only insulin) do not respond similarly to the addition of prolactin (*3*). The action of hydrocortisone that endows mammary epithelial cells with the capacity to respond to prolactin in terms of casein synthesis has been heretofore unknown. This report describes an effect of hydrocortisone on the ultrastructure of the alveolar epithelial cells.

Alveolar epithelial cells in explants that have been cultured in the presence of insulin for 4 days have only a small amount of rough endoplasmic reticulum (RER) (Fig. 1). This ultrastructural appearance was very constant in cultured tissue from eight animals, and is also representative of the morphology of the majority of alveolar cells in uncultured tissue from C3H/HeN mice midway through pregnancy. When explants from the same animals were cultured for 4 days in the presence of both insulin and hydrocortisone a dramatic alteration was observed. The alveolar epithelial cells developed an extensive RER system (Fig. 2).

This marked difference in the structure of these cells is not reflected in their ability to synthesize some nonsecretory proteins, for it has been demonstrated (*4*) that insulin causes a fourfold increase in the formation of a nonmilk protein fraction, and that hydrocortisone exerts no effect on such synthesis in the presence of insulin. It appears, however, that the presence of the hydrocortisone-induced RER is a prerequisite for the formation of secretory protein, such as casein, under the influence of prolactin.

Work by others has implicated steroids in the formation of smooth membranes in a variety of systems (*5*). Rough endoplasmic reticulum has been shown to be associated with secretory proteins in a number of tissues (*6*). The observations presented here suggest that the formation of RER, under the influence of hydrocortisone, in mammary alveolar cells is necessary for full expression of the genetic potential of these cells. Prolactin, a terminal agent in the differentiation of mammary epithelium, elicits milk protein synthesis only in cells which possess this membrane system.

ELINOR S. MILLS
YALE J. TOPPER
National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland 20015

References

1. W. G. Juergens, F. E. Stockdale, Y. J. Topper, J. J. Elias, *Proc. Nat. Acad. Sci. U.S.* **54**, 629 (1965).
2. R. W. Turkington, D. H. Lockwood, Y. J. Topper, *Biochim. Biophys. Acta* **148**, 475 (1967).
3. D. H. Lockwood, F. E. Stockdale, Y. J. Topper, *Science* **156**, 945 (1967).
4. D. H. Lockwood, R. W. Turkington, Y. J. Topper, *Biochim. Biophys. Acta* **130**, 493 (1966).
5. A. H. Conney, K. Schneidman, M. Jacobson, R. Kuntzman, *Ann. N.Y. Acad. Sci.* **123**, 98 (1965); A. L. Jones and D. W. Fawcett, *J. Histochem. Cytochem.* **14**, 215 (1966); M. W. Rancourt and G. Litwack, *Exp. Cell Res.* **51**, 413 (1968).
6. L. G. Caro and G. E. Palade, *J. Cell Biol.* **20**, 4 (1964); S. Ito and R. J. Winchester, *ibid.* **16**, 541 (1963); S. L. Wissig, *J. Biophys. Biochem. Cytol.* **7**, 419 (1960); *J. Cell Biol.* **16**, 93 (1963).
7. M. J. Karnovsky, *J. Cell Biol.* **27**, 137A (1965).
8. B. O. Spurlock, V. C. Kattine, J. A. Freeman, *ibid.* **17**, 203 (1963).
9. E. S. Reynolds, *ibid.*, p. 208.

23 May 1969; revised 30 June 1969

Uptake of Isolated Chloroplasts by Mammalian Cells

Abstract. *Mouse fibroblasts (L cells) in suspension culture incorporated isolated chloroplasts of spinach and African violets and isolated mitochondria of chicken liver. The organelles resided in the cell cytoplasm and were not contained in vacuoles or digestion vesicles. Green cells divided like normal cells. Green chloroplasts were followed for five cell generations or 5 days, at which time hybrid cells were greatly outnumbered by nongreen progeny cells. The ingested chloroplasts retained their structural integrity as determined by electron microscopy of organelles and hybrid cells and by analysis of photochemical activity and DNA in chloroplasts reisolated from cells after 1 or 2 days in culture.*

The introduction of exogenous genetic material or DNA-containing structures into animal cells can be achieved by diverse techniques. These include infection of cells with bacteria and viruses, formation of hybrids of two cell types by fusion (*1*), transplantation of isolated single nuclei in eggs and embryos (*2*), microinjection of nuclear and mitochondrial fractions (*3*), and pinocytic uptake of purified DNA (*4*). We have been interested in the problem of the degree of independence which can be achieved by various cytoplasmic organelles that contain DNA, such as mitochondria and chloroplasts.

