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Relation between ribosomal RNA genes and the DNA satellites of *Phaseolus coccineus*

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The main band DNA of *Phaseolus coccineus* has a buoyant density of 1.692 g/ml. In roots, shoots, integuments and suspensors there is a DNA satellite with a buoyant density of 1.700 g/ml. The satellite of the roots, shoots and integuments represents approximately 28.2 %, 29.4 % and 34.7 % respectively of the total DNA. In suspensors, where polyteny occurs, besides the 1.700 g/ml satellite there is a second one at 1.696 g/ml. They represent about 32.9 % and 13.1 % of the total DNA.

H^3-25S and H^3-18S ribosomal RNA of *Phaseolus coccineus* were hybridized separately with DNA of shoots from CsCl gradient fractions. In both hybridizations the peak of labelling coincides with the position of the DNA satellite with a buoyant density of 1.700 g/ml. Thus the genes for 25S and 18S are mainly located in this DNA component.

Hybridization experiments at saturation inputs of H^3-25S ribosomal RNA with DNA of shoots, integuments, roots and suspensors give saturation values of 0.72 %, 0.64 %, 0.51 % and 0.42 % respectively.

The lower saturation value in the suspensors may indicate an underreplication of ribosomal genes in this tissue. This is partly cancelled out by the amplification in another DNA: that of the second satellite at 1.696 g/ml which does not seem to be part of the ribosomal DNA.

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In animals the amount of DNA, the number of given genes and the type of DNA sequences have been studied in different cell types and tissues of many organisms in an attempt to bring the study of cell differentiation to the molecular level. Repetitive DNA and gene redundancy is a feature observed in many species (reviews in BRITTEN and DAVIDSON 1971; WALKER 1971 and FLAMM 1972). Gene amplification has been mainly studied in two organisms: *Xenopus* (reviews in BROWN and DAWID 1968; GALL 1969, BIRNSTIEL et al. 1971) and in *Acheta* (LIMA-DE-FARIA et al. 1969; CAVE 1972; PERO et al. 1973; LIMA-DE-FARIA 1974). Gene magnification occurs in *Drosophila* (RITOSSA 1973) and sequence analysis of DNA has been carried out in several species (SOUTHERN 1973; GALL and ATHERTON 1974; PEACOCK et al. 1974).

In plants a similar study has been delayed mainly due to difficulties in extracting DNA caused primarily by the presence of cellulose cell walls in these organisms. But plants are yielding other types of molecular information so far not avilable from animal tissues (STERN and HOTTA 1969; AVANZI et al. 1972, 1973, INGLE et al. 1973; SCOTT and INGLE 1973).

In the present paper we report on the occurrence of two DNA satellites in *Phaseolus coccineus* and their relation to ribosomal RNA genes. In this species the chromosomes of the suspensor cells show a high degree of polyteny (NAGL 1962). In connection with this phenomenon the number of ribosomal genes was investigated in four different tissues.

Material and methods

Four different tissues of *Phaseolus coccineus* were used in the present work: roots, shoots, integuments and suspensors. The roots and shoots

were collected from seedlings. The integuments were obtained from adult plants and the suspensors were dissected out by hand under the large field binocular microscope. The suspensors are nearly microscopic each measuring on the average 300 × 600 microns. For each DNA extraction 1,000 to 2,000 suspensors were used and over 10,000 were employed in the present work. The suspensors and integuments were used frozen or lyophilized, the other tissues were used fresh or frozen (−28°C).

1. DNA extraction from nuclei of roots, shoots and integuments

A. *Isolation of nuclei*

DNA has been prepared from these three tissues from isolated nuclei rather than from whole cells to avoid contamination with mitochondria or chloroplast DNA.

The method of KIRK et al. 1970, was followed, with some modifications, to isolate the nuclei. The frozen tissue was homogenized in the Sorvall Omni-mixer (140 seconds) in 0.4 M Sucrose, 0.05 M Tris and 10 m M NaCl (pH = 7.8). The solution volume was about twice that of the tissue. The homogenate was strained through a double layer of muslin and then centrifuged at 300 g for 2 minutes in the Sorvall Superspeed RC2-B. The pellet (cell fragments) was discarded and the supernatant was centrifuged once more at 600 g for 10 min. The pellet thus obtained was resuspended in 10 ml of: 0.5 M Sucrose, 0.05 M Tris, 0.1 M EDTA (pH = 7.23) containing 3.3 percent (v/v) of the non-ionic detergent, Triton X-100. Triton X-100 is known to selectively disrupt chloroplasts but not nuclei (SPENCER and WILDMAN 1964). The suspension was centrifuged for 10 minutes at 600 g and then the pellet (nuclei plus starch grains) was washed twice in the same medium by careful resuspension on the Whirlimixer followed by centrifugation (at 6,000 g for 15 minutes). The final clean pellet and supernatant were centrifuged at 12,000 g for 40 minutes. The DNA was extracted from the nuclei present in this last pellet. All steps were carried out at 0–4°C.

B. *DNA extraction*

The DNA was extracted by homogenizing the nuclear pellet (on ice or in the cold room at 4°C) in 2 ml Kontes glass homogenizers with 0.4 M sucrose, 0.003 M $CaCl_2$, 0.1 M NaCl. An equal volume of saline-EDTA (0.2 M EDTA, 0.1 M NaCl) is added after homogenization. To the suspension was added 25 % sodium dodecyl sulfate (0.08 ml; this volume and those of the following solutions refer to 1 ml of homogenized material). This solution was incubated at 60°C for 15 min. After chilling the solution to room temperature 0.5 ml of 5 M $NaClO_4$ and 0.5 ml of 1 M Tris pH = 8.0 were added. Subsequently the mixture was extracted with 3 ml of chloroform-amyl alcohol (24: 1), shaken gently for 30 minutes and centrifuged for 10 minutes at 12,100 g in a Sorvall Superspeed RC2-B centrifuge. To the supernatant was added 2 1/2 volumes of 99 % ethanol (MARMUR 1961). The DNA was collected on a glass rod and dissolved in 0.1 × SSC. Previously boiled RNase (1 mg/ml) was added and allowed to act overnight in 1 × SSC at 20°C. α-amylase was subsequently added to the DNA in 1 × SSC (100 μg/ml) acting for 2 hours at room temperature. Predigested pronase (100 μg/ml) acted on the DNA solution for 3 hours at 35°C. After that this solution was dialysed overnight in 1 × SSC and was subsequently gently shaken for 10 min. with an equal volume of chloroform-amyl alcohol (24: 1) centrifuged at 12,000 g for 10 min. This step was repeated two or three times. The DNA was precipitated with 2 1/2 volumes of 99 % ethanol, collected on a rod and dissolved in 0.1 × SSC. After this procedure the DNA was purified by fractionation in CsCl gradients.

