Changes in Lysosomal Associated Structures in Human Fibroblasts Kept in Resting Phase (35974)

A. MACIERA-COELHO, E. GARCIA-GIRALT, AND M. ADRIAN

Institut de Cancérologie et Immunogénétique, Hopital Paul Brousse, 94-Villejuif, France

Cell division can be induced within 24 hr after the addition of fresh medium to several different cell types in the resting phase (1–7). Human fibroblasts respond in the same fashion if stimulated soon after the cells reach the resting phase (5). However, when the cells are kept for a few days in the resting phase before stimulation, fewer cells enter the division cycle and eventually the whole cell population fails to divide. The present work investigates the physiological and structural changes associated with the delay between stimulation and initiation of cell division.

Materials and Methods. Cell culture. The WI-38 human embryonic diploid line was maintained in Eagle's essential medium supplemented with 10% calf serum as described by Hayflick (8). Cultures were considered to be in the resting phase when cell division had virtually ceased following proliferation in the same medium after a 1:2 split (9). For each experiment, cells were pooled and distributed evenly into culture flasks.

Biochemical analyses. Deoxyribonucleic acid (DNA) synthesis was measured adding 0.1 μC/ml of tritium-labeled thymidine (^{3}H-TdR) to the cultures which were treated with trichloroacetic acid (TCA) 24 hr later as described by Levine *et al.* (10). The acid precipitate was dissolved in 1 ml of soluene (Packard Co.) added to 12 ml of Liquifluor (Packard Co.) and the radioactivity was measured in a liquid scintillation spectrometer. Quenching corrections were made when necessary. Protein determinations were performed by the method of Lowry *et al.* (11) and acid phosphatase measurements were made according to De Mars (12). Enzyme activity is expressed as micromoles of *p*-nitrophenol produced per hour. In all measurements, each value represents the mean of duplicate samples.

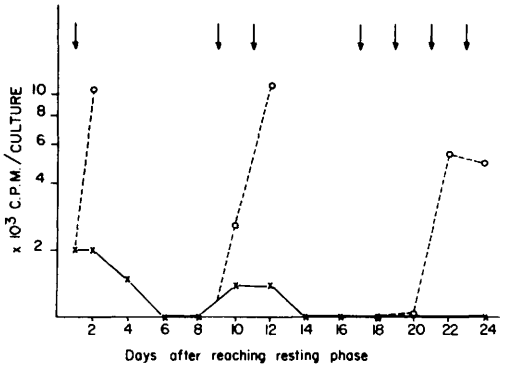

FIG. 1. ^{3}H-TdR incorporation during 24-hr periods in the control cultures (+—); and after medium changes (o - - -) in cultures kept 1, 9, and 17 days in resting phase. Each arrow indicates a medium change.

Electron microscopy. Cell monolayers grown in 30 ml plastic Falcon flasks were fixed *in situ* with glutaraldehyde (5% in cacodylate buffer) for 1 hr; treated with osmium tetroxide (2% in cacodylate buffer) for 30 min; and dehydrated with 50, 75, 95%, and finally absolute ethanol. The monolayer was detached from the plastic substrate with propylene oxide (13) and included in Epon 812 (14). To stain acid phosphatase, the Gomori technic (15) was used following fixation with glutaraldehyde. After staining, the cell layer was dehydrated, detached, and included as described above. The ultrathin sections were made with a MT2 Sorvall microtome and stained with uranyl acetate for 20 min and lead citrate for 5 min (16). Cultures used for electron microscopy and enzyme studies were not labeled with ^{3}H-TdR.

