The Isolation of Nucleoli from Ungerminated Pea Embryos*,‡

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PLATES 28 AND 29

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ABSTRACT

A method is described for the isolation of nuclei and nucleoli from ungerminated pea embryos. Electron micrographs of the nucleolar preparation indicate a high degree of purity but only a partial preservation of composition, because of a loss of material from the nucleoli which occurs during the isolation procedure.

INTRODUCTION

This paper describes the location of nuclei and nucleoli from ungerminated embryos of the pea, *Pisum sativum* (var. Arthur). Vincent (1) and Dounce (2) have prepared nucleoli from starfish oocytes and rat liver respectively; isolation of plant nucleoli has not yet been reported. The procedure followed is based on that previously used in the isolation of nuclei from viable wheat embryos (3, 4).

Methods

One hundred-gram lots of dry peas were ground as such in a Waring blendor for 8 to 10 seconds at room temperature. In this way two fragments, one consisting of plumules and the other of hypocotyls and radicles, were split from the cotyledons without grinding more finely much of the material. Not all varieties of peas were similarly broken by such treatment, but of those that were the variety "Arthur" was chosen because it was available in large quantities as a fairly pure stock.

The ground material, which consisted largely of endosperm, was sifted through a pair of 10- and 28-mesh sieves and the embryo fragments remaining in the 10 to 28 portion were floated off on a mixture of carbon tetrachloride:cyclohexane (3:1 by volume), air-dried, and freed of small pieces of seed-coat by means of a seed blower. The two types of embryo fragments may be separated at the time of sifting by inserting a 14-mesh sieve which passes only plumules. In these studies, however, nucleoli were prepared from a mixture of both fragments.

Five-gram portions of embryos were wetted with a 2.0 M sucrose solution and cut into 25-micron sections on a freezing microtome. Unless otherwise indicated, all subsequent steps were performed within a temperature range of 0–5°C. The sections were collected in a solution of 2.0 M sucrose and 0.01 M $CaCl_2$, about 10 ml. per gm. of embryo, and stirred in a stainless steel blendor jar ("omnimixer") for a few seconds at 35 volts to disperse the tissue. The suspension was then allowed to stand for 30 minutes in order to harden the nuclei, calcium ion being the effective agent (4). After standing, the suspension was ground for 20 minutes at 35 volts and filtered through a single thickness of muslin and a double thickness of flannelette to retain the clumps of unbroken cells. The residue was immediately reground for 1 minute at 90 volts, filtered as above and the combined filtrates spun in an angle-head centrifuge for 20 minutes at 12,000 *g* to separate nuclei from most cytoplasmic components. The resulting sediment was resuspended in about 2.4 ml. of a supersaturated solution of sucrose (205 gm. of sucrose dissolved in 100 ml. of water at room temperature and cooled to 0°C. for use) for each original 1 gm. of embryo (4). At this concentration of sucrose, nuclei floated when centrifuged for 2 hours at 140,000 *g* and were thus freed from clumps of unbroken cells and starch grains which sedimented or remained dispersed in the medium. The floating material was suspended in a small volume of 2.0 M sucrose and centrifuged at 12,000 *g* for 20 minutes to sediment the nuclei and further remove cytoplasmic contaminants. Centrifugation in 2.0 M sucrose was repeated two or three times. A light micrograph of the nuclear suspension is shown in Fig. 1.

In order to prepare nucleoli, the volume of the packed nuclei was estimated, and to each ml. of nuclear pellet were added 2 ml. of the supersaturated sucrose solution, 13 ml. of a 1:2 mixture of 2 M: supersaturated