C. *Isopycnic centrifugation of DNA in CsCl*

DNA was dissolved in 2 ml of 0.1 × SSC containing 0.2 ml of 2.5 % SDS. To 5.1 g of CsCl (Analar) were added the DNA solution and 0.1 × SSC to give a final weight of 9.10 g (density 1.69–1.70 g/ml). The samples were overlaid with liquid paraffin (specific gravity 0.830–0.870) and centrifuged for 65 hours at 42,000 rpm and 25°C in the No. 50 angle rotor of the L2-65B Beckman Ultracentrifuge (FLAMM et al. 1966, 1969). 10 drop fractions were collected by puncturing the bottom of the centrifuge tube (approximately 25 fractions). Each fraction was diluted with 0.5 ml of 0.1 × SSC and its optical density at 260 mμ was determined in the Beckman Acta III Spectrophotometer. The fractions containing the main

band DNA (buoyant density 1.692 g/ml) and the heavy satellite DNA region (buoyant density 1.700 g/ml) were collected, pooled and dialysed at 4°C in 0.1 × SSC overnight. As an extra check the DNA solutions were assayed for RNase activity as described in PERO et al. (1973). No RNase activity was present in the DNA solutions. This DNA was loaded on filters for the hybridization experiments at saturation. DNA fractionated from CsCl gradients was also used to hybridize the DNA of every fraction with 18S and 25S RNA. In this case the DNA of each fraction was loaded on filters.

D. *DNA extraction for analytical ultracentrifugation*

The DNA isolated from nuclei of shoots and roots and purified on CsCl gradients in the preparative ultracentrifuge as described above under A), B) and C) was analysed in the analytica ultracentrifuge Model E. Also total DNA extracted from shoots, roots and integuments by the modified Marmur procedure was analysed in the Model E.

2. DNA extration from suspensors

Due to their microscopic size and the difficulties involved in collecting large numbers of suspensors the DNA of this tissue had to be prepared following other procedures.

A. *Marmur's method*

The method described above for roots, shoots and integuments, was used but without previous isolation of nuclei. In the case of suspensors, the extraction took place directly from the tissues. This DNA was also treated with RNase, α-amylase and pronase, but due to the small amount of DNA which was collected no purification on CsCl gradients was carried out. Instead the DNA was cleaned carefully four times with water saturated redistilled phenol (containing 0.1 % 8-hydroxyquinoline) and after precipitation in alcohol was dialysed over night in 0.1 × SSC (at 4°C). This DNA was used in the first analytical ultracentrifugation studies and in the hybridization with H^3-25S RNA.

B. *Grisvard and Guille's method*

The DNA extraction method of GRISVARD and GUILLÉ (1973) was followed with minor modifications.

Circa 1,500 suspensors (0.057 g of lyophilized tissue) were ground in a mortar. The material was suspended and agitated with a magnetic stirrer for 3 hours at room temperature in 20 ml of 4 % (w/v) sodium dodecyl sarcosinate, 0.08 M EDTA and 5 % (v/v) ethanol (SEE), pH = 7.8. Solid NaCl was then added until a 2 M concentration was reached and this solution was kept under agitation for 5 hours. Dialysis took place overnight in a solution of 1 × SSC at 4°C. The mixture was incubated with RNase (Sigma, Type A bovine pancreas) at a concentration of 100 μg/ml for 1 hour at 37°C in a dialysis tube immersed in 1 × SSC. This was followed by pronase from Koch-Light Laboratories, England (previously digested for 2 hours) at a concentration of 500 μg/ml, overnight at room temperature (dialysed in 1 × SSC). The solution was centrifuged at 30,000 g in the Sorvall Superspeed RC2-B for 10 minutes at 4°C. The supernatant was collected, the pellet was washed with 1 × SSC, centrifuged again and both supernatants added and dialysed for 1 hour in 0.1 × SSC at 4°C. The volume was reduced by evaporation in a dialysis tube at 4°C and the solution was once more dialysed in 0.1 × SSC for 25 hours. After that the DNA was purified by CsCl gradient centrifugation in the L2-65B Beckman ultracentrifuge at 15°C for 24 hours at a speed of 42,000 rpm. The fractions were pooled, dialysed in 0.1 × SSC for 1 hour, the volume was reduced by evaporation at 4°C and the solution was dialysed once more against 0.1 × SSC for 1 hour. The DNA sample was then prepared for CsCl gradient centrifugation in the second series of studies carried out with the analytical ultracentrifuge Model E as described below.

C. *Extraction including Triton-X*

The DNA was extracted following the modified procedure of GRISVARD and GUILLÉ (1973) just described but in this case Triton-X-100 was introduced into the method. After the material had been agitated for 3 hours in SEE (20 ml), sucrose (0.5 M) and Triton-X-100 (3.3 %) were added and the solution stirred for 10 minutes.

Solid NaCl was then added, the rest of the procedure being as described above. The DNA was also purified on preparative CsCl gradients and after the steps mentioned above was prepared for the Model E analytical ultracentrifugation (3rd series of runs).

3. RNA labelling and extraction

Hypocotyls of sterile darkgrown five-day-old *P. coccineus* seedlings were excised. Ten grams of this tissue were grown in 100 ml of sterile White's medium containing 3.5 mCi of 5-H^3-uridine (The Radiochemical Centre, Amersham, specific activity 24 Ci/m mol). The culture was kept under agitation at 25°C in the dark for 3 days, after which time the hypocotyls were washed in sterile distilled water and transferred to 100 ml of fresh White's medium containing nonradioactive uridine in a concentration 100-fold that of the radioactive precursor. The tissue was allowed to develop further for 6 hours.

For RNA isolation, the method of KIRBY (1965) was followed with modifications. The hypocotyls were rinsed with distilled water and homogenized in a mortar with Tris buffer (0.1 M, pH 7.6) and sodium dodecylsulphate in a 1: 1 ratio (v/w). To the homogenate an equal volume of water-saturated redistilled phenol was added and the mixture was stirred for 30 minutes at 5°C and then centrifuged at 7000 r.p.m. for 10 minutes. The supernatant was removed, an equal volume of phenol added and the mixture centrifuged again. H^3-RNA in the supernatant was precipitated with two volumes of cold ethanol and NaCl 0.1 M and the mixture was kept at −20°C overnight. The H^3-RNA was collected by centrifugation and dissolved in Tris buffer at pH 7.6. Further treatments with phenol and precipitations with ethanol were made until a 2: 1 ratio of A_{260}:A_{280} was ascertained in a spectrophotometer.

