THE INCORPORATION OF RADIOACTIVE PROLINE INTO CULTURED CELLS

Interpretations Based On Radioautography and Electron Microscopy

H. W. ISRAEL, M. M. SALPETER, and F. C. STEWARD

From the Laboratory of Cell Physiology, Growth, and Development and the Section of Neurobiology and Behavior (Division of Biological Sciences) and the Department of Applied Physics, Cornell University, Ithaca, New York 14850

ABSTRACT

Cultured carrot explants, stimulated to grow rapidly in a medium containing coconut milk, were labeled with radioactive proline. After an initial period of absorption (8 hr for proline-^{3}H; 24 hr for proline-^{14}C) the tissue was allowed to grow for a further period of 6 days in a similar medium free from the radioactivity. Samples were prepared for electron microscopy and radioautography at the end of the absorption period and also after the further growth. The distribution of the products from the radioactive proline in the cells is shown by high-resolution radioautography and is rendered quantitative for the different regions of the cells. The results show that the combined label, which was present in the form of proline and the hydroxyproline derived from it, was all in the protoplasm, not in the cell walls. Any combined label that appeared to be over the cell walls is shown to be due to scatter from adjacent cytoplasmic sites. Initially the radioactivity was concentrated in nuclei, even more so in nucleoli, but it subsequently appeared throughout the ground cytoplasm and was also concentrated in the plastids. The significance of these observations for the general concept of a plant cell wall protein and for the special problem of growth induction in otherwise quiescent cells is discussed.

INTRODUCTION

In 1948 the means were described (2) by which small explants of secondary phloem from carrot roots could be caused to grow very rapidly under aseptic conditions. Later the fine structural changes which accompany the induction of growth in the individual cells of the explants were examined (8). The present paper describes the results of an investigation which has applied to such actively growing explants the combined techniques of radioautography and electron microscopy in ways which permitted the localization of certain radioactive substances, proline-^{3}H and proline-^{14}C, and their products in the cells. The background of knowledge which motivated this work and against which the results should be interpreted is as follows.

The otherwise quiescent carrot tissue was originally caused to grow rapidly in a basal nutrient medium by the stimulus imparted by coconut milk (or coconut water) which is the liquid endo-

sperm of the coconut. Later, it was also shown (16) that potato tuber tissue could be induced to grow under similar conditions by the combined action of coconut milk (10% by volume) and 2,4-dichlorophenoxyacetic acid (2,4-D) at 5–10 ppm. As the carrot and potato cells grew, they synthesized protein; and when this protein was analyzed in bulk in the form of an alcohol-insoluble residue it was found, unexpectedly, to be enriched with hydroxyproline (25).

When proline-^{14}C was furnished to the rapidly growing carrot or potato explants, it was detectable in the alcohol-insoluble fraction after short periods (order of 15 min). The ratio of combined proline-^{14}C to hydroxyproline-^{14}C declined with time in a manner which suggested that it—the proline—was hydroxylated *after* it was bound. If hydroxyproline was supplied exogenously to the growing tissue, it antagonized the growth, suppressed the proline incorporation, and behaved as a competitive proline antagonist, a property shared in varying degrees by many hydroxyproline derivatives that were subsequently tested (22).

Once the carbon of proline-^{14}C was incorporated into the alcohol-insoluble hydroxyproline-rich complex of the tissue, it remained fixed; it did not turn over or become dispersed throughout other compounds and it appeared as proline-^{14}C and hydroxyproline-^{14}C. Thus, proline behaved as though it entered a structural, nonmetabolizable protein moiety.

Although, at the outset, the analogy with collagen was suggestive, nevertheless the material in question was not, chemically, a plant collagen for its content of glycine in particular seemed too low relative to hydroxyproline. Moreover, carrot cell homogenates, freed from large particles at 100,000 *g*, also contained hydroxyproline-rich substances, much of which, not surprisingly, remained in the large fragments and cellular debris which were removed by slow speed centrifugation (12). The 100,000 *g* supernatant contained several soluble, electrophoretically separable entities with different proline-hydroxyproline ratios as well as more immobile material that remained at the point of application in the electrophoretic systems then employed (17). These hydroxyproline-containing substances had, however, been obtained in ways which precluded their presence in the cell walls or its fragments, all of which remained in the cellular debris. In fact, the early picture was that proline-^{14}C first appeared as hydroxyproline in more soluble and mobile entities and later seemed to accumulate in the larger less mobile components referred to.