In order to introduce isolated heterospecific cytoplasmic organelles into mammalian cells with technical facility and high yield of hybrid cells, advantage was taken of the phagocytic properties of mouse fibroblasts (L cells) grown in suspension culture. These cells incorporated isolated chloroplasts and mitochondria within 30 minutes after addition of the organelles to the cell culture. We have first concentrated on chloroplasts because they are easier to follow than mitochondria. Unlike the fate of DNA-containing gelatin particles, which were hydrolyzed in digestion vacuoles within an hour after uptake by L cells (*5*), green chloroplasts were seen in L cells for at least 5 days or five cell generations after ingestion. Green cells divided like normal cells, randomly segregating chloroplasts into progeny cells. After six divisions, green cells were greatly outnumbered by cells of subsequent generations, limiting further observations on their fate.

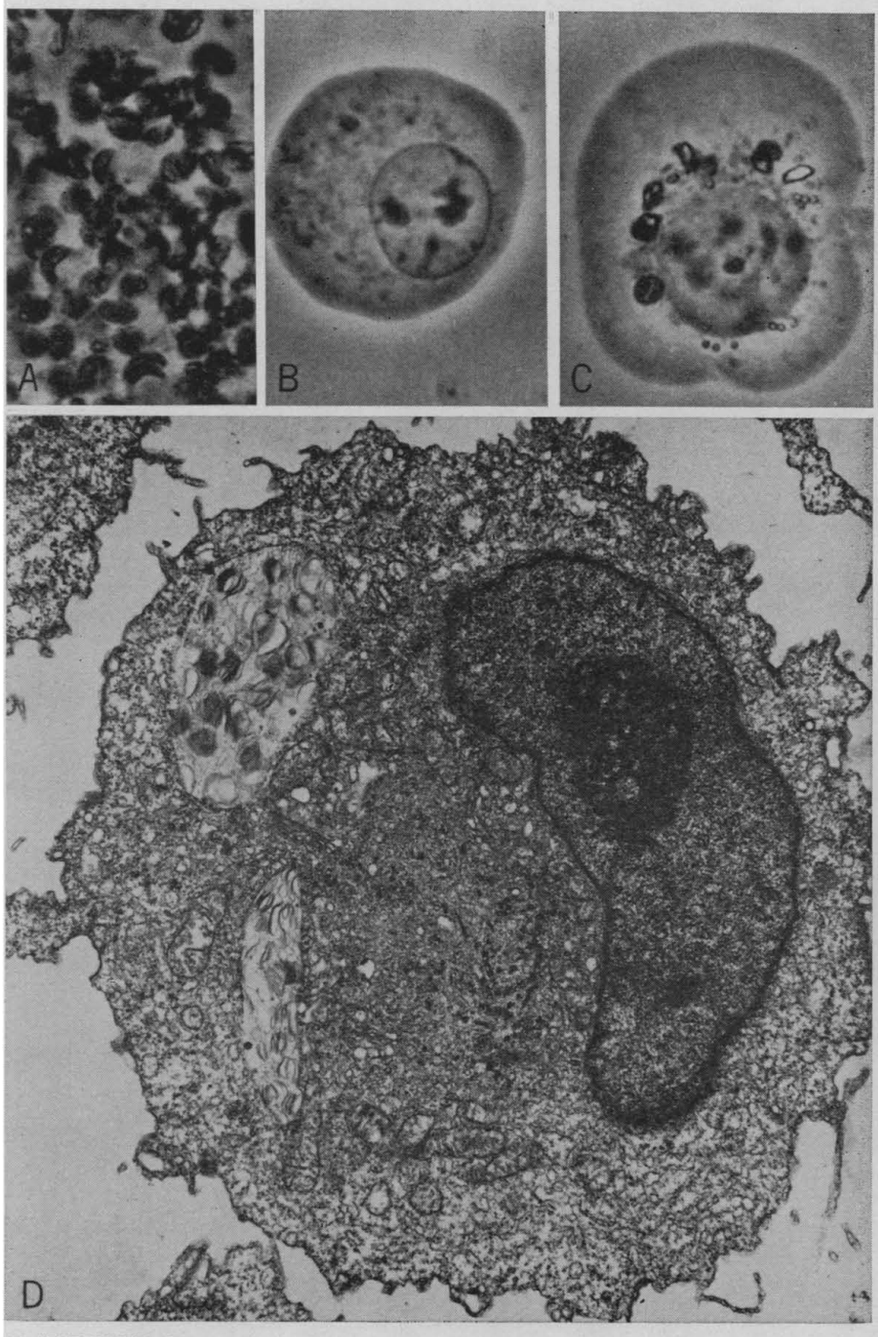
The ingested chloroplasts retained a considerable degree of structural integrity. With electron microscopy it was seen that the chloroplasts residing in the cytoplasm were only partially surrounded by a membrane. No phagocytosis vesicles surrounded the chloroplasts. The organization of chloroplast lamellae was similar to that in freshly isolated organelles. Chloroplasts reisolated after 1 or 2 days in cell culture showed light-stimulated Hill reaction activity (reduction of dichlorophenolindophenol) and CO_2 fixation; the chloroplast DNA was found to be in macromolecular form with a molecular weight in the range of 10^7 to 10^8 daltons.