4. Sucrose gradient centrifugation

The *Phaseolus* H^3-RNA was layered onto a linear 5 to 40 % sucrose gradient made up of 0.1 M NaCl, 0.001 M EDTA, 0.02 M Na-acetate pH = 5.0. The tubes were centrifuged in an L2-65B Beckman Ultracentrifuge employing the rotor SW 25.2 at 25,000 rpm at 4°C for 18 hours. Fractions (2 ml) were collected through a hole pierced in the bottom of the tube and the optical density at 260 mμ determined. The heavier peak (25S) of *Phaseolus* RNA contained approximately twice as much RNA as the 18S peak (Fig. 2). Five μl aliquots were taken from each fraction of the gradient and were pippetted over Whatman GF/C filters. These were dried under infra-red light for 20 minutes and put in bottles with liquid scintillation fluid (toluene-PPO-POPOP). The peaks of radioactivity coincided with the optical density values (Fig. 2). The RNA from the 25S and 18S fractions was precipitated separately in ethanol and these RNA components were further cleaned (LIMA-DE-FARIA et al. 1969) by adding an equal volume of phenol-hydroxyquinoline to the RNA previously dissolved in 0.15 M NaCl, 0.05 M Tris pH = 7.5, and shaking the solution at 70°C for 3 minutes. After cooling the mixtures on ice the 25S and 18S RNA were precipitated again in ethanol and after centrifugation washed 3 times with 99 % ethanol. The pellets were dissolved in 0.1 × SSC. The specific activity of *Phaseolus* 25S and 18S H^3 RNA varied from 40,000 to 43,000 cpm/μg.

5. Molecular annealing on filters

In the saturation experiments the same amount of DNA from a given tissue was loaded on every filter. Depending on the experiment and tissue, 12 to 20 μg of DNA were used per filter. HAWP Millipore filters (HA, 0.45 μ, 13 mm) were presoaked overnight in 2 × SSC. During all steps plastic gloves were used. In every experiment four control filters without DNA were treated in the same way as those containing DNA to check the background. The procedure used to load the DNA on filters was that of Gillespie and Spiegelman (1965) with minor modifications. To each 1.0 ml fraction of DNA in 0.1 × SSC was added 1.0 ml of 1 N NaOH by mixing vigorously. The fractions were allowed to stand for 30 minutes. The filters were mounted on Millipore filter holders (No. XX3001203) to which was added a plastic cylinder with a 6 ml capacity. The filters were rinsed once more with 2 × SSC. The samples were neutralized by the addition of 4 ml of a medium containing 1.0 N HCl, 1.0 M Tris pH = 8.0, and 3 M NaCl in a ratio 1: 1: 2, and immediately poured into the filter holders. The solution was filtered under gravity.

To reduce background the filters were treated according to DENHARDT (1966). Each filter was washed for 10 minutes in 3×SSC and then incubated for 1 hour and 30 minutes at room temperature in a mixture of 0.02 % Ficoll (Pharmacia), 0.02 % Polyvinylpyrrolidone (Sigma), 0.02 % Bovine Albumin (Armour, Fraction V) 0.2 g of each in 1 liter of 3×SSC. The filters were dried in the vacuum oven for 2 hours at 80°C. These were either used immediately for molecular hybridization or were stored at −20°C in containers over blue silica gel.

The following 25S RNA concentrations were prepared: 4, 2, 1, 0.5, 0.25 and 0.125 μg/ml and for 18S: 7.0, 3.5, 1.8, 0.9, 0.4 and 0.2 μg/ml. When non-radioactive 25S RNA was added to H^3-18S RNA their amounts were 2: 1 respectively. The molecular hybridization was carried out according to MCCONAUGNY et al. (1969) the incubation mixture consisting of RNA in 5×SSC and 50 % formamide. The molecular hybridization took place in plastic tubes with a central rod which can accommodate up to 5 filters in a volume of 1 ml (QUETIER, pers. comm.) for 17 hours at 45°C (MILLER and KNOWLAND 1970). The hybridization tubes had well-fitting stoppers which prevented evaporation. After the hybridization the filters were washed twice in a 50 % formamide, 5×SSC mixture at 45°C for 10 minutes and subsequently washed 3 times in 2×SSC during stirring (at room temperature). The filters were incubated with RNase A (20 μg/ml) in 2×SSC at 37°C for 1 hour, rewashed as before and dried over Kleenex and in the vacuum oven at 80°C for 10 minutes. The radioactive samples were counted in 5 ml of toluene-PPO-POPOP mixture. Three times 20 minute-counts were taken for every sample and the values averaged. The four control filters, without DNA, which were treated in the same way (prepared for every experiment) had a background which averaged 10 cpm and did not exceed 15 cpm. A Packard Tri-Carb Liquid Scintillation Spectrometer Model 3320 is used. All the results were corrected for counts sticking to the blank filters. The amount of DNA which remained on the filters after hybridization was assayed by the method of BROWN and WEBER (1968) which involves acid hydrolysis. The filters were washed with chloroform 3 times, dried and hydrolized in 1.0 N HCl for 15 minutes at 100°C in stoppered test tubes. The optical density at 260 mμ of 1 mg hydrolyzed DNA/ml in 1.0 N HCl is 27.8. The measurements were made in a Beckman Spectrophotometer ACTA III.

6. Analytical ultracentrifugation

An analytical ultracentrifuge Beckman Model E which is equipped with a monochromator and electronic speed control is used. Two single 4° sector cells, with 12 mm KEL-F centerpieces, are placed in a rotor AN-D. The two cell assemblies have quartz windows. The upper windows are minus 1° (green), and of the lower ones, one is flat (black) and the other is a side wedge 1° up (blue) according to SZYBALSKI (1968). *Micrococcus lysodeikticus* DNA was prepared from actively growing cultures by the method of MARMUR (1961) and was used as a marker (buoyant density 1.731 g/ml). 1.7 μg *Micrococcus* DNA was added per analytical cell. *Phaseolus* DNA solutions containing amounts of DNA ranging from 7.8 to 17.0 μg had a refractive index of 1.3990–1.4008. CsCl of optical grade from the Harshaw Chemical Company, Cleveland, Ohio was employed in preparing this solution (MANDEL et al. 1968). The density gradient centrifugation was carried out at 44,000 r.p.m. at 25°C for 22 hours. The wave length was set at 265.4 mμ since this is the emission maximum of the U.V. light source. Photographs were taken with Kodak Commercial Fine Grain Film CF7. Tracings of the negatives were made on a Beckman Acta III spectrophotometer, after its gel scanner had been adapted for this purpose. An inexpensive film holder was built in the workshop for tracing the films from the analytical ultracentrifuge in the Acta III. The magnification of the tracing was calculated by separate centrifugation using a counterbalance. Buoyant densities and GC content of the DNA were calculated according to MANDEL et al. (1968).