Proline-^{14}C was also furnished to particulate preparations obtained from the actively growing cells (23). Although these preparations were made by techniques then supposed to yield mitochondria, they proved to be (cf. Fig. 1b, loc. cit.) vesiculate fragments of cytoplasm with adhering granules (ribosomes). The proline-^{14}C readily entered the alcohol-insoluble (protein) fraction of such preparations but it was *not* hydroxylated. Thus the hydroxylation of the combined proline depended on some feature of the intact cells which was not reproduced in the isolated particles. These varied observations seemed the more interesting because the formation of the nonmetabolizable, structural, hydroxyproline-rich moiety was so conspicuous in cells undergoing a recrudescence of growth and, as shown later, these cells also exhibited their innate totipotency as they formed embryoids and generated whole plants from free cells (15, 21).

Work by Lamport on another but somewhat similar system localized in the cell walls not only 95% of the combined hydroxyproline derived from proline, but also as much as 27% of the cell nitrogen or approximately 40% of the total cell protein (for review see reference 10). The results to be presented bear upon the generality of Lamport's interpretations.

The cultured tissue system used by Lamport originated from cambium of *Acer pseudoplatanus* which was first prompted to grow (like the potato tuber tissue referred to above) and has since been maintained by the combined effects of coconut milk and 2,4-D. Those who have worked with, or cited, this material have reached the conclusion that the bound proline-hydroxyproline was virtually all in the cell walls (3–7, 10, 11). Consequently they maintained that measuring the combined hydroxyproline of a tissue was tantamount to measuring cell wall protein. Lamport (10, 11), in advance of a definitive isolation and analysis, has even named the protein moiety in question "extensin."

The present study was, therefore, undertaken to localize the bound products of radioactive proline in the cells of the cultured tissue system which provided the first evidence of a hydroxyproline-rich, structural, nonmetabolizable moiety.

TABLE I

Distribution of Developed Grains from Proline-^3H in Cultured Carrot Cells after 8 Hr and after 6 Days

(Columns 2a, 3a, and 4a, which are based on the distribution of 3776 grains in 70 electron micrographic fields, represent tissue sampled immediately after an 8 hour labeling period; Columns 2b, 3b, and 4b, which are based on 3831 grains in 133 fields, represent tissue sampled 6 days after the 8 hour labeling period.)

Column 1	Column 2		Column 3		Column 4	
Regions of the cell	Per cent of the total grains observed		Per cent of total area as region in question		Relative concentration: Per cent total grains / Per cent total area	
	a	b	a	b	a	b
Cell wall	5.42	4.00	9.94	12.37	0.55	0.32
Protoplasm	84.00	90.52	41.58	56.15	2.02	1.61
Cytoplasm	43.20	79.90	28.72	49.66	1.50	1.61
Ground cytoplasm*	32.66	69.00	20.50	40.72	1.59	1.69
Plastids	6.70	5.85	4.68	2.54	1.43	2.30
Mitochondria	2.48	3.05	2.04	3.50	1.22	0.87
Vesicles‡	1.36	2.00	1.50	2.90	0.91	0.69
Nucleus	40.80	10.62	12.86	6.49	3.17	1.64
Nucleolus	16.20	1.10	3.46	0.54	4.68	2.04
Vacuole	10.58	5.48	48.48	31.48	0.22	0.17

Data are given first (column 2) as percentages of the total number of developed grains observed in the specific regions of the cells (column 1), and then as relative concentrations (column 4) established by weighting these percentages against the percentages of the total area (column 3) occupied by each of the specified regions.

* Cytoplasm excluding all membrane-bounded organelles.

‡ Golgi bodies, lipid droplets, etc.

MATERIALS AND METHODS

Radioactive proline was applied for 8 hr[1] or 24 hr[2] to actively growing carrot explants during the exponential phase of their growth, i.e. after 5 days of aseptic culture in a basal medium supplemented with coconut milk. Throughout these experiments, whenever the proline was not radioactive it was replaced with unlabeled proline at the same concentrations as those given. After the tissue explants had received the radioactive proline, they were transferred to culture tubes containing fresh volumes of the culture medium and allowed to grow for a further period, up to 6 days. During this time the tissue grew substantially in accordance with the time course of fresh weight and cell number which has previously been described in the form of growth curves in papers published from this laboratory (see reference 20).