Chloroplasts were isolated from leaves of fresh spinach (*Spinacia oleracea*) and African violets (*Saintpaulias*) by grinding the thoroughly washed leaves at 4°C in a mortar with a modified Honda (*6*) medium (0.25*M* sucrose, 2.5 percent Ficoll, 5 percent Dextran-40, 0.01 percent bovine serum albumin, 6 m*M* mercaptoethanol, 25 m*M* tris-(hydroxymethyl)aminomethane-HCl, *p*H 7.8). The solutions and glassware were sterile. The suspension was filtered through cheesecloth, centrifuged twice at 400*g*, and the bulk of chloroplasts was sedimented at 1000*g*. The pellet was washed twice in the same medium. Electron microscopy of the isolated organelles showed that approximately 40 to 60 percent of spinach chloroplasts and 10 to 30 percent of African violet chloroplasts retained at least part of their surface membrane. Further

purification of chloroplasts by density gradient centrifugation was omitted because it caused substantial loss of structural integrity and photochemical activity. The final pellet of chloroplasts was suspended at a concentration of 1 to 2 mg of chlorophyll per milliliter as determined by the method of Arnon (*7*).

The L cells were grown in suspension culture as described (*8*), except that streptomycin and penicillin were omitted from the medium to avoid possible damage to chloroplast functions. Cells in the logarithmic phase of growth (5×10^5 cell/ml) were incubated with isolated chloroplasts for 30 to 60 minutes at 37°C at a ratio of 20 to 25 chloroplasts per cell. The reaction was monitored by phase-contrast microscopy. Figures 1A and 2A show phase micrographs of isolated spinach and African violet chloroplasts, respectively. African violet chloroplasts are three to four times larger than the organelles of spinach. After 30 minutes of incubation with L cells, one to six spinach chloroplasts or one to two large African violet chloroplasts were present inside 70 to 90 percent of the cells. The cells were sedimented, resuspended in fresh medium, and grown in culture for several days, being exposed to about 12 hours of daylight per day. The cells were diluted with fresh medium after each division. Figures 1 and 2 show phase micrographs of L cells before incubation (Figs. 1B and 2B) and 1 to 3 days after incubation with spinach or African violet chloroplasts (Figs. 1C and 2, C to E). The chloroplasts were vivid green, and their size and structure appeared similar to that of freshly isolated chloroplasts (Figs. 1A and 2A). The uptake of chloroplasts was generally accompanied by a significant increase in cell volume from about 1500 μm^3 to 2500 μm^3, as measured in the Coulter counter.