Abbreviations. rRNA, ribosomal RNA; rDNA, DNA which contains the segments (cistrons) coding for 28S (25S in plants) and 18S ribosomal RNA together with sequences not homologous to this RNA ("spacer" and/or other DNA stretches accompanying these cistrons); SSC, 0.15 M NaCl, 0.015 M Na Citrate, pH = 7.0; SDS, sodium dodecyl sulfate; toluene-PPO-POPOP (5 g 2.5 diphenyloxazole and 0.3 g 1.4 bis [2-(5-phenyl-oxazolyl)]-benzene in 1 litre of toluene); TCA, trichloroacetic acid; EDTA, ethyl-

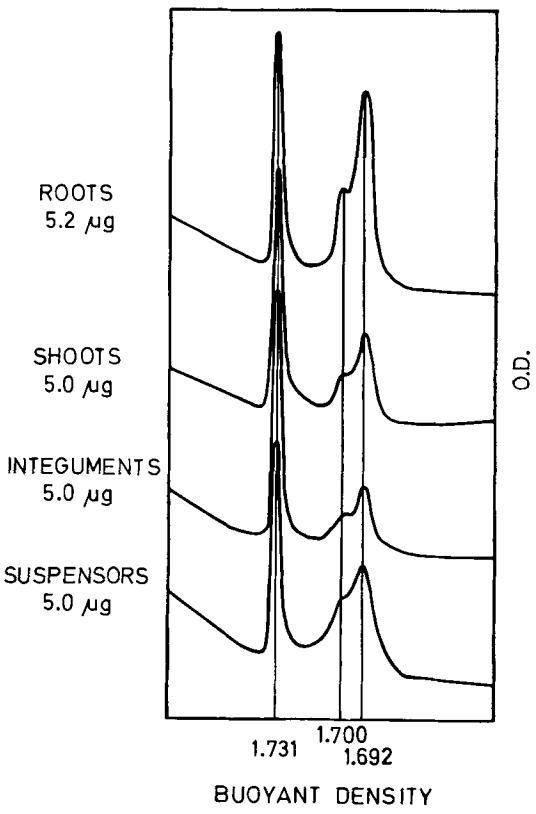

Fig. 1. *Phaseolus coccineus* DNA from roots, shoots, integuments and suspensors was centrifuged to equilibrium in CsCl at 44,000 rpm at 25°C for 22 hours in the model E. Beckman ultracentrifuge. The density marker is *Micrococcus lysodeikticus* (1.731 g/ml). The main band of *P. coccineus* DNA has a buoyant density of 1.692 g/ml and the DNA satellite present in the four tissues has a buoyant density of 1.700 g/ml. That this satellite contains the cistrons for ribosomal RNA is shown in Figs. 3 and 4. O.D., optical density. The DNA employed in this centrifugation was total DNA.

enediaminetetraacetate; O.D., optical density; SEE, 4 % (w/v) sodium dodecyl sarcosinate, 0.08 M EDTA and 5 % (v/v) ethanol pH = 7.8.

Results

1. Characterization of *Phaseolus coccineus* DNA

In preparative CsCl gradients, with inputs of DNA up to 5.0 O.D., the DNA of *P. coccineus* forms one main band when centrifuged to equilibrium. However, in the analytical ultracentrifuge, besides the main band, a large satellite, heavier than the main band is present. This satellite is so close to the main band that it forms a shoulder. The buoyant density of the main band is 1.692 g/ml and that of the satellite is 1.700 g/ml, corresponding to 32.7 % and 40.8 % guanine plus cytosine respectively.

This satellite is found in the DNA of roots, shoots, integuments and suspensors and it has the same buoyant density in these four tissues (Fig. 1). The DNA used in these experiments was total DNA prepared by the modification of the Marmur method described above.

The magnitude of the satellite does not seem to be the same in all tissues. In roots the satellite is circa 25.5 % of the total root DNA and in shoots, integuments and suspensors this figure is circa 31.8 %, 34.7 % and 38.0 % respectively. The satellite in the suspensors is about 1.5 times larger than that found in the roots. These estimates are approximate because the satellite is closely located to the main band and because there is a variation in the molecular weight of the DNA from different tissues.

2. Conditions of molecular hybridization

DNA-RNA hybridization experiments were carried out to find out: (1) whether the ribosomal genes were present in this satellite, and (2) to determine the amount of DNA homologous to ribosomal RNA (25S and 18S, i.e. the 1.3 and 0.7×10^6 Mol.Wt. rRNAs).

Hybridization was performed between DNA of different tissues and rRNA in the following way. (1) The DNA from the fractions of CsCl gradients was hybridized with a constant amount of ribosomal RNA. (2) A constant amount of DNA was fixed on membrane filters whereas the amount of RNA varied. (3) The hybridization with 25S and 18S RNA species was analysed in separate experiments.

The 25S, 18S and 5 + 4S peaks of RNA were well defined in the sucrose gradients. Moreover, the radioactivity peaks coincided with the optical density values. The ratio of 25S to 18S RNA was about 2: 1, an indication of little degradation of the RNA (Fig. 2). Another indication of the purity of the RNA was furnished by the hybridization experiments (see below).