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sucrose, and 0.16 ml. of 1 M sodium citrate. The concentration of sucrose achieved in this way, although not at all critical for nuclear rupture, was necessary for an effective separation of nucleoli from unbroken nuclei. Beyond rather narrow limits of sucrose concentration, nucleoli and intact nuclei would either sediment or float together when centrifuged. Citrate was added to complex calcium ions; in its absence only a small proportion of nuclei were ruptured under the conditions indicated. The suspension was ground in an omnimixer at 90 volts until the nuclei were thoroughly broken. The time required for this varied from 12 to 30 minutes, depending upon the sample of peas used, and this was determined by periodically examining the nuclear suspension with the light microscope. The suspension was then cooled to 0°C. and centrifuged at 140,000 *g* for 10 minutes to sediment residual intact nuclei and starch grains. Nucleoli remained in the supernatant fluid and were sedimented by centrifuging at the same speed for an additional 90 minutes. Further purification was effected by resuspending the nucleoli in 2 M sucrose and centrifuging two or three times for 20 minutes at 12,000 *g*. Fig. 2 is an optical micrograph of the product.

For electron microscope observation, pellets of isolated nucleoli were broken into pieces of about 0.5 $mm.^3$ and fixed for 12 hours at 0°C. in buffered 2 per cent osmium tetroxide (pH, 7.3) which was 2.0 M with respect to sucrose. Following fixation, the pellet fragments were washed several times with 1.3 M sucrose and transferred to 70 per cent alcohol. After warming to room temperature, they were dehydrated and embedded in methacrylate (4 parts butyl to 1 part methyl). Sections were cut on a Porter-Blum microtome and observed with embedding medium present in a modified RCA EMU I microscope. During sectioning the pellet fragments were oriented so that the sections passed through the thickness of the pellet at various angles. For observation of nucleoli *in situ*, isolated pea embryos were fixed and sectioned as described previously (5).

RESULTS AND DISCUSSION

The purpose of this study has been to provide a method for isolating plant nucleoli. The effectiveness of the method just described may be judged from the following considerations.

The identity of the isolated particles with nucleoli was established in a number of ways. The nucleoli of isolated nuclei maintained in sucrose solution are refractive, and have been identified as such adding a mixture containing 0.2 per cent pyronine and 0.5 per cent methyl green in 0.1 M acetate buffer (pH, 4.1). Under these conditions the nucleoli gradually stain red while the rest of the nucleus remains greenish blue; it is not necessary to differentiate the respective staining properties with alcohol (Fig. 3). If citrate is added to an unstained sucrose suspension and a droplet of this squeezed between coverslip and microscope slide, rupture of nuclei and freeing of nucleoli may be directly observed. In the case of pea embryo cells no other subnuclear body of comparable size or shape appears to be released. While it is conceivable that dense particles of non-nucleolar material form at undetectable speed when the nuclei are broken, the probability of this occurring is remote. The most reasonable interpretation of our observations is that the bodies released on nuclear rupture which stain red in a methyl green–pyronine mixture (Fig. 2) are the same as the red-staining intranuclear ones. The only difference noted between the two is the somewhat larger diameter of the free nucleoli, but it is obvious from microscopic studies that whenever the isolated nucleus is even partially disrupted, the nucleolus begins to swell. Indeed, unless an adequate concentration of sucrose is present in the pyronine–methyl green stain, the nucleoli swell and nuclei disrupt when the stain is added to the sucrose suspension.

It would be most difficult to attribute the origin of the presumed nucleoli to the cytoplasm, not only because comparable structures cannot be found there, but also because few of them are found free in suspension unless nuclei are ruptured. The presence of some free nucleoli may be explained by the fact that a small percentage of nuclei is continually broken as a result of mechanical manipulations. Furthermore, apart from whole cells, nuclei, and starch grains, the nucleolar pellet contained all of the microscopically visible nuclear bodies present on nuclear rupture, and electron microscope analysis of it showed it to be quite homogeneous throughout its thickness. The composition of the pellet is illustrated in Fig. 4. It contained almost exclusively free nucleoli, with only occasional plastids (*P*) or mitochondria (*M*). The very little debris present appeared to be mainly fragmented nucleoli (*F*). The non-nucleolar bodies were readily identified by the presence of membranes, since isolated nucleoli, like those *in situ*, possess none. The fragmented material was concentrated toward the top of the pellet but, even so, constituted only a small proportion of the total fraction. Occasionally material that may have been of nuclear origin was found in association with nucleoli, but this formed an insignificant portion of the pellet. From the electron microscope exami-

nation it was therefore concluded that the fractionation procedures were effective in separating nucleoli from other cellular structures.