Results. Figure 1 shows the results of an

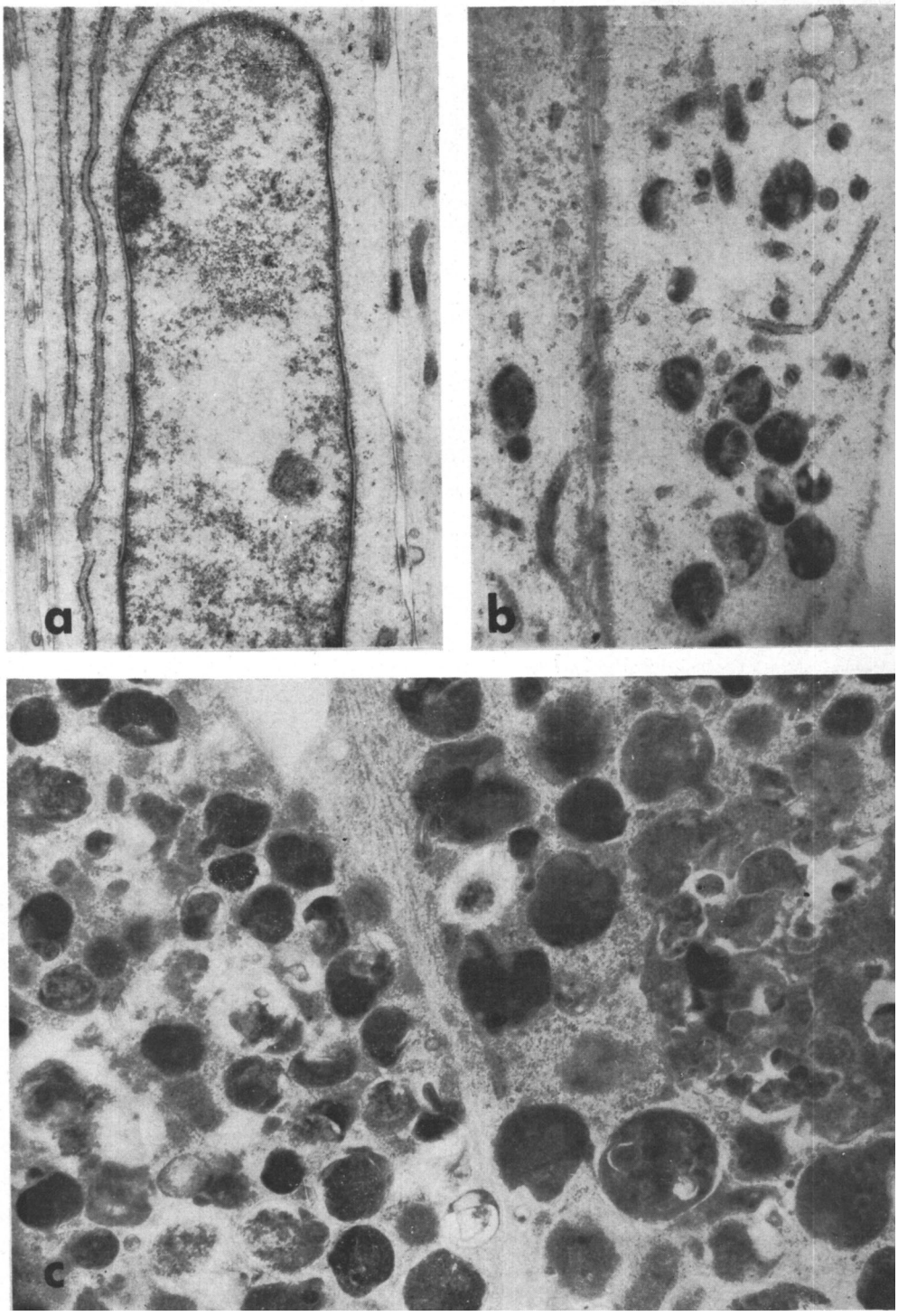

FIG. 2. Morphology of human fibroblasts on day: (a) 1; (b) 6; and (c) 20 after reaching resting phase; ×18,000.