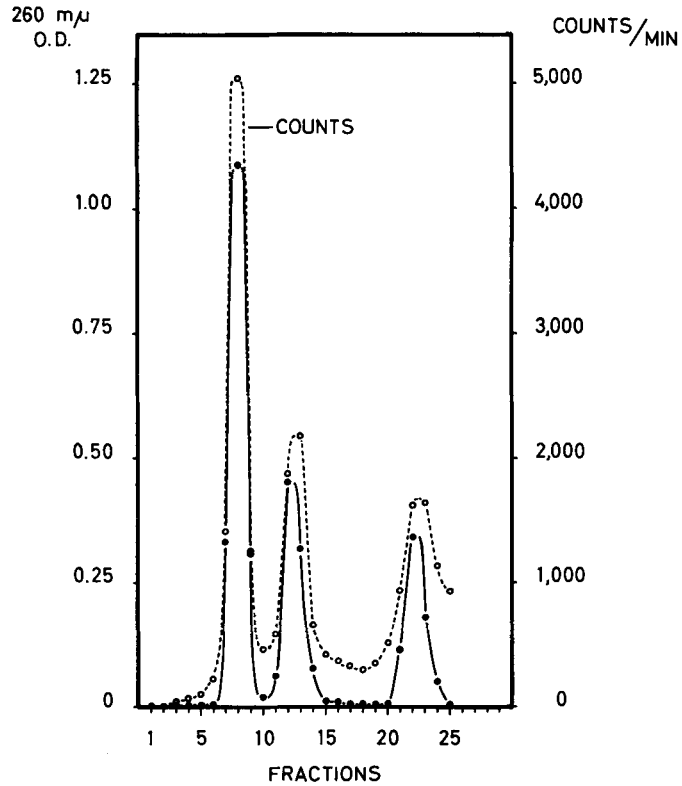

Fig. 2. *Phaseolus coccineus* RNA fractionated on sucrose gradient 5 to 40 %. Solid line = optical density, dashed line = H^3 counts. Centrifugation in L2-65B Beckman ultracentifuge, rotor SW25.2 at 25,000 rpm at 4°C for 18 hours. From left to right 25S, 18S and 5+4S RNA components. The counts per min. are from 5 μl aliquots.

To minimize the thermal degradation of the nucleic acids, formamide was included in the hybridization mixture (McConaughy et al. 1969).

To decrease background the DNA containing filters were treated with a solution described by Denhardt (1966), which prevents non-specific sticking of single strand nucleic acids to the filters. When after rRNA-DNA hybridization single filters showed up as much as 800 c.p.m., the blank control filters treated similarly throughout the whole hybridization process did not display more than 15 counts per minute.

3. Hybridization of ribosomal RNA with the DNA from CsCl gradient fractions

Phaseolus coccineus DNA extracted from nuclei of shoots was fractionated together with *Micrococcus lysodeikticus* DNA (used as a marker) in CsCl gradients in the preparative ultracentrifuge. The DNA from fractions 5 to 20 was fixed to membrane filters and hybridized with radioactive *P. coccineus* 25S RNA (Fig. 3). The peak of labelling coincides with the position of the DNA satellite of buoyant density 1.700 seen in Fig. 1. Thus the genes for 25S are mainly located in this DNA component.

If DNA from the same source is used for a similar experiment but the RNA used in the hybridization is H^3-18S the result confirms the previous location of the ribosomal genes, showing the peak of hybridization at the position of the DNA satellite (Fig. 4).

These experiments also disclose that the RNAs employed have negligible amounts of DNA-like RNA since the hybridization curves are in both

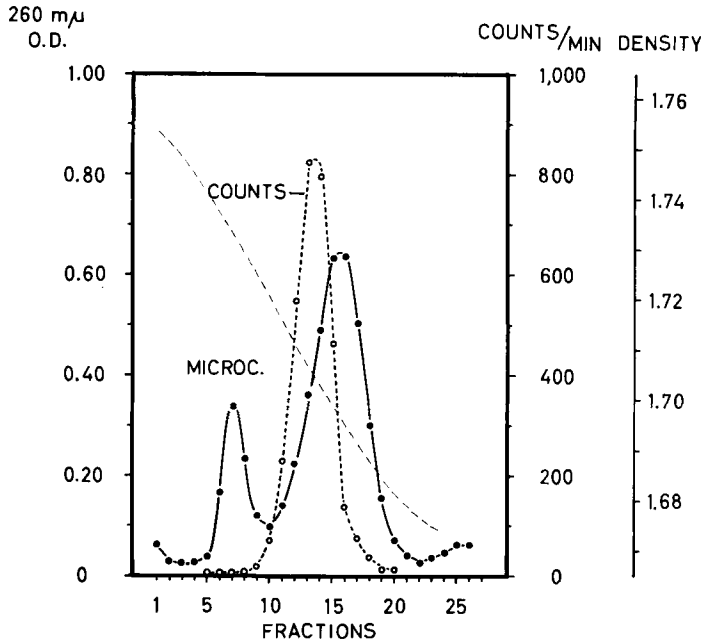

Fig. 3. Hybridization between unlabelled DNA of *P. coccineus* shoots, *Micrococcus lysodeikticus* DNA and 25S H^3 labelled RNA of *P. coccineus*. Fractions 5 to 20 of the CsCl gradient were selected for hybridization with 25S RNA. The hybridization curve peaks at fractions 13—14. This is the position which corresponds to the location of the DNA satellite seen in the analytical centrifuge, where the DNA of *Micrococcus lysodeikticus* was also used as a marker (Fig. 1). Gradient centrifugation in the L2-65B ultracentrifuge No. 50 angle rotor, 42,000 rpm, 25°C, 65 hours. Hybridization conditions 5×SSC, 50 % formamide, 45°C, 17 hours, O.D., optical density.

cases symmetric showing an almost equal number of counts in the region of the *Micrococcus* DNA as on the side of the main band *Phaseolus* DNA (Fig. 3, 4).

4. Saturation of DNA with 25S ribosomal RNA

In these experiments four sets of filters containing the same amounts of DNA from: (1) shoots, (2) roots, (3) integuments and (4) suspensors were incubated with increasing amounts of 25S H^3-RNA. In the three first tissues the DNA was extracted from previously isolated nuclei; in the case of the suspensors the DNA was extracted from whole tissue. In all four cases the DNA was thoroughly cleaned through CsCl gradients or through several phenol extractions (suspensors). The saturation values obtained for shoots, integuments, roots and suspensors are 0.72 %, 0.64 %, 0.51 % and 0.42 % respectively (Fig. 5).

These results show that the suspensors which have the highest degree of polyteny have the lowest saturation value.