It cannot be assumed, however, that the procedures used are generally applicable to plant embryos. Ruptured pea nuclei show few, if any, microscopic bodies other than nucleoli; it may be presumed, therefore, that the conditions of nuclear rupture are such as to disintegrate other nuclear structures. The heavy sediment removed prior to nucleolar sedimentation consists largely of small starch granules and intact nuclei, while the supernatant fluid remaining after removal of the nucleoli contains only some membranous elements floating at the surface of the liquid. When wheat nuclei are ruptured, on the other hand, the nucleoli become associated with many fine filaments, probably chromosomal threads, which adhere strongly to their surface. Rupture of other types of nuclei was not attempted.

The degree of preservation of the isolated nucleoli may be judged from a comparison of electron micrographs of isolated nucleoli (Figs. 4 to 6) with those of nucleoli *in situ* (Fig. 7) (5). Isolated nucleoli have a much lower general electron density and an internal structure which is considerably less homogeneous than *in situ* ones. It may, therefore, be inferred that the isolation procedure produces changes in nucleolar composition, and it may be supposed that such changes result from a leaching out of nucleolar material. The main change in appearance occurs during disruption of the nuclei; nucleoli of isolated nuclei (Fig. 8) have about the same electron density and structure as nucleoli *in situ*. The electron density and heterogeneity of individual isolated nucleoli is variable (Figs. 4 to 6) and may be partially due to a correspondingly variable "loosening" of the nucleolar material. In section, nucleoli *in situ* never exceeded 5 μ in diameter, while isolated nucleoli showing a loosened structure often reached 6 to 7 μ, a behaviour consistent with observations made under the light microscope. The loss of material from isolated nucleoli as inferred from electron micrographs is consistent with the absence of a nucleolar membrane, which makes it likely that materials not firmly attached to the structural framework would be washed out by the sucrose solution. Isolated nucleoli may, therefore, represent no more than structural skeletons of their living counterpart.

In passing, it might be noted that neither *in situ* nor isolated nucleoli show evidence of nucleonemata (6) or of the small dense granules reported in nucleoli of plant roots (7). The absence of these structures may be a reflection of the physiological state of these nucleoli. Certainly if nucleonemata were major structural components of pea embryo nucleoli they might be expected to be more clearly visible in the loosened isolated nucleoli.