Samples of the tissue were taken immediately after the 8- or the 24-hr labeling periods (i.e. after a total of 5⅓ days of culture for the proline-^3H or after 6 days of culture for the proline-^{14}C) and at the conclusion of the experiments (i.e. after a total of 11 days of culture), and were prepared for electron microscopy by several of the procedures which have yielded a detailed knowledge of the fine structure of carrot cells under these conditions of growth (see references 8 and 9). Briefly, the explants were fixed successively in unbuffered 2.5% aqueous solutions of glutaraldehyde and osmium tetroxide, dehydrated successively in ethanol and propylene oxide, and embedded in Epon 812 resin. These methods do not extract combined (i.e. alcohol-insoluble) label. Actual tests showed that the solvents and fixatives used to prepare the tissue for electron microscopy did not remove appreciable amounts of the combined radioactivity (50 explants labeled with proline-^{14}C recorded 35,000 cpm per explant prior to fixation and 32,600 afterwards at a stage just prior to embedding, whereas 10 explants labeled with tritiated proline did not lose any detectable amount of radioactivity). Moreover, the radioactivity, whether supplied as proline-^3H or as proline-^{14}C, remained in the form of combined proline and hydroxyproline

[1] L-proline-3,4-^3H; specific activity: 5 c/mM; concentration: 1.78 μg/ml (56 μc/ml).

[2] L-proline-UL-^{14}C; specific activity: 204 mc/mM; conc.: 1.425 μg/ml (2.5 μc/ml).

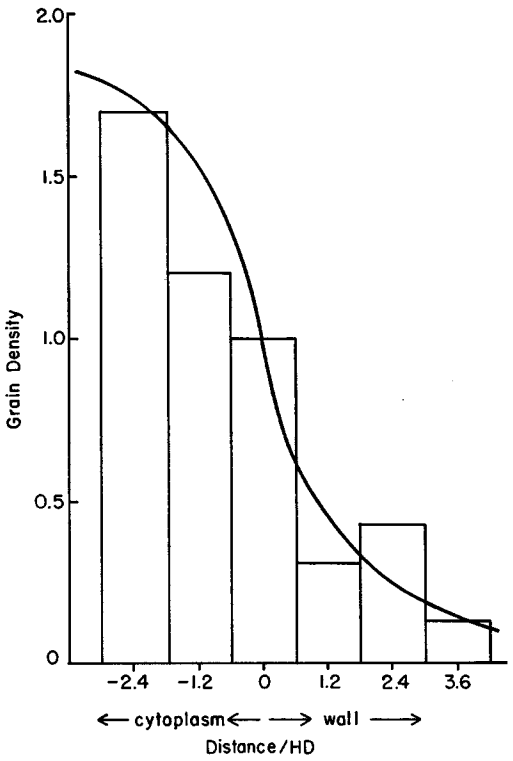


FIGURE 1 The distribution of developed silver grains overlying cell walls and adjacent cytoplasm in tissue sampled 6 days after it had absorbed proline-^{3}H during an 8 hr period.

A plot of the density of silver grains at different distances from the cytoplasm-cell wall interface is shown in the form of a histogram. The distances are recorded in multiples of a resolution unit (HD). The distribution fits the theoretical curve which describes the scatter of grains, formed by radioactive sites uniformly distributed in a broad band, on one side only of an interface.[3] This radioactive band is, in these specimens, on the cytoplasmic side of the cytoplasm-cell wall interface. (For further details, see text.)

in the general ratio of 1:1 even after the tissue had been fully prepared for electron microscopy.