Since the DNA satellite of high GC-content contains the ribosomal genes, as demonstrated by the hybridization with the gradient fractions, and since the satellite DNA seems to be larger in suspensors than in shoots one would expect the number of genes for 25S RNA to be higher in the suspensors. This does not turn out to be the case. Thus we looked for the source of this contradiction. The simplest answer was that we were not dealing with one DNA satellite but with two which were very closely located: one which

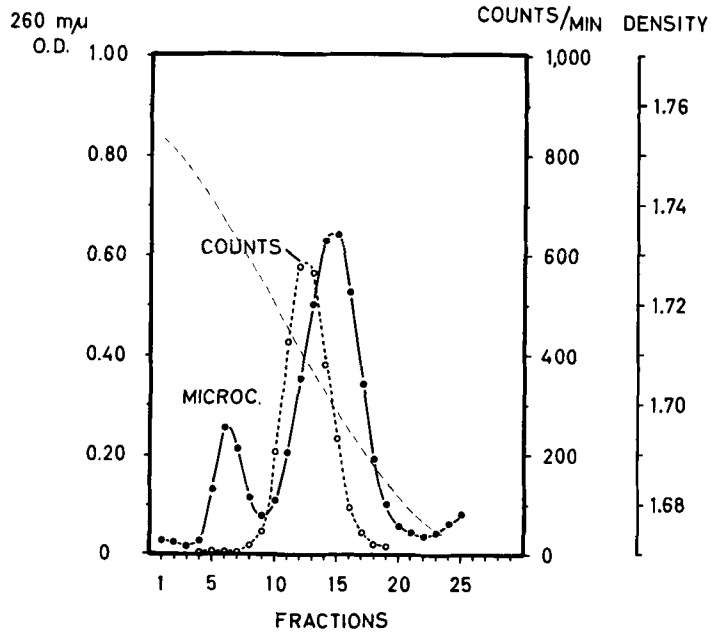

Fig. 4. Same experiment as Fig. 3 but in this case the hybridization was carried out with 18S H^3RNA.

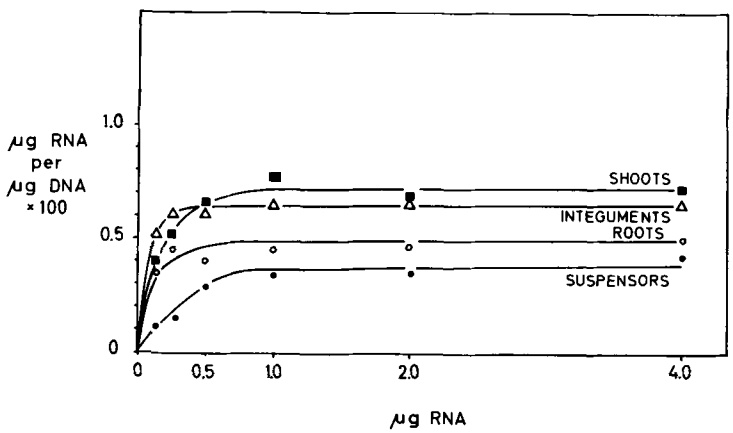

Fig. 5. Hybridization at saturation between H^3-25S *Phaseolus coccineus* RNA and homologous DNA of shoots (squares), integuments (triangles), roots (open circles) and suspensors (solid circles). Filters containing a constant amount of DNA were incubated with increasing concentrations of RNA. The hybridization was carried out with H^3-25S RNA (42,000 cpm/μg) in 5×SSC, 50 % formamide, for 17 hours at 45°C. The amount of DNA on each filter was measured before and after hybridization. Blank filters treated in the same way as those containing DNA averaged 10 cpm and did not exceed 15 cpm.

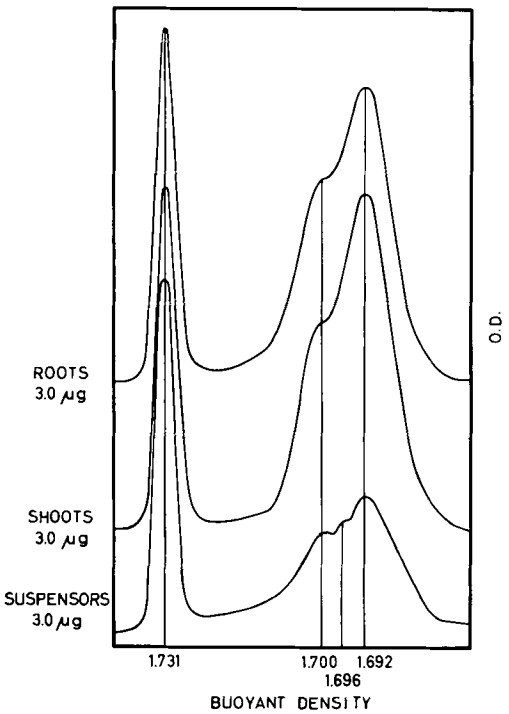

Fig. 6. *Phaseolus coccineus* DNA from roots, shoots and suspensors was centrifuged in the model E ultracentrifuge as in Fig. 1 but in this case the DNA of roots and shoots was prepared from isolated nuclei, and that of suspensors was prepared according to the method of GRISVARD and GUILLÉ (1973). Besides the DNA satellite present in the three tissues (with a buoyant density of 1.700 g/ml) a second DNA satellite appears in the suspensors with a buoyant density of 1.696 g/ml.

contained the ribosomal genes and another present mainly in suspensors which was not part of the ribosomal DNA. This proved to be true.

5. Analysis in the Model E

When DNA is extracted from suspensors following the method of GRISVARD and GUILLÉ (1973), the DNA is better preserved from shearing forces and membrane-bound DNA also seems to be kept relatively intact.

Suspensor DNA extracted by this procedure was run in the analytical ultracentrifuge Model E simultaneously with DNA extracted from isolated nuclei of shoots and roots, which also means an improvement on the earlier centrifugations.

Three micrograms of each tissue DNA, shoots, roots and suspensors, were added to CsCl solutions for Model E ultracentrifugation. The results showed the same satellite DNA found previously in all three tissues, which has a buoyant density of 1.700, but in addition in suspensors a second satellite appeared between this last one and the main band. This second satellite has so far only been identified in suspensors, its buoyant density being 1.696 (Fig. 6).

The 1.700 satellite of the roots and shoots represents approximately 30.8 % and 26.9 % respectively of the total DNA. In the early centrifugations made from total DNA the values were 25.5 % and 31.8 % respectively. The average of these values is 28.2 % for roots and 29.4 % for shoots. This means that this satellite is approximately the same size in both tissues. The error involved arises because the satellite is not heavy enough to appear as a separate entity but remains close to the main band.