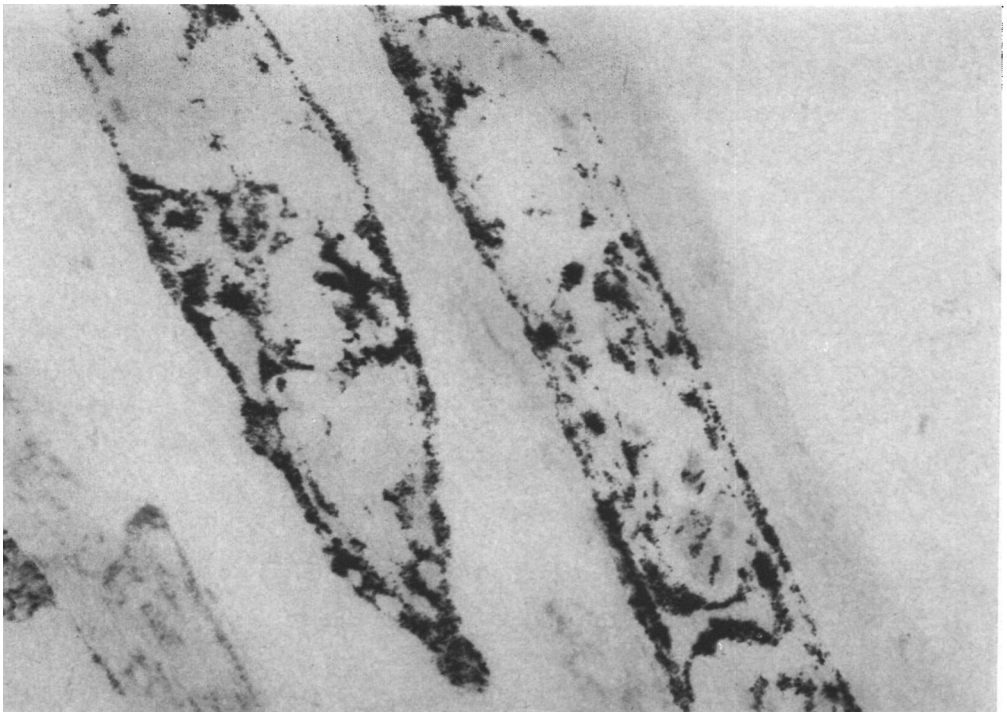

FIG. 3. Acid phosphatase stain in cells kept 20 days in resting phase; Same experiment as Fig. 2; ×18,000.

experiment where medium changes were performed on three groups of cultures that had been maintained 1, 9, and 17 days in the resting phase. Medium was changed once in the first group; twice in the second group; and each second day during 6 consecutive days in the third group, starting on day 17 after the cells had attained the resting phase. ^{3}H-TdR was added after each medium change. As shown in Fig. 1 only the second stimulation significantly increased the ^{3}H-TdR incorporation in cultures kept 9 days in resting phase. In the last group, only the third stimulation increased ^{3}H-TdR incorporation which nevertheless did not reach the same level as observed in the other two groups.

Cells kept in the nondividing stage were observed under electron microscopy before and after medium change. Figure 2 shows the morphology of cells on days 1, 6, and 20 after reaching resting phase. A progressive accumulation of cytoplasmic vacuoles filled with unidentified material could be seen. The vacuoles finally filled the whole cytoplasm (Fig. 2c). The Gomori stain revealed an increase in acid phosphatase mainly around the vacuoles but also inside them (Fig. 3). The increase in acid phosphatase activity was also observed with light microscopy (Fig. 4a).

Starting on day 21, medium was changed daily in the remaining cultures and duplicate samples were fixed at different days thereafter for examination by electron and light microscopy. Twenty four hours after changing the medium the acid phosphatase decreased considerably in the cytoplasm of these cells as shown under light microscopy (Fig. 4b). A pronounced decrease in the number of vacuoles was also seen 24 hr after the first medium change (Fig. 5a). Cultures fixed in the following days revealed that the cytoplasmic changes disappeared progressively. Four days after initiating the medium renewals occasional empty vacuoles were still seen but most cytoplasm was occupied by the endoplasmic reticulum (Fig. 5b).