The ingestion of chloroplasts by the L cells was verified with electron microscopy. At intervals, cells were fixed with 2 percent osmium tetroxide in 0.1*M* phosphate buffer at *p*H 7.4, and then, to enhance preservation of membranes, fixed with 0.5 percent uranyl acetate in Zetterqvist buffer as described (*9*). The samples were embedded in Araldite and sections were stained with lead citrate. Within seconds after the additions of chloroplasts to the cell suspension, projections of the cell surface contacted the organelles. After 5 to 30 minutes chloroplasts resided in the cytoplasm of most cells. Figure 1D shows an ultrathin section of an L cell 24 hours after ingestion of two spinach chloroplasts. As in isolated chloroplasts, the lamellae and grana were well distinguished but slightly disorganized. A higher magnification of the structure of lamellae is shown for African violet chloroplasts after having resided intracellularly for 1 and 2 days (Fig. 2, G



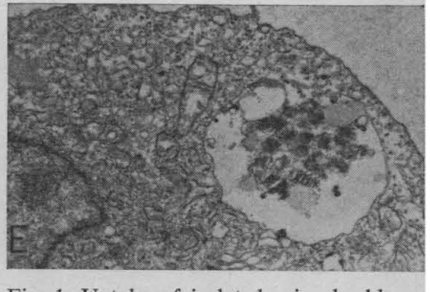


Fig. 1. Uptake of isolated spinach chloroplasts by mouse L cells. (A–C) Phase micrographs. (A) Isolated chloroplasts; (B) L cell; (C) L cell with ingested chloroplasts after 30-minute incubation and growth for 1 day. Cells dilated in hypotonic saline. (D, E) Electron micrographs. (D) Cell with two ingested chloroplasts, as described for (C). The chloroplasts are not surrounded by digestion vesicles. (E) Cell with heat-denatured chloroplasts, which are degraded in digestion vesicles after 1 day of growth (A–C, × 1700; D–E, × 8000).

and H). The organization of lamellae was similar to that of freshly isolated chloroplasts (Fig. 2F). Many organelles appeared twisted. This might have been the result of a strong cytoplasmic flow, which was also reflected in the presence of highly pleomorphic nuclei and mitochondria. The osmiophilic, probably lipid-containing granules of isolated chloroplasts (Fig. 2F) were less osmiophilic in intracellular chloroplasts. Perhaps addition of certain lipids or lipid precursors to the medium could have restored the granules. Ribosome-like particles could be seen in the stroma of both types of chloroplasts at higher magnifications. Characteristically, most chloroplasts were confluent with the cytoplasm of the cell because they were only partially limited by an outer membrane. They were not surrounded by membrane-bound phagocytosis or digestion vesicles. Such vesicles, however, were formed in control experiments in which inactive material in the form of 0.55-μm polystyrine particles or spinach chloroplasts heated at 95°C for 10 minutes (Fig. 1E) was ingested. This material was collected in membrane-bound vesicles within 1 day after phagocytosis.

After 5 days or five cell generations, one to three green chloroplasts were still present in many cells of the greatly diluted green cell population. Cells containing chloroplasts divided normally, and chloroplasts were apparently distributed into daughter cells at random, as judged by cell and chloroplast counts. It appears that chloroplasts were not rejected by the cells. The average chlorophyll content after four cell divisions was correspondingly reduced from 8.0×10^{-10} mg of chlorophyll per cell (one to two spinach chloroplasts per cell) to 1.8×10^{-10} mg of chlorophyll per cell. It is not yet known, however, whether chlorophyll of degraded chloroplasts is also retained by the cell.

