The purpose of the present investigation was not that of stimulating or inducing mitotic activity in lemon fruit explants as an end in itself but rather to investigate the relationship between the endogenous and exogenous nutrient factors associated with the transformation of quiescent cells in vivo into mitotically active cells in vitro. Thus the principle of thought underlying the present investigation is well expressed by the following quotation from FILNER[21]: 'Wouldn't it be nice to have systems in which a developmental event is initiated by a metabolite, particularly one whose biochemistry is well known? Then, with a little bit of luck, perhaps the site of initiation and the chain of biochemical steps leading to the developmental event would be found in the known biochemistry'[22].

Zusammenfassung. Nachweis einer Mitose-Aktivität bei Zitronenfrucht-Gewebeschnitten, die auf einem Calcium-Kalium-Saccharose-Medium ohne andere bekannte Nährsubstanzen bebrütet wurden.

H. A. KORDAN

Department of Botany, University of Birmingham, P.O. Box 363, Birmingham B15 2TT (England), 11 January 1974.

[21] P. FILNER, *Communication in Development, Developmental Biology*, Supplement 3 (Ed. ANTON LANG; Academic Press, New York and London 1969), p. 206.

[22] I thank the Dr. HADWEN, Trust For Humane Research and the Air Chief Marshall The Lord Dowding Fund For Humane Research for funds received in support of this research.

Observations on Nucleolar Staining with Osmium Tetroxide

A simple technique for nucleolar staining in plant cells, based on the osmium tetroxide (OsO_4) fixation, has been reported by BATTAGLIA and MAGGINI[1]. The application of this technique has allowed us to study the topographic distribution of the osmiophilic component in normal and segregated nucleoli of *Allium cepa* meristematic cells[2,3]. The mechanism of nucleolar staining with OsO_4 remains unknown, although a reaction between OsO_4 and unsaturated lipids has been suggested[1,4]. We here present some observations in relation to a mechanism which could account for nucleolar staining with OsO_4.

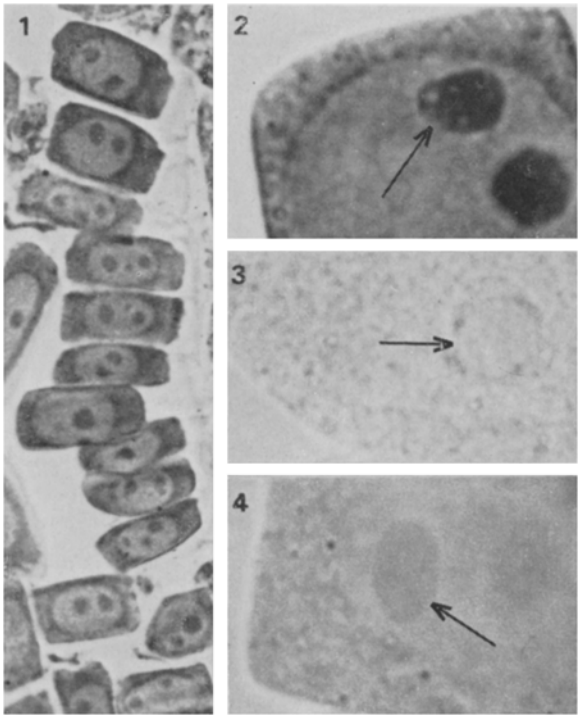

Fig. 1. *Allium cepa* root-tip cells after osmium staining.
Fig. 2. Greater magnification of an osmium-stained cell, showing the high contrast of the nuleolus (arrow).
Fig. 3. RNase digestion before OsO_4 fixation. Notice the unstained nucleolus (arrow).
Fig. 4. Ethanol fixation and tryipsin digestion before osmium staining. The nucleolus appears slightly stained (arrow).

Materials and methods. Roots of *Allium cepa* bulbs growing in tap water at room temperature were fixed with OsO_4 for 3–10 min in one of the following solutions: a) 1% in distilled water; b) 5% in distilled water; c) 2.5% in 0.1 *M* phosphate buffer at pH 7.0, and d) 2.5% in 0.4 *N* NH_3. Other fixatives were also used: e) 5% formaldehyde in 0.1 *M* phosphate buffer at pH 7.0 for 30 min, followed by OsO_4 as in b); f) 1 h in 96% ethanol, followed by 50% and 25% ethanol, distilled water and then OsO_4 as in b). After fixation and washing in distilled water the samples were heated: g) at 60 °C during 15 min; h) at 80 °C during 5 min; i) at 100 °C during 2 min, or j) treated 10 min with 1% *p*-phenylendiamine in 70% ethanol[5].