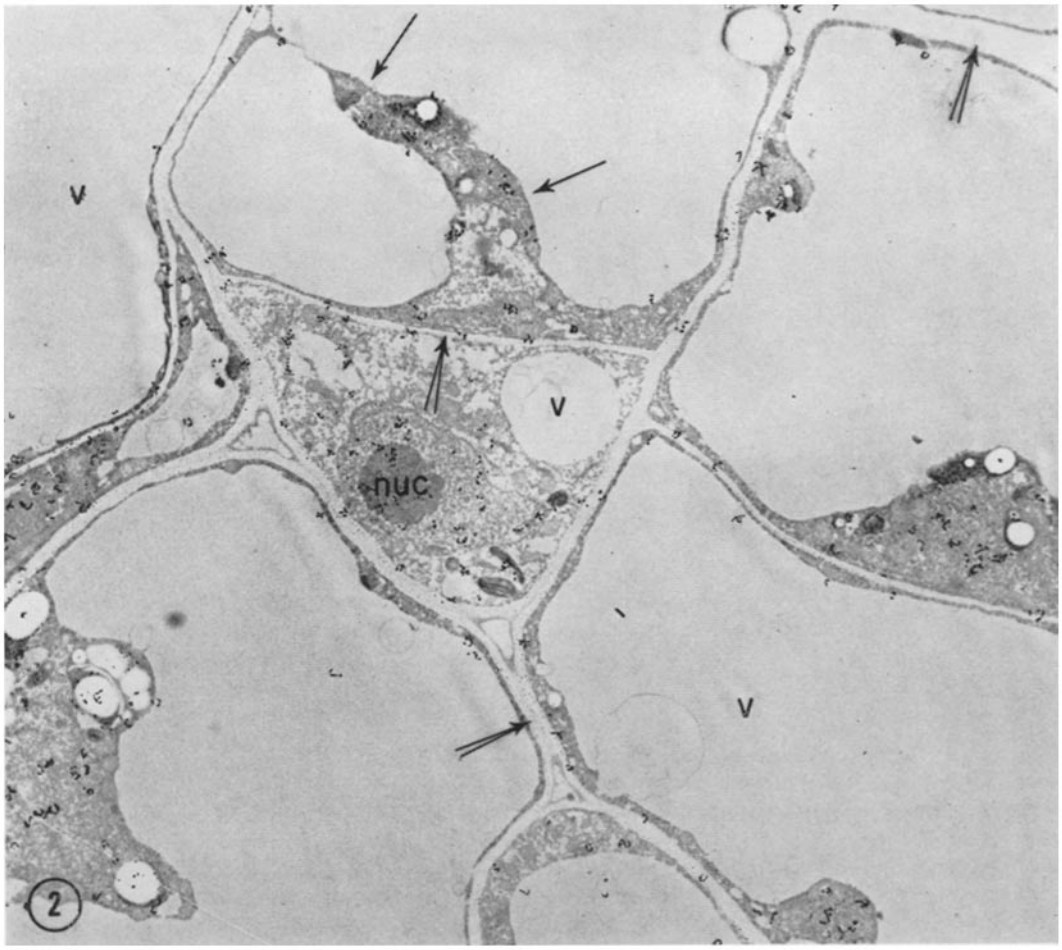
To localize the products of the proline-^{3}H and the proline-^{14}C in the tissue, the radioautographic technique used (cf. Salpeter, 14) was based on work of Bachmann and Salpeter (1). In the application of this technique two procedures of specimen preparation (SP) were employed. In the one (SP-1), sections of a pale gold interference color were coated with Ilford L4 emulsion and developed with Kodak Microdol-X. This produced relatively large irregular silver grains at points where radioactive disintegrations affected the emulsion (see Fig. 3). In the other (SP-2) silver grey sections were coated with Kodak NTE emulsion and developed with Kodak Dektol. This procedure produced grains in the form of compact aggregates of dots (see Fig. 4) and its resolution is better[3] than that of SP-1 by a factor of 2.0 (1). For both SP-1 and SP-2 the sections were stained with solutions of uranyl acetate and vacuum coated with carbon before being coated with the respective emulsions. One of the factors which limit the inherent resolution of any radioautographic technique is the energy of the radioactive source in the specimen. Although the resolution of SP-1 is somewhat reduced if ^{14}C rather than ^{3}H is the emitting source,[3] nevertheless the resolution obtained with ^{14}C was found to be sufficient to distinguish between the radioactivity in the cytoplasm and that in the cell wall. Because uniformly labeled proline-^{14}C had been used in earlier biochemical work (13), it was used here in order to support the data obtained by the use of tritiated proline.

In the outcome, the sites of radioactive material could be located with known degrees of precision. One could examine simultaneously on the photographic print both the fine structure of the specimen and the distribution of the developed silver grains. These grains are due to the radioactivity which is known to be present in the form of combined proline and hydroxyproline in the approximate ratio of 1:1 for the cell as a whole (13).

The distribution of radioactivity in the cells was made quantitative in the following manner. The percentage of grains which occurred in a given region of a field was compared with the percentage of the field area which that same region represents; in so doing, relative concentrations of the labeled material in the different parts of the cell were established. This was done by locating a "center of gravity"[3] for each developed grain on photographic prints of the electron micrographs and then assigning the grain in question to a cellular region as indicated in Table I. The enlarged photographic prints were then cut and weighed, so that relative areas (assumed proportional to the weight of paper) occupied by the regions in question could be measured. In this way, data which were first assembled on an absolute basis could be weighted against the surface area of the cellular components in question. (See also reference 19.)