The two satellites found in the suspensors with buoyant densities of 1.700 and 1.696 constitute approximately 32.9 % and 13.1 % respectively of the total DNA. This shows that the large satellite found previously in suspensors was a composite of these two satellites. The heavier satellite has the same buoyant density (1.700) and is present in about the same amount in suspensors as in roots and shoots (32.9 %, 28.2 % and 29.4 % respectively). It is the new satellite at 1.696 which makes the total satellite DNA as high as 46.0 % of the total suspensor DNA. The 1.700 satellite is in all probability the one containing the ribosomal genes for three reasons. (1) This is the satellite that has approximately the same size in all tissues and the RNA-DNA saturation values are not higher in suspensors; on the contrary, they are lower. (2) The hybridization of 25S and 18S H^3 RNA with DNA from CsCl fractions in both cases has a position which is closer to the 1.700 than to the 1.696 satellite (2 to 2.5 fractions from the main band). The buoyant density of the fractions showing the hybridization peaks is actually 1.702 which is on the heavier side of the 1.700 satellite. (3) The hybridization with the gradient fractions was carried out with DNA of shoots extracted from nuclei, after triton-X treatment, and this same DNA when studied in the analytical ultracentrifuge shows only the 1.700 satellite (Fig. 6).

Thus, the present evidence supports the con-

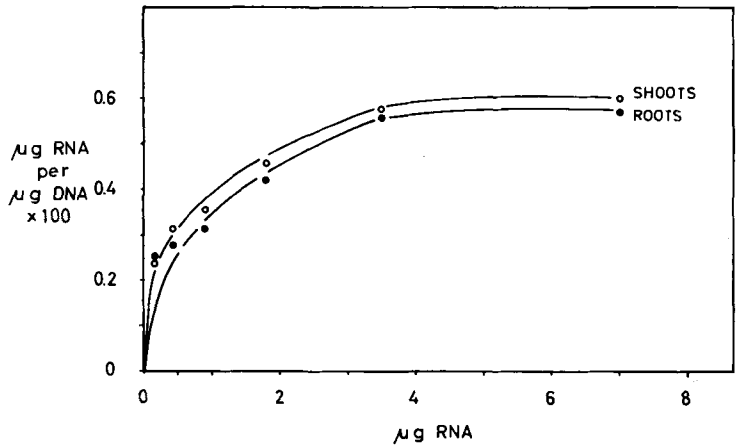

Fig. 7. Hybridization at saturation between H^3-18S *P. coccineus* RNA and homologous DNA of shoots (open circles) and roots (solid circles). The DNA was extracted from isolated nuclei. Same conditions as in Fig. 5.

clusion that the ribosomal genes are present in the DNA of the heavier satellite.

6. Saturation of DNA with 18S ribosomal RNA

In this case the saturation values for shoots and roots were nearly identical 0.61 % and 0.58 % respectively (Fig. 7). The saturation value is reached at a concentration of 3.5 µg RNA. In the case of 25S it was already reached at 1.0 µg RNA. The same sample of DNA extracted from isolated nuclei was used in both types of experiments. This means that the values obtained for roots and shoots with 25S RNA, 0.51 % and 0.72 % represent experimental variation. The average of these two values is 0.61 % which is very close to the average of the saturation values for 18S in roots and shoots, i.e. 0.60 %. This result discloses that the saturation value of 18S is practically the same as that for 25S in these two tissues, instead of half the value of 25S. For instance in *Xenopus* (BIRNSTIEL et al. 1966) and in *Acheta* (PERO et al. 1973) the relation between 28S and 18S is 2: 1.

This high value for 18S saturation was already suggested by the number of counts in the hybridizations with DNA from CsCl gradient fractions. As may be seen in Fig. 3 and 4, where two identical DNA gradients (2.0 O.D. in each) were run simultaneously in the preparative ultracentrifuge, the total number of counts in the 25S experiment is only 1.2 times higher than in the 18S hybridization.

To check whether this high saturation value of 18S was due to contamination of this RNA component with 25S RNA an experiment was carried out where an excess of 25S non-radioactive RNA was added to the 18S H^3 RNA (one part 18S plus 2 parts 25S, Fig. 8). The saturation value for shoots turned out to be the same as without the excess of 25S RNA, i.e. 0.62 %. Thus the high saturation value obtained for 18S RNA is not due to contamination with 25S RNA. This result is reinforced by the information on the purity of the RNA components as disclosed by the sucrose gradient separation and the hybridizations with DNA fractions from gradients (Fig. 2–4).

Discussion

1. The buoyant density of the DNA satellites

The DNA of *Phaseolus* has previously been studied in the Model E. *P. vulgaris*, *P. multiflorus*, *P. lunatus* and *P. aureus* were analyzed by BERIDZE (1972) who found the buoyant density of the main band to be 1.694 g/ml and a satellite present in the four species at 1.703 g/ml. *Phaseolus*

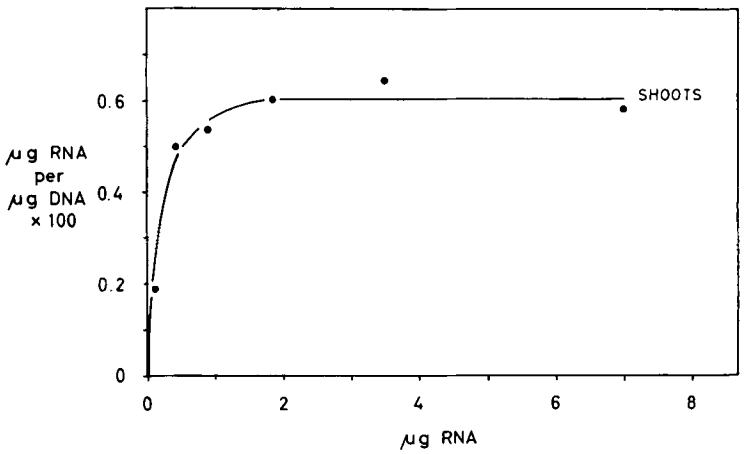

Fig. 8. Hybridization at saturation between 18S H^3-RNA plus non-radioactive 25S RNA of *P. coccineus* and homologous DNA of shoots. DNA extracted from isolated nuclei. Same conditions as Fig. 5.

vulgaris was also studied by MEYER and LIPPINCOTT (1967). Their buoyant density calculations were different from those of BERIDZE. The main band had a calculated value of 1.691 g/ml and they noticed two satellites at 1.696 and at 1.705 g/ml. INGLE et al. (1973) found the values of 1.693, 1.692 and 1.694 g/ml for the main bands of *coccineus*, *vulgaris* and *aureus* respectively. The heavy satellite was 1.702, 1.703 and 1.705 in these same species respectively.

Our calculations in *P. coccineus*, using electronic speed control in our Model E, give the values of 1.692 g/ml for the main band and 1.696 and 1.700 g/ml for the two satellites. A comparison of INGLE et al. and our results shows for *coccineus* the main band at 1.693 versus 1.692 and the heavy satellite at 1.702 versus 1.700. This is a difference of one and two units which is within experimental error.