Figure 6 illustrates the amount of acid

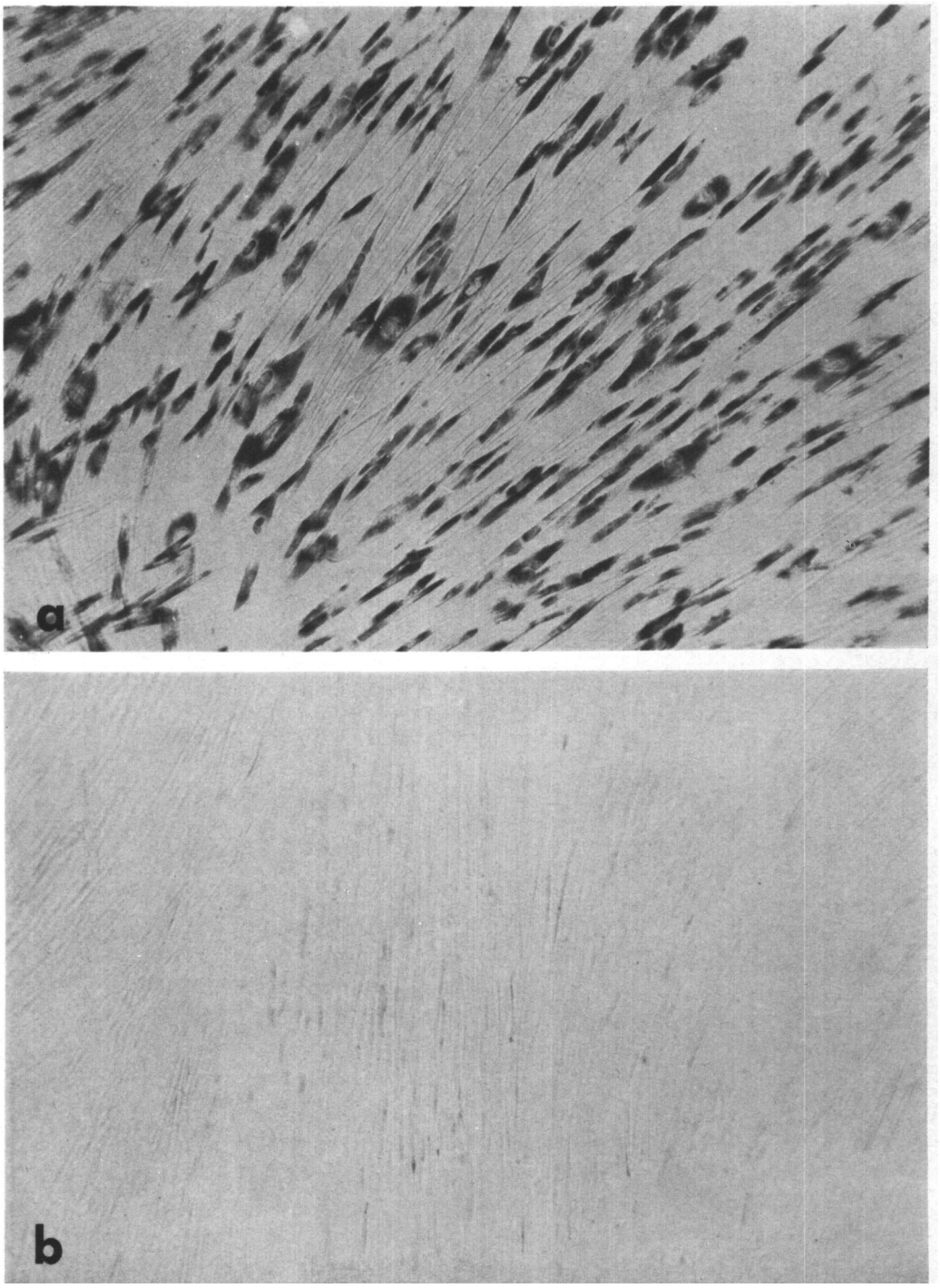

FIG. 4. Acid phosphatase stain under light microscopy in cells kept 20 days in resting phase (a); and in sister cultures 24 hr after medium change (b); same experiment as Figs. 2 and 3; ×700.