The integrity of ingested spinach chloroplasts was examined in organelles reisolated from green L cells after 1 to 2 days in culture. Hill reaction activity, as reflected by the light-dependent reduction of dichlorophenolindophenol, was determined as described (*10*). Illumination of 2000 foot candles (22,000 lu/m^2) of light was provided by a 300-watt photoflood lamp. At a concentration of 0.2 to 0.5 μg of chlorophyll per milliliter of reaction mixture, values of 200 to 400 μmole of dye reduced per milligram of chloro-

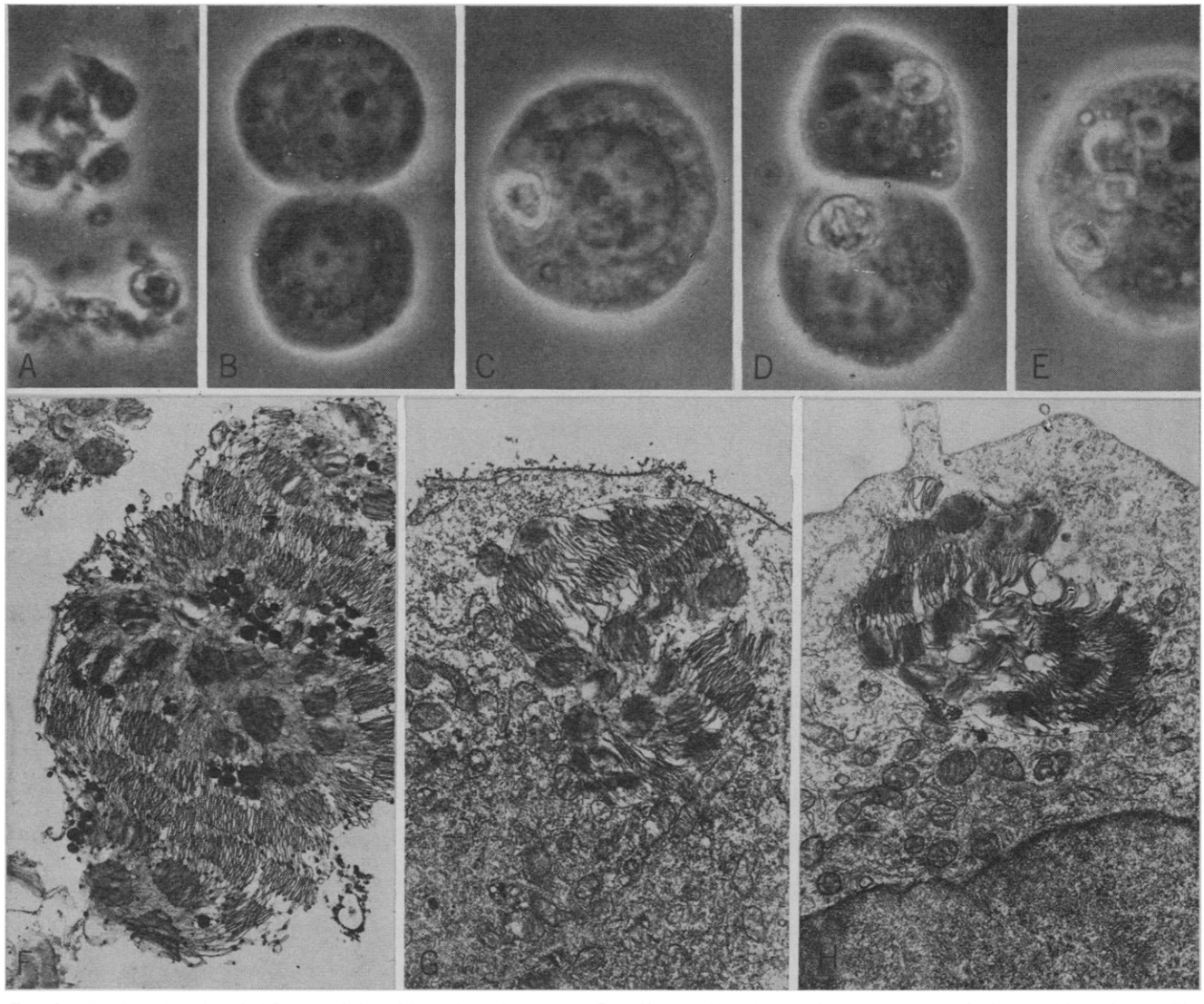


Fig. 2. Uptake of isolated African violet chloroplasts by mouse L cells. (A–E) Phase micrographs. (A) Isolated chloroplasts; (B) control L cells; (C) L cell with ingested chloroplast after growth for 1 day, (D) 2 days, and (E) 3 days. (F–H) Electron micrographs. (F, lower left) Longitudinal section of an isolated chloroplast; (G, lower center) transverse section of an ingested chloroplast after growth of L cell for 1 day and (H, lower right) 2 days. (A–E, × 1700; F–H, × 12,000).

phyll per hour were obtained for chloroplasts after 1 day in cell culture as well as for freshly isolated chloroplasts. Jagendorf and Evans (*10*) reported a similar value of about 300 μmole/mg per hour for spinach chloroplasts. Fluctuation of values was mostly due to a considerable dye reduction rate in the dark, which was subtracted from the light reaction. Contaminating L cell mitochondria were responsible for most of the dark reaction. The results suggest that the chloroplast lamellae retained some functional as well as structural integrity.

Tests of light-dependent, endogenous CO_2 fixation ($NaHC^{14}O_3$) of chloroplasts reisolated from L cells after 24 hours were carried out as described (*11*). The radioactivity was measured in acidified chloroplasts (*11*) and in a hot, 20 percent ethanol extract of the organelles, which would contain phosphoglyceric acid if present (*12*). The radioactivity in extracts