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Explanation of Plates

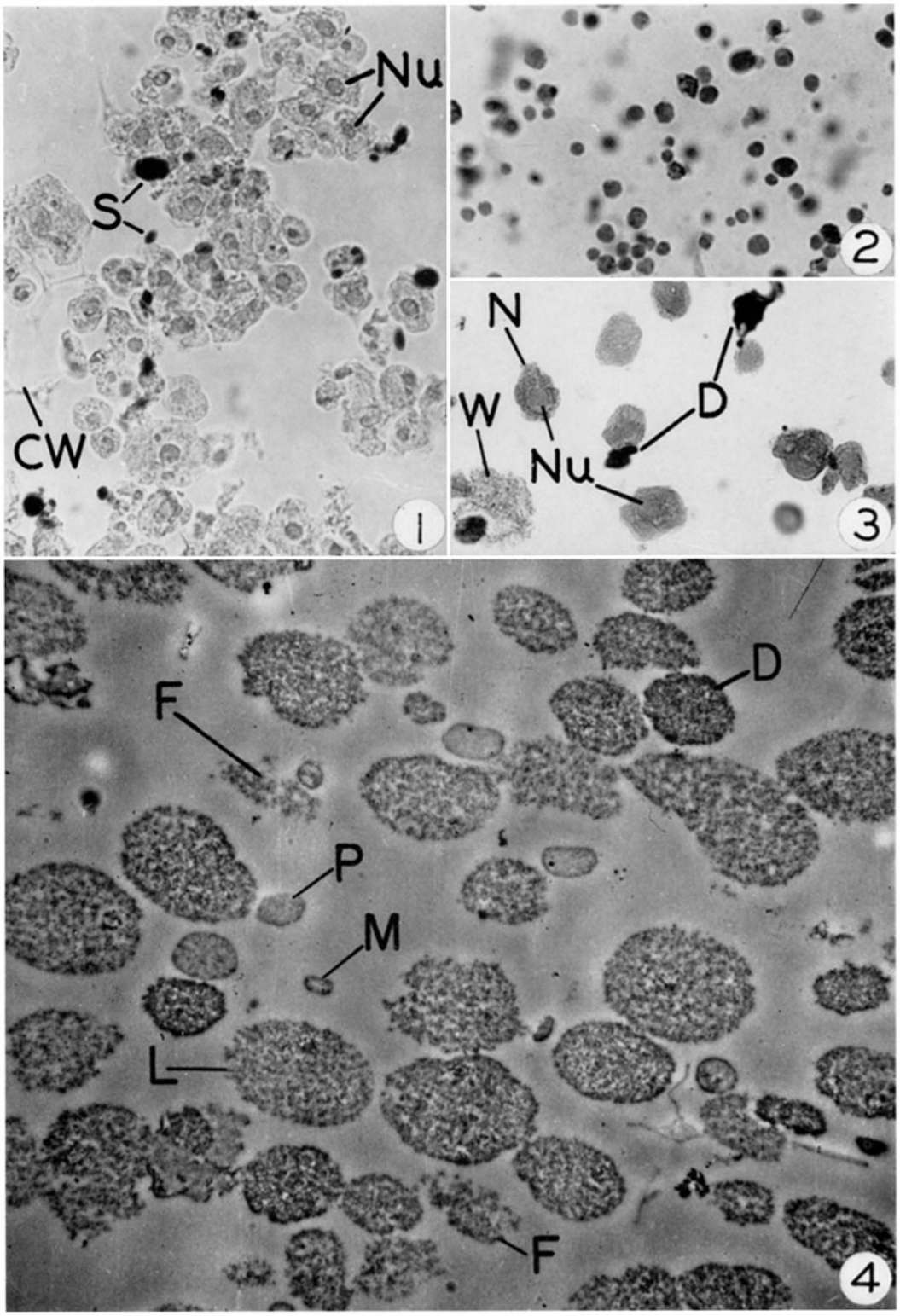
Plate 28

Fig. 1. Brightfield micrograph of a suspension of isolated nuclei. Nuclei and nucleoli (*Nu*) were stained by adding iodine solution to the sucrose suspension. This method of fixing and staining has been found to be most effective for isolated plant nuclei. Starch grains (*S*) are readily identified by their intense staining. Cell wall (CW). × 740.

Fig. 2. Brightfield micrograph of a sucrose suspension of isolated nucleoli treated with pyronine and methyl green. The nucleoli appear red in a green-tinted medium. × 740.

Fig. 3. Sucrose-suspended nuclei (*N*) treated with pyronine–methyl green (for conditions see text). Nucleoli (*Nu*) stain red against a blue-green nuclear background. The nuclear fraction was not freed of "heavy" contaminants and a whole cell (*W*) as well as other debris (*D*) may be seen. Although reasonably pure preparations of nuclei can be obtained, they are not essential for isolating nucleoli, since the contaminants shown, along with whole cells are removed in the course of centrifugation. × 740.

Fig. 4. Low-magnification electron micrograph of a typical area of a section through pellet of isolated nucleoli. Note the moderately dense (*D*) and diffuse (*L*) nucleoli. Nucleolar fragments (*F*) and a few contaminating plastids (*P*) and mitochondria (*M*) are also present. × 4,000.