Before the OsO_4 treatment, extractive procedures and enzymatic digestions were carried out as follows: k) RNase, 1 mg/ml in 0.1 *M* phosphate buffer at pH 7.0, 1–2 h at room temperature or at 37 °C, followed by washing in 5% cold perchloric acid for 10 min; l) trypsin, 1 mg/ml in distilled water at pH adjusted to 8 with 0.01 *N* NaOH, 1 h at 37 °C; m) 5% perchloric acid, 16 h at 4 °C; n) absolute ethanol 1 h, followed by chloroform for 3–48 h, and then absolute, 96%, 70% and 50% ethanol; o) 5 *N* HCl, 1 h at room temperature; p) DNase, 1 mg/ml in 0.1 *M* phosphate buffer at pH 7.0, 2–4 h at 37 °C.

After all these procedures the root-tips were flattened in 1–2 drops of distilled water. The Feulgen reaction was carried out in roots previously fixed for 10 min as in b). After a 10 min hydrolysis in 1 *N* HCl at 60 °C, the roots were treated with the Schiff's reagent for 30 min. Some samples fixed as in c) and treated as in g) or j), were dehydrated in alcohols and embedded in Maraglas. Thin sections were obtained with a Porter Blum microtome and observed in a Zeiss 9A electron microscope without staining. Other sections were stained with uranyl acetate and lead citrate as usually.

Results and discussion. The observations are summarized in the Table. Any of the OsO_4 fixations, followed by any of the heating procedures or by the treatment with

[1] E. BATTAGLIA and F. MAGGINI, Caryologia *21*, 287 (1968).
[2] M. E. FERNÁNDEZ-GÓMEZ and J. C. STOCKERT, Nucleus *13*, 149 (1970).
[3] M. E. FERNÁNDEZ-GÓMEZ, M. C. RISUENO, G. GIMÉNEZ-MARTÍN and J. C. STOCKERT, Protoplasma *74*, 103 (1972).
[4] M. L. BIRNSTIEL and M. I. H. CHIPCHASE, Biochim. biophys. Acta *76*, 454 (1963).
[5] J. M. LEDINGHAM and F. O. SIMPSON, Stain Tech. *45*, 255 (1970).

Osmium staining in light microscopy after several experimental procedures

Fixations or/and pretreatments	OsO_4 treatment	Post-treatment	Staining degree		
			Nucleolus	Chromatin	Cytoplasm
	(a, b, c, d)	(g, h, i, j)	+++	−	++
	(b)		±	−	−
Formaldehyde (e)	(b)	(i)	+++	++	+
Ethanol (f)	(b)	(i)	+++	−	++
RNase (k)	(b)	(i)	±	−	−
Ethanol (f), RNase (k)	(b)	(i)	−	−	±
Ethanol (f), perchloric acid (m)	(b)	(i)	−	+++	±
Formaldehyde (e), trypsin (l)	(b)	(i)	+	−	+
Ethanol (f), trypsin (l)	(b)	(i)	++	−	+
Ethanol (f), chloroform (n)	(b)	(i)	+++	±	++
Ethanol (f), HCl (o)	(b)	(i)	−	+++	−
Ethanol (f), DNase (p), HCl (o)	(b)	(i)	−	−	−

Letters refers to the treatments detailed in material and methods.

p-phenylendiamine, results in a dark brown staining of the nucleoli (Figures 1 and 2). The cytoplasm appears brown and the nuclear content remains unstained. Within telophase nuclei, the occurrence of brown-stained prenucleolar bodies can also be observed. Mitotic chromosomes appear unstained but they are outlined by the stained cytoplasm.