Since the heavier satellite is present in four tissues of *coccineus* and in five different species it probably represents the same type of DNA; the differences in buoyant density may be due to difficulties in calculating the exact buoyant density. For instance in the case of the main band DNA of *vulgaris* three different laboratories have obtained different values: 1.694 (BERIDZE 1972), 1.693 (INGLE et al. 1973), 1.692 (MEYER and LIPPINCOTT 1967).

2. The nuclear nature of the satellites

In plant material the question has arisen previously whether some satellites are not due to bacterial contamination or to chloroplast and mitochondrial DNA. In the first studies we performed with the analytical ultracentrifuge a crude nuclear extraction was carried out which revealed the satellite at 1.700 g/ml (Fig. 1). Later, to make sure that no mitochondria or chloroplast DNA was present, a nuclear extraction was carried out including Triton-X, which is known to bring chloroplasts but not nuclei into solution (KIRK et al. 1970). The purity of the nuclear pellet was monitored in the light microscope at every stage of nuclear extraction. In the DNA from nuclei prepared with these precautions the satellite appeared as before and with the same buoyant density both in shoots and roots (Fig. 6). This leaves little doubt that the heavy satellite is nuclear.

In suspensors, due to their microscopic size, the same method of extraction could not be easily applied. Besides the heavy satellite a lighter satellite appears at 1.696 g/ml. It is unlikely that it represents chloroplast DNA due to the fact that this satellite is very large (circa 13 % of the total DNA) and that in suspensors there are no chloroplasts, and only a few leucoplasts

may be present as documented by electron microscopy (SCHNEPF and NAGL 1970). Moreover, an extraction of suspensor DNA was carried out including Triton-X. When this DNA was analysed in the Model E, this satellite appeared as before and at the same buoyant density.

3. The size of the DNA satellites

According to BERIDZE (1972) the size of the heavy satellite in *P. vulgaris* is 30 % of the total nuclear DNA. For the same species INGLE et al. (1973) estimate its size to be 19 % of the total DNA. In *P. coccineus* INGLE et al. give a value of 24 % of the total DNA for the heavy satellite and our value for the same species and satellite is 28.2 % (for roots) and 29.4 % (for shoots). The differences in size may be due to extraction procedures, and to the fact that the satellite is a shoulder, which increases the calculation error. However, all the values agree in showing the very large size of this satellite.

When the heavy satellite is added to the light satellite in *coccineus* the total size of both is 46.0 % of the suspensor DNA. This is the largest satellite DNA mass so far recorded in plants if one compares it with the data on 69 species described in the table of INGLE et al. (1973). The closest value is in the cucumber (*Cucumis sativus*) where both satellites represent 44 % of the nuclear DNA.

The satellite containing the ribosomal cistrons in *P. coccineus* is also among the largest single satellites so far found in plant species (28–29 %). Other large satellites are present in *Cucumis sativus* (28 %), in *C. melo* (25 %) and in citrus species (24 to 19 %, INGLE et al. 1973).

4. The amount of DNA homologous to 25S ribosomal RNA in polytene cells

The saturation experiments gave the values of 0.72 % for shoots and of 0.42 % for suspensors for the DNA homologous to 25S ribosomal RNA. The average degree of polytene in suspensors cells has been calculated by NAGL (1962) to be 512n, whereas in shoots it is mainly or solely 2n. The amount of 25S ribosomal genes seems to be less in suspensors, in any case it is not higher. Thus, there is no indication of amplification of ribosomal genes in the suspensors and the results point to a certain amount of underreplication – circa 300n. One should, however, take into account that a certain experimental error may be involved due to difficulties in extracting suspensor DNA of the same quality as shoot DNA. The cytological studies of BRADY (1973) showed no indication of amplification or underreplication in the suspensors of *P. coccineus* but the photometric method employed has in this respect more limitations than the hybridization analysis, and the extra satellite DNA present in the suspensors may cancel out the underreplication of ribosomal DNA. Ribosomal DNA is underreplicated in the polytene cells of *Drosophila hydei* (HENNIG and MEER, 1971), *Rhynchosciara* (GAMBARINI and MENEGHINI 1972) and *Drosophila melanogaster* (SPEAR and GALL 1973). Thus the evidence from *P. coccineus* indicates that the same phenomenon may occur in the polytene chromosomes of plants.

5. The extra satellite in the suspensors

The light satellite at 1.696 g/ml has so far been detected only in the suspensors where polyteny reaches its extreme level in *Phaseolus coccineus* and it represents an appreciable fraction of the total suspensor DNA (circa 13 %). The nature of this DNA cannot be ascertained at present but it is tempting to speculate that it represents the amplified DNA present during production of DNA puffs at some chromosomal regions and which does not hybridize with ribosomal RNA as demonstrated by *in situ* hybridization (AVANZI et al. 1972).

As pointed out by INGLE et al. (1973) the plant satellites that they studied were more complex than mouse satellite and several of them contained two or more components.

6. The 18S hybridization values

The saturation value for 25S and 18S RNA was 0.72 % and 0.62 % respectively. This means that instead of being half of 25S the 18S value was as high as 0.86. That this figure was not the result of contamination of 25S was ascertained by the experiment with excess of non-radioactive 25S RNA. A similarly high value for the hybridization with plant 18S was found by SCOTT and INGLE (1973). From their experiments they are inclined to believe that it is due to hybridization of the 18S RNA with "spacer" regions.

7. The cistronic and "extra-cistronic" DNA

The saturation value for 25S + 18S (0.72 % + 0.62 %) is 1.34 % in shoots. This means that about 2.68 % of the shoots DNA codes for rRNA.

According to LOEING (1968) all higher plants have ribosomal RNA with molecular weights of approximately 1.3 and 0.7 million.

Since circa 2.68 % of the shoot DNA codes for ribosomal RNA (double strand DNA) and since about 29.4 % of the genomic DNA of shoots is satellite DNA it follows that 2.68/29.4 or 9.1 % of the satellite mass is cistronic. Therefore approximately 90 % is extracistronic DNA, or DNA which contains sequences non-homologous to mature ribosomal RNA. In *P. coccineus* these values can only be considered approximate and preliminary for two reasons. (1) The ribosomal DNA appears as a shoulder of the main band, a badly defined entity which may contain a certain amount of main band DNA. (2) The high value of the 18S saturation experiments is at present difficult to explain and may represent a certain amount of non-homologous hybridization. This high value may also be caused by heterogeneity of nucleotide sequences in 18S RNA as found by HASHIMOTO and MURAMATSU (1973).

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