(Johnston *et al.*: Isolation of nucleoli)

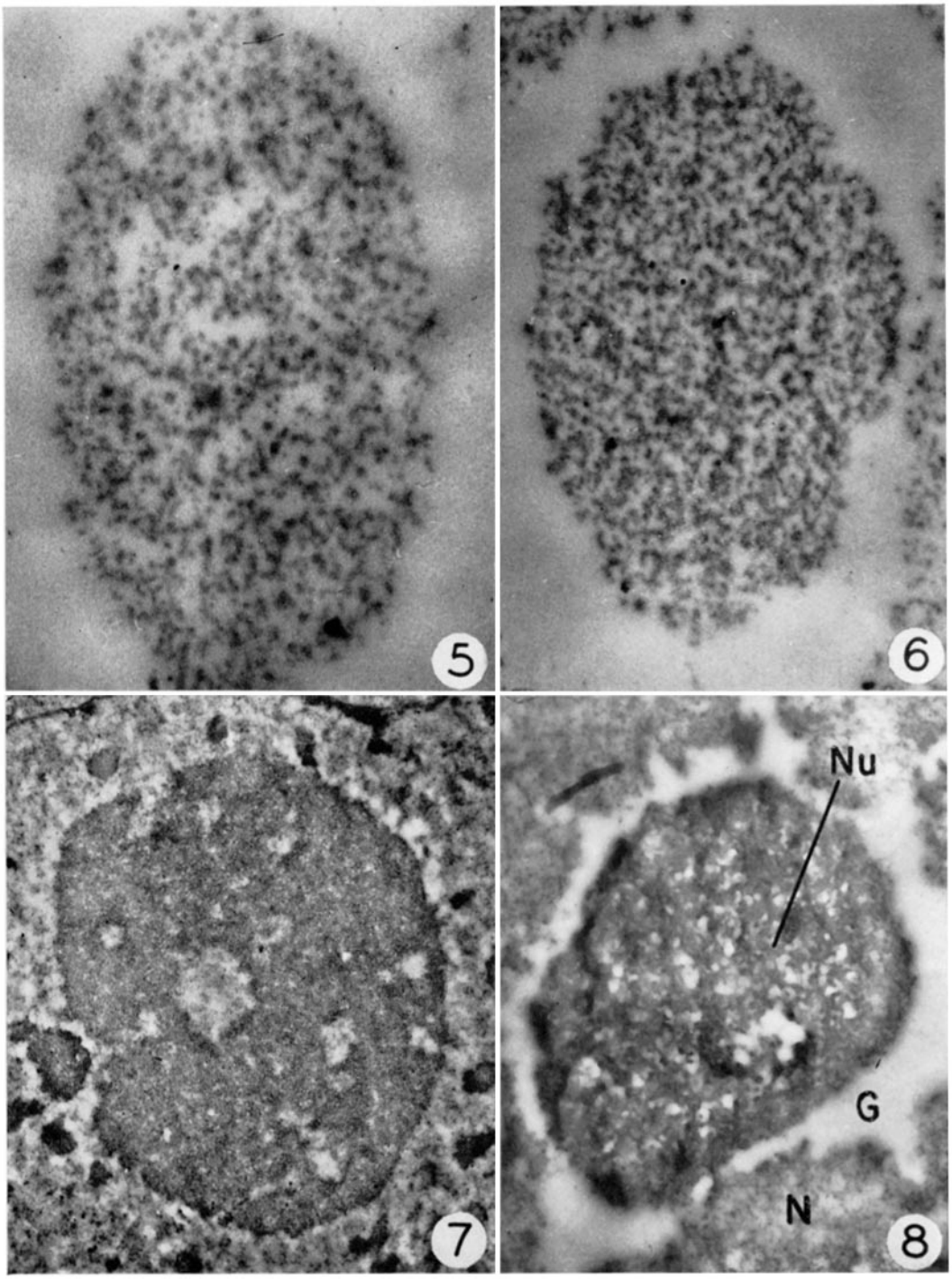
PLATE 29

FIG. 5. Section of an isolated nucleolus showing a fairly diffuse structure.

FIG. 6. Section of an isolated nucleolus with denser structure than that in Fig. 4.

FIG. 7. Section of a nucleolus *in situ*. Low magnification electron micrographs showing the entire nucleus have been presented previously (5). × 16,000.

FIG. 8. Section of a nucleolus in an isolated nucleus. In the isolated nuclei the nuclear material (*N*) was usually pulled away from the nucleolus (*Nu*) leaving a clear area (*G*). × 12,000.



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