The lack of heating after the OsO_4 treatment results in a very pale staining of the nucleolus. Prefixation can modify the staining pattern (formaldehyde) or not (ethanol). Treatments with RNase, cold perchloric acid or HCl strongly decrease the osmiophilia of the nucleolus and the cytoplasm (Figure 3), while trypsin digestion results in a slightly decreased staining of both structures (Figure 4). Chloroform extraction does not modify the affinity of nucleoli and cytoplasm for OsO_4, as revealed by light microscopy.

Under the electron microscope, the same osmium staining distribution shown in Figures 1 and 2 is observed. A slight increase in the general contrast can be obtained by staining with uranyl acetate and lead citrate, but the contrast between nucleoli and chromatin remains constant.

The capacity of OsO_4 to react with the lipidic components of cells and tissues is actually well known. In addition, experimental evidence for a mechanism of OsO_4 fixation which implies hydrogen bonding with proteins has recently been reported by LITMAN and BARNETT[6]. Under the electron microscope, several authors have observed the high contrast of ribonucleoprotein containing structures after OsO_4 fixation, as well as the low staining degree of the chromatin[7–9]. Our results agree with all these observations. After OsO_4 fixation, nuclei and chromosomes remain Feulgen positive in flattened preparations, suggesting that there is no appreciable DNA loss during the OsO_4 treatment (ref. [1]).

Our results indicate that a reaction with RNA is probably implied in the nucleolar staining by OsO_4. The 'pars granulosa' of the nucleolus – which seems to be the region of greatest RNA concentration[4] –, appears as the more osmiophilic region in light microscopy[3,10–12]. Treatments with RNase or perchloric acid which eliminate the nucleolar and cytoplasmic basophilia in meristematic cells[3], strongly reduce the osmium staining of nucleoli and cytoplasm. OsO_4 has been considered unreactive with respect to nucleic acids[13], but it could react by producing an unblackened but bound form of osmium[14,15]. According to ZOBEL and BEER[16] OsO_4 reacts with pyrimidines of denatured DNA and the ethylenic bond of the pyrimidines (only in singlestranded nucleic acids) can condense with OsO_4 to form 4, 5-diol osmates[11]. This reaction – similar to that which occurs between OsO_4 and other aromatic compounds –, could presumably account for the osmiophilia of nucleoli. The high staining degree of the chromatin by OsO_4 after hydrolysis in 5 *N* HCl (which is removed by a previous DNase digestion) also suggests the participation of pyrimidines of the apurinic DNA in the reaction with OsO_4. The present results allow us to assume that RNA, or at least ribonucleoproteins, is one of the more reactive osmiophilic components within the nucleus[17].

Resumen. La fijación con tetroxido de osmio origina una tinción selectiva del nucleolo y citoplasma en células meristemáticas de *Allium cepa*. Las observaciones efectuadas indican que esta tinción se basa en la reacción entre el tetroxido de osmio y estructuras que contienen ribonucleoproteínas.

J. C. STOCKERT[18] and ONDINA D. COLMAN[18]

Centro de Investigaciones en Reproducción. Facultad de Medicina, Buenos Aires (Argentina), 14 January 1974.

[6] R. B. LITMAN and R. J. BARRNETT, J. Ultrastruct. Res. *38*, 63 (1972).
[7] R. CHARRET, Expl Cell Res. *54*, 353 (1969).
[8] R. SIMARD and A. M. DUPRAT, J. Ultrastruct. Res. *29*, 60 (1969).
[9] E. ROBBINS, J. Biochem. biophys. Cytol. *11*, 449 (1961).
[10] F. FABBRI, Caryologia *16*, 715 (1963).
[11] K. BURTON and W. T. RILEY, Biochem. J. *98*, 70 (1966).
[12] V. B. WIGGLESWORTH, Proc. R. Soc., Lond. B *147*, 185 (1957).
[13] G. F. BAHR, Expl. Cell Res. *7*, 457 (1954).
[14] R. CRIEGE, Ann. Chem. *522*, 75 (1936).
[15] M. WOLMAN, Expl Cell Res. *12*, 231 (1957).
[16] C. R. ZOBEL and M. BEER, Int. Rev. Cytol. *18*, 363 (1965).
[17] The work was partially supported by a grant No. 4840/71 from the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.
[18] Present address: Max-Planck-Institut für Biologie, Abteilung Beermann, Spemannstrasse 34, 7400 Tübingen (W. Germany).