 14% after 15 days was due to the decreased rate of mainband accumulation. Thus, during germination the percentage satellite DNA synthesis in the cotyledon appeared to be

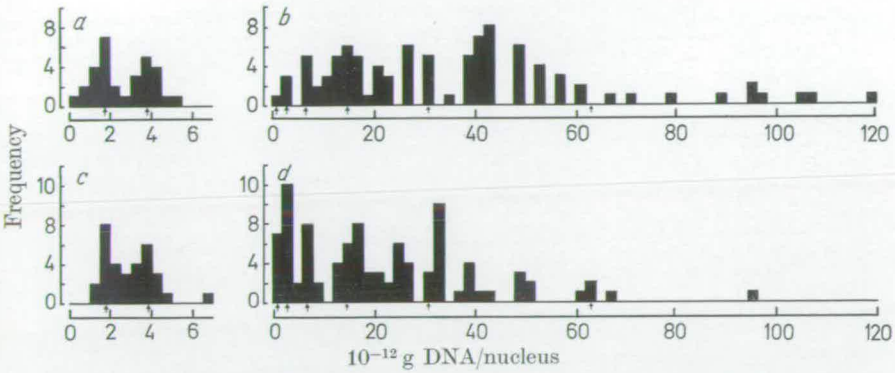


Fig. 4. The nuclear DNA content in tissues of cucumber and melon. Nuclei were isolated from root tip (a) and fruit tissue (b) of cucumber and root tip (c) and fruit tissue (d) of melon, and DNA content was determined by Feulgen spectrophotometry. The arrows indicate the positions of a doubling series based on the 2c and 4c values from the root nuclei

constant at 8%, much less than that involved during their development in the seed (25%).

There was a similar decrease in the percentage of satellite DNA during the development of the first leaf, from 24 to 16% (Fig. 3b). The mechanism of the decrease was, however, quite different from that in the cotyledons, since there was no large initial amount of DNA present in the leaf (Fig. 3d). The change in percentage satellite therefore reflected the different rates of accumulation of satellite and mainband DNAs. Initially satellite DNA was accumulated at 24% of the total, but between 15 and 20 days there was a relative increase in the rate of accumulation of mainband, with consequent decrease in the percentage satellite. These data suggest, therefore, differential replication of mainband and satellite DNAs during the development of the first leaf.

Nuclear Development in Fruit Tissue

An attempt was made to correlate the difference in percentage satellite DNA between meristematic tissue and mature fruit tissue with the state of nuclear development. Root tips (and seed cotyledons) from cucumber and melon showed a bimodal distribution of 2c and 4c nuclei, (Fig. 4a and c), the 2c nucleus containing approximately 2×10^{-12} g of DNA. The nuclear complement of the cucumber fruit was very different, with few 2c and 4c nuclei, the majority being between 16c and 64c (Fig. 4b). The melon fruit similarly contained a high proportion of polyploid nuclei (Fig. 4d). These distributions suggest that the lower