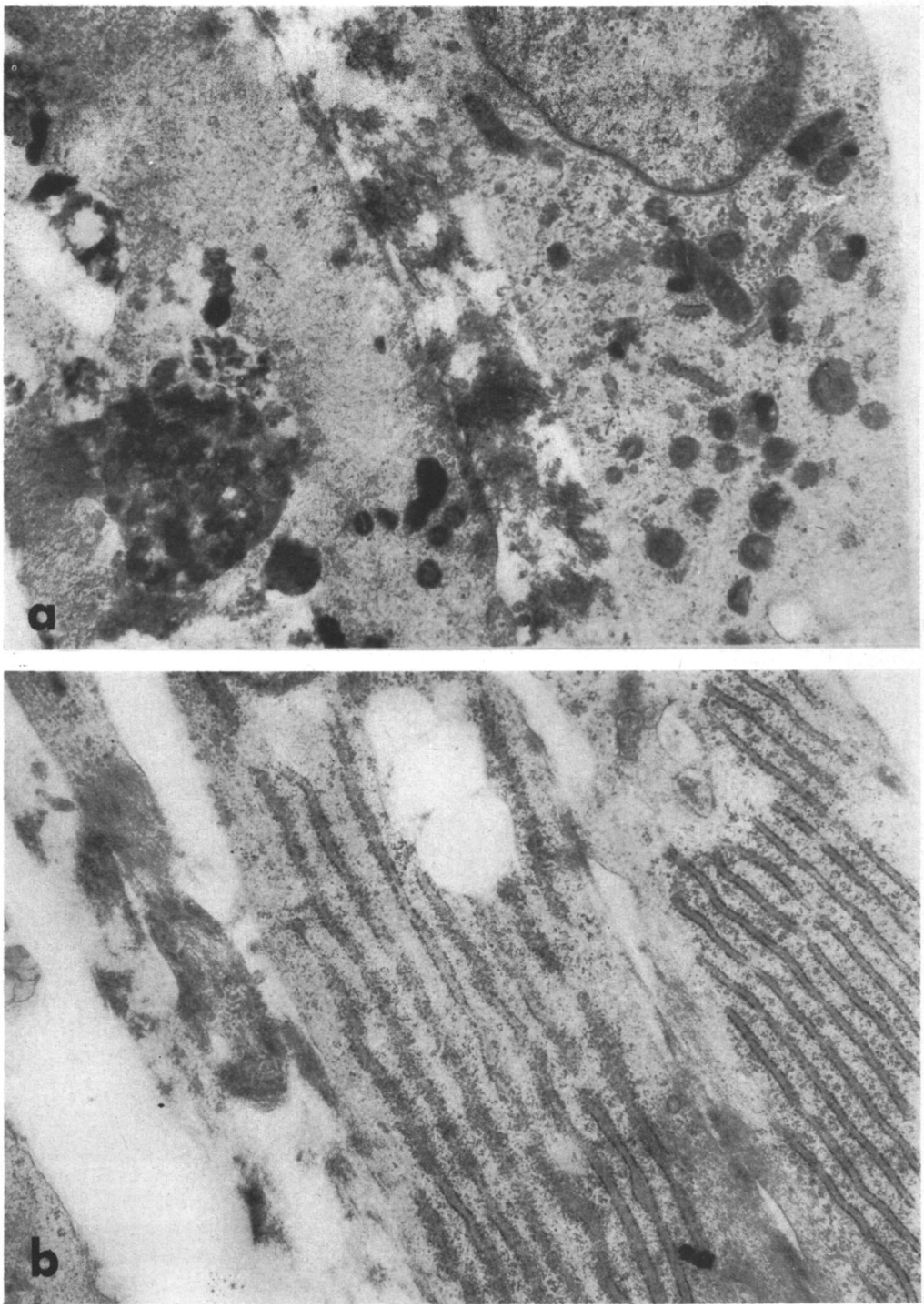

FIG. 5. Morphology of human fibroblasts: (a) 24 hr; and (b) 4 days after medium changes in cultures kept for 20 days in the resting phase; ×18,000.

TABLE I. Amount of Acid Phosphatase Found in Human Fibroblasts Kept for 19 Days in Resting Phase (μmoles of *p*-nitrophenol produced/hr/culture).

Expt. I		Expt. II	
Before medium change	24 hr after medium change	Before medium change	4 hr after medium change
4.2×10^{-3}	2.4×10^{-3}	3.3×10^{-3}	1.1×10^{-3}

phosphatase (μmoles of *p*-nitrophenol produced) found on days 1, 6, 12, and 14 after another group of cultures had reached resting phase. A significant increase in acid phosphatase was seen on days 12 and 15, which is in agreement with the histochemical observation. A quantitative assay of the enzyme was also undertaken on cultures kept for 19 days in resting phase, before and after medium change. Four hours after a medium change a significant decrease in the amount of acid phosphatase was observed (Table I).

Discussion. The experiments described above show that the induction of DNA synthesis by medium renewal in nondividing human fibroblasts, is delayed when the cells are kept for several days in the resting phase. It was also found by Van't Hof (17) that the initiation of DNA synthesis in pea root meristems is increasingly delayed with extended time in stationary phase.

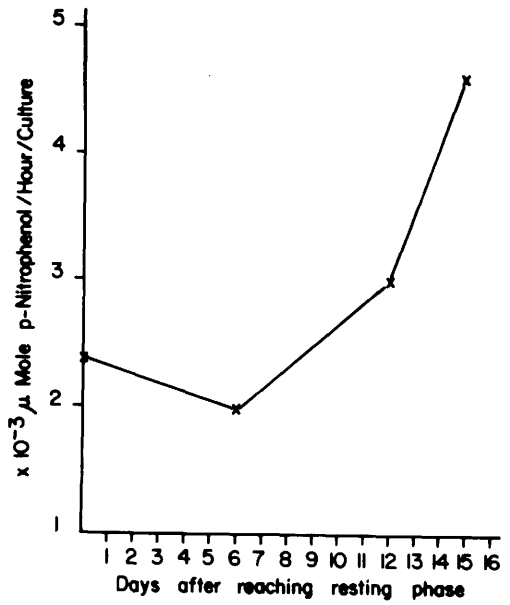

FIG. 6. Acid phosphatase assayed quantitatively at different days after human fibroblasts had reached resting phase.