of dark-kept chloroplasts was subtracted from that of illuminated preparations. Values corresponding to about 0.2 μmole of CO_2 fixed per milligram of chlorophyll per hour were obtained as total endogenous activity, and values of 0.05 to 0.1 μmole of CO_2 fixed per milligram of chlorophyll per hour were calculated from radioactivity in hot, 20 percent ethanol extracts. Similar activities were found for freshly isolated chloroplasts. Correspondingly, Spencer and Unt (*11*) reported a rate of total endogenous CO_2 fixation in the light of 0.23 μmole of CO_2 fixed per milligram of chlorophyll per hour for spinach chloroplasts without their outer membrane. Because of the lack of a limiting outer membrane of the chloroplasts, radioactive material seemed to diffuse from the chloroplasts into the medium. This was also apparent in autoradiographs of illuminated chloroplasts prepared for autoradiography as described (*13*).

The integrity of the DNA of chloroplasts after they resided intracellularly for 2 days was also studied. Chloroplasts were isolated as described above and were further purified by centrifugation in sucrose gradients (*8*). DNA was visualized after the chloroplasts were spread on a protein film by the osmotic shock technique (*8*). Clusters of DNA molecules, showing some free ends, were associated with ruptured membranes (Fig. 3). The DNA was resolved in some cases into linear molecules with molecular weights between 10^7 and 10^8 daltons. Similar values were obtained for the linear DNA isolated from purified fresh chloroplasts by osmotic shock techniques and centrifugation in cesium chloride to equilibrium.

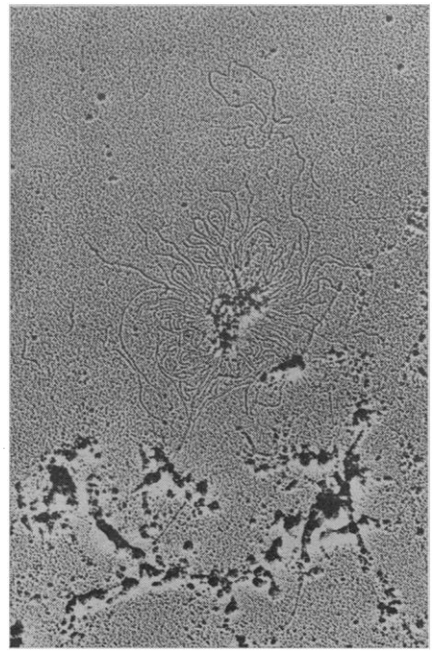


Fig. 3. Electron micrograph of the DNA of a spinach chloroplast reisolated after residing in an L cell for 2 days. Chloroplasts were lysed by osmotic shock on a protein film and rotary shadow-cast with vaporized platinum-iridium. The DNA is linear and several free ends are seen (× 20,000).