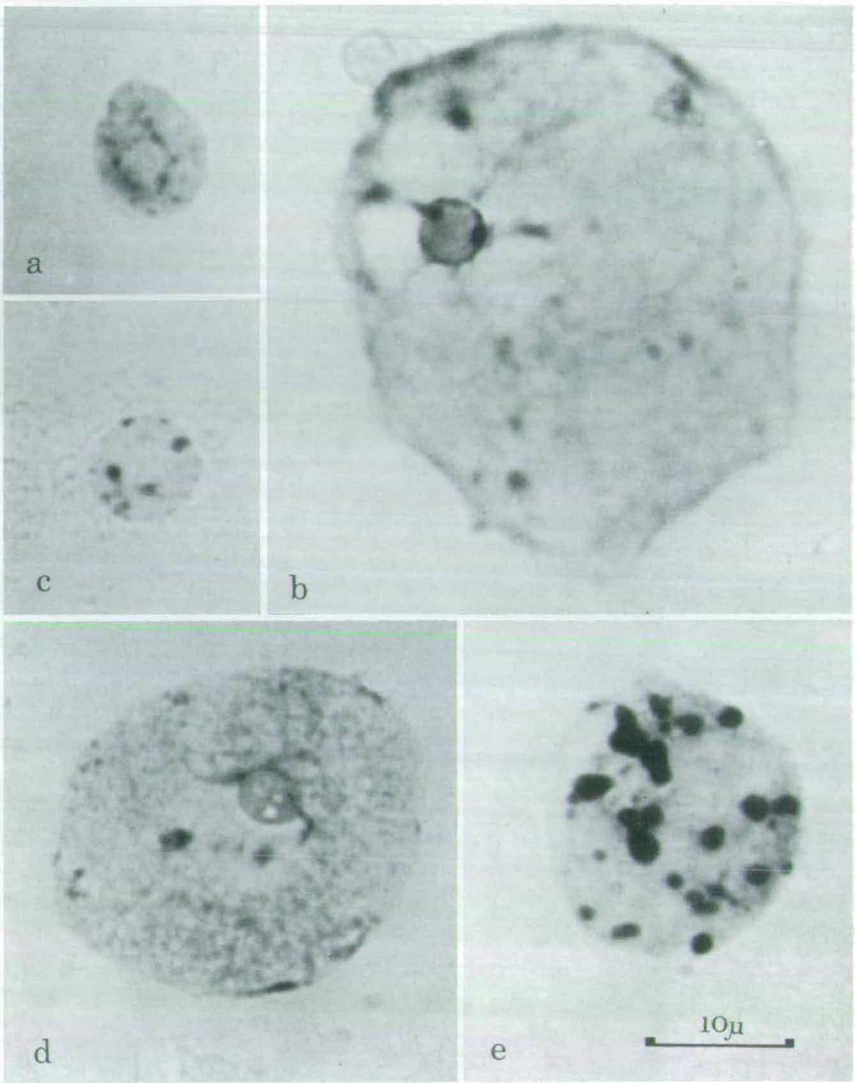


Fig. 5. Nuclei isolated from cucumber and melon tissues. Feulgen stained nuclei from melon root tip, 4c (a); melon fruit, 32c (b); cucumber root tip, 4c (c); cucumber fruit, euchromatin 16c heterochromatin 4c (d), euchromatin 16c heterochromatin 32c (e)

percentage satellite DNA present in the fruit may be due to the higher ploidy levels of these nuclei (compare Fig. 5a with b, and c with d, and e). This would be consistent with the lower percentage of satellite present

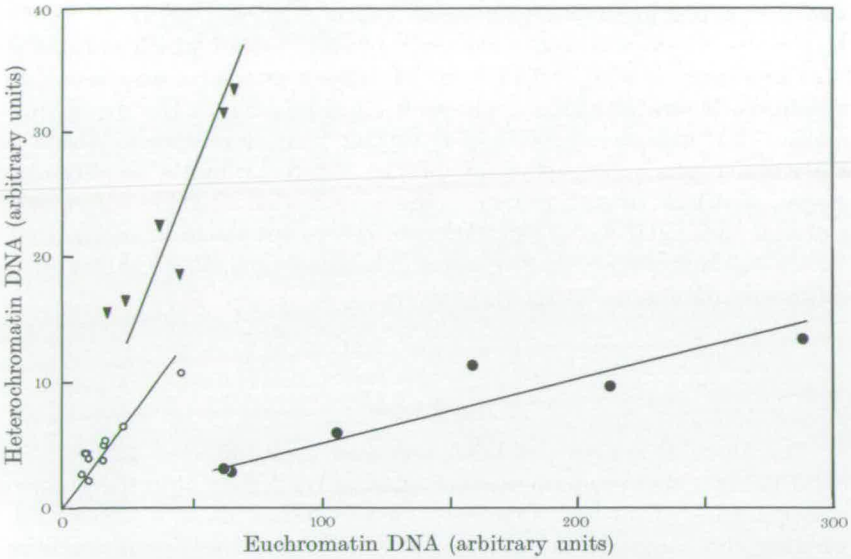


Fig. 6. Heterochromatin content of nuclei isolated from cucumber tissues. Nuclei were isolated from root tips and fruit, stained with Feulgen and photographed. The heterochromatin and euchromatin distribution was estimated from the area of heterochromatin and the different densities of the euchromatin and heterochromatin determined from microdensitometer scans of photographs of 9 root tip and 12 fruit nuclei. The root nuclei contained 25% heterochromatin (-o-). Fruit nuclei fell into two classes, containing 37% (-▾-) and 5% (-•-) heterochromatin

in mature cotyledon and leaf tissue, which both contain a higher proportion of polyploid nuclei.