Our results also show a progressive accumulation of cytoplasmic vacuoles in cells kept in resting phase. The vacuoles are associated with lysosomes as shown by the increased acid phosphatase activity. The enzyme however, is mainly increased outside the vacuoles. The increase in acid phosphatase was confirmed by light microscopy and with a quantitative enzymic assay. Medium renewals could reverse these changes. A decrease in acid phosphatase was observed as soon as 4 hr after the first medium changes. Then the vacuoles disappeared progressively. Finally the cytoplasm of the cells became normal with occasional empty vacuoles. The morphologic changes and the decrease in acid phosphatase, preceded the stimulation of DNA synthesis after medium change. The findings with acid phosphatase suggest the involvement of lysosomes in the cytoplasmic changes that follow the medium renewal.

Lysosomes have been previously implicated in the control of cell division (18–20). In the present experiments, the mobilization of lysosomal enzymes could facilitate the digestion of the content of the vacuoles and thus supply energy needed for cell division. The delay in DNA synthesis could be due to the time needed for the latter process to occur. The delay could also be due to the time needed for the cell metabolism to switch from metabolic pathways needed for secretion to those needed for cell division. In fact, it has been shown that human fibroblasts have an increased production of collagen (21) in the resting phase and the structural changes we observed are compatible with an increased excretion in nondividing cells. Regardless of the eventual association between

the structural changes and the initiation of DNA synthesis, the phenomenon described herein provides an excellent model to study the factors controlling the release of acid phosphatase from lysosomes.

1. Earle, W. R., Fed. Proc., Fed. Amer. Soc. Exp. Biol. 17, 967 (1958).

2. Temin, H. M., J. Nat. Cancer Inst. 35, 679 (1965).

3. Todaro, G. J., Lazar, G. K., and Green, H., J. Cell. Comp. Phys. 66, 325 (1966).

4. Bryant, J. C., Ann. N.Y. Acad. Sci. 139, 143 (1966).

5. Maciera-Coelho, A., Proc. Soc. Exp. Biol. Med. 125, 548 (1967).

6. Becker, Y., and Levitt, J., Exp. Cell Res. 51, 27 (1968).

7. Yoshikura, Y., and Hirokawa, Y., Exp. Cell Res. 52, 439 (1968).

8. Hayflick, L., Exp. Cell Res. 37, 614 (1965).

9. Hayflick, L., and Moorhead. O. S., Exp. Cell Res. 25, 221 (1961).

10. Levine, E. M., Becker, Y., Boone, C. W., and Eagle, H., Proc. Nat. Acad. Sci. U.S.A. 53, 350 (1965).

11. Lowry, O. H., Rosebrough, N., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

12. De Mars, R., Nat. Cancer Inst. Monogr. 13, 181 (1964).

13. Adrian, M., and Garcia-Giralt, E., Rev. Fr. Etud. Clin. Biol. 14, 622 (1996).

14. Luft, J. H., J. Biophys. Biochem. Cytol. 9, 409 (1961).

15. Gomori, G., "Microscopic Histochemistry, principles and practice." Univ. of Chicago Press, Chicago (1952).

16. Reynolds, E. S., J. Cell Biol. 17, 208 (1963).

17. Van't Hof, J., Exp. Cell Res. 61, 173 (1970).

18. Allison, A. C., and Malluci, L., Lancet 2, 1371 (1964).

19. Hirschhorn, R., Kaplan, J. M., Goldberg, A. F., Hirschhorn, K., and Weissmann, G., Science 147, 55 (1956).

20. Ryan, W. L., and Cardin, C., Proc. Soc. Exp. Biol. Med. 126, 112 (1967).

21. Green, H., and Goldberg, B., Proc. Soc. Exp. Biol. Med. 117, 258 (1964).

Received April 19, 1971. P.S.E.B.M., 1971, Vol. 138.