Isolated mitochondria of chicken liver, labeled with H^3-thymidine, were also taken up by L cells in 30 minutes, as observed by electron microscopy and by incorporation of the label into cells. The radioactivity of the cells declined in direct proportion to the increase of cell numbers during four cell generations.

Functional chloroplasts have been reported to occur naturally as symbiotic organelles in the digestive gland of the slug *Elysia* (*14*). We now see that animal cells can incorporate chloroplasts and mitochondria from other organisms. The degree to which these heterospecific organelles can function and multiply in the animal cells under various conditions of culture is being studied.

MARGIT M. K. NASS
Department of Therapeutic Research, University of Pennsylvania School of Medicine, Philadelphia, 19104

References and Notes

1. G. Barski, S. Sorieul, F. Cornefert, *C. R. Hebd. Séances Acad. Sci. Paris* **251**, 1825 (1960); M. C. Yoshida and B. Ephrussi, *J. Cell Physiol.* **69**, 33 (1967).
2. R. Briggs and T. J. King, *J. Exp. Zool.* **122**, 485 (1953).
3. E. G. Diacumakos, L. Garnjobst, E. L. Tatum, *J. Cell Biol.* **26**, 427 (1965).
4. E. R. M. Kay, *Nature* **191**, 387 (196 L. Gibb and E. R. M. Kay, *J. Cell Biol.* **38**, 452 (1968); A. B. Robins and D. M. Taylor, *Nature* **217**, 1228 (1968).
5. K. Bensch, G. Gordon, L. Miller, *Trans. N.Y. Acad. Sci. Ser. 2*, **28**, 715 (1966).
6. S. I. Honda, T. Hongladarom, G. G. Laties, *J. Exp. Bot.* **17**, 460 (1966).
7. D. I. Arnon, *Plant Physiol.* **24**, 1 (1949).
8. M. M. K. Nass, *Proc. Nat. Acad. Sci. U.S.* **56**, 1215 (1966); *J. Mol. Biol.* **42**, 521 (1969).
9. —— and S. Nass, *J. Cell Biol.* **19**, 593 (1963).
10. A. T. Jagendorf and M. Evans, *Plant Physiol.* **32**, 435 (1957).
11. D. Spencer and H. Unt, *Aust. J. Biol. Sci.* **18**, 197 (1965).
12. A. A. Benson and M. Calvin, in *Meth. of Enzymol.* **4**, 890 (1957).
13. A. Gibor, *Proc. Nat. Acad. Sci. U.S.* **54**, 1527 (1965).
14. D. J. Taylor, *J. Mar. Biol. Ass. U.S.* **48**, 1 (1968).
15. Supported by NIH grant 5-P01-AI07005 and PHS career development award 1-K03-AI-0830. I thank Mrs. A. Hathaway, Mrs. H. Williams, J. R. W. Hobbs and W. Colquhoun for technical assistance.

1 April 1969

Nerve Endings: Rapid Appearance of Labeled Protein Shown by Electron Microscope Radioautography

Abstract. *Electron microscope radioautographs of nerve ending fractions from mouse cerebrum show that radioactive protein is associated with nerve endings 15 and 90 minutes after intracerebral injection of tritiated leucine and lysine. The results are consistent with synthesis of protein at nerve endings, or with extremely rapid transport of particulate protein to nerve endings in the mouse cerebrum, or both.*

For days after intracerebral injection of a pulse of radioactive leucine, labeled soluble protein progressively appears in nerve endings (*1*, *2*). This delay is believed to be due to transport of soluble protein through axons from a perikaryal site of synthesis. Although there is very little labeled *soluble* protein in the nerve ending fraction 1 hour after injection, there is already substantial labeled *particulate* protein in this fraction at this time (*1*, *2*). Some of this appears to be mitochondrial protein made at nerve endings (*2*, *3*), but some is not associated with mitochondria (*2*). Since the nerve ending fraction is known to be