Although the melon fruit nuclear distribution contained peaks corresponding to the doubling series of the diploid nucleus, many nuclei were intermediate between the 16 and 32c and the 32 and 64c values (Fig. 4d). The cucumber showed a more continuous distribution, with the bulk of the nuclei being outside of the doubling series. particularly those above the 16c value (Fig. 4b). One possible reason for this observation is that, even in the mature fruit, nuclei were still replicating their DNA in which case final ploidy levels would not have been reached. However, the continuous distribution of DNA content in cucumber fruit nuclei was due, at least in part, to the presence of two or more classes of nuclei differing in heterochromatin content (compare Figs. 5d and e). The semiquantitiative estimations on root tip nuclei indicated approximately 25% heterochromatin. The majority of the fruit nuclei (95%), however, contained only 5% heterochromatin (Fig. 6). From a comparison with the heterochromatin-euchromatin distribution in root tip 2c and 4c nuclei it was calculated that whereas the euchromatin in these fruit

nuclei was 16c to 64c, the heterochromatin was only at 2c or 8c respectively. There was also a minority of fruit nuclei which contained 37% heterochromatin, and such nuclei appeared to have undergone an additional heterochromatin replication (16c) relative to the 8c euchromatin. This analysis suggests that during fruit development there is both under and over-replication of the heterochromatin in different nuclei, with the overall change being a reduction of heterochromatin material. A similar under-replication of heterochromatin in melon fruit nuclei was indicated (Fig. 5b), but the heterochromatin areas were too diffuse to allow a quantitative analysis.

Discussion

The Model E analyses of DNA prepared from different tissues of a plant indicate that the proportion of satellite DNA present in the genome varies during plant development. These studies do not distinguish whether this variation arises from differential synthesis or differential stability of satellite and mainband DNAs. Although stability to DNA is normally assumed, the apparent reversion of heterochromatin-rich to normal nuclei in orchid tissue treated with hydroxyurea to inhibit DNA synthesis (Nagl, 1972) could be interpreted in terms of heterochromatin turnover.

There is a general indication that meristematic cells contain a higher proportion of satellite DNA than those cells which have undergone endopolyploidization during tissue development. This would be consistent with a role for satellite DNA in chromosome contraction and organisation during mitosis (and meiosis) (Walker, 1971). Differential replication of satellite DNA therefore appears to be associated with general nuclear development, and is not restricted to the formation of polytene nuclei, as may be inferred from the *Drosophila* studies.

The magnitude of the variation in the percentage of satellite DNA, and the small but significant changes in buoyant density of the satellite, suggest that differential replication may be preferential for certain sequences within the satellite fraction. Renaturation studies on melon, cucumber, flax and bean satellites have indicated the presence of at least two components, differing in their renaturation rates, within the satellite peak (Ingle, Pearson and Sinclair, 1973). The variation in buoyant density of the mainband DNA suggests that differential replication is not limited to those repetitious sequences in the satellite peak. This would be consistent with the observation of under-replication of mainband repetitious sequences during the production of polytene nuclei in *Drosophila* (Hennig, 1972).

Although the chromosomal distribution of satellite DNA has not been established in plants, from analogy with animal cells it is probably associated with heterochromatin. Certainly all those plant species containing satellite (Ingle *et al.*, 1973) do contain heterochromatin, although the reverse is not true, even within dicotyledonous species. Satellite DNA was not observed in any of the monocots examined. Although many monocotyledons contain heterochromatin it would appear to be of a different type from the centromeric form observed in the majority of dicotyledons (Dyer, 1964). The lower percentage of satellite DNA in the polyploid fruit tissue may be correlated with the smaller percentage of heterochromatin in these nuclei. The correlation is certainly not quantitaitve, since satellite DNA decreases from 41 to 34% compared with a change from 25 to 8% heterochromatin (after correcting for the two populations of nuclei in the cucumber fruit). This indicates that not all heterochromatin contains satellite DNA, and a similar conclusion, that heterochromatin cannot always be equated with repetitious sequence DNA, was reached from studies on the sex chromosomes of *Drosophila* (Hennig, 1972b).

These analyses of satellite DNA and heterochromatin distribution suggest that differential replication may be a common, though complex phenomenon associated with the differentiation of non-dividing cells.

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Dr. G. G. Pearson, Dr. J. N. Timmis, Dr. J. Ingle
Department of Botany
University of Edinburgh
King's Building
Mayfield Road
Edinburgh EH9 3JH
Scotland, U.K.