The distribution of developed grains in relation to the cell walls was further analyzed by obtaining a density gradient of the grains on *both* sides of, and near to, the cytoplasm-cell wall interface. This was done because Salpeter et al.[3] have been able to show the theoretical distribution of developed grains that should arise from various tri-

[3] Salpeter, M. M., L. Bachmann, and E. E. Salpeter. In preparation.



Key to abbreviations in Figs. 2–24

m, mitochondrion	*p*, plastid
n, nucleus	*v*, vacuole
ne, nuclear envelope	*vs*, vesicle
nuc, nucleolus	*w*, wall

FIGURES 2–4 The fine structure of the cells and the radioautographic technique.

FIGURE 2 A view, at low magnification, of growing cultured carrot cells from tissue sampled 5½ days after inoculation and immediately after it absorbed proline-^{3}H during an 8 hr period. The parenchyma cells, bounded by cell walls of varied thicknesses (double-stemmed arrow), contain vacuoles and protoplasm in proportions that indicate their individual states of growth and metabolic activity (cf. reference 8). The figure shows some large vacuolated cells (lower right) with scant layers of parietal cytoplasm, cells with intravacuolar strands (upper center, arrow) along which cyclosis typically occurs, and small dense cells (center) that could have originated by recent, unequal division from larger vacuolated cells. It may be noted that most of the developed silver grains in this radioautograph coincide with the protoplasm. (SP-1). × 4,000.

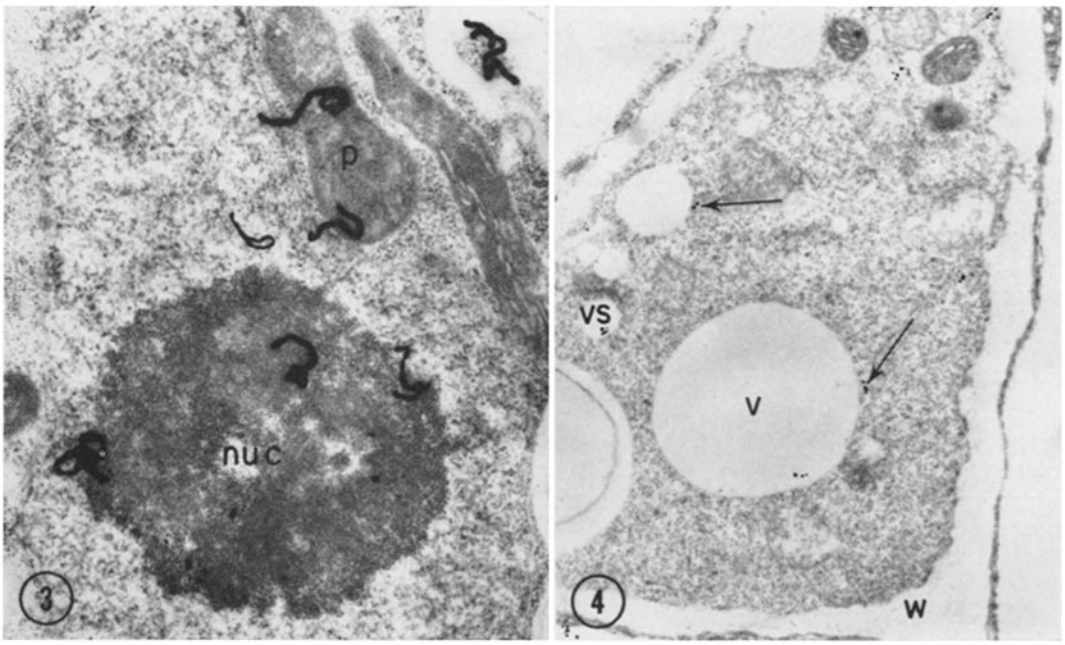


FIGURE 3 A portion of a nucleus with adjacent cytoplasm in a radioautograph that shows the typical developed grains rendered by the lower-resolution technique (SP-1). The tissue here was sampled 6 days after it had absorbed proline-^{3}H during 8 hr. × 17,500.

FIGURE 4 A section through a dense cell, with its cell wall, from tissue fixed 6 days after labeling with proline-^{3}H. The sizes of developed silver grains (arrow) produced by the higher-resolution technique (SP-2) are illustrated in this radioautograph. × 15,500.

tiated sources. The shapes of such theoretical distributions are universal although the spread is a function of the resolution obtained. Therefore, the data obtained by the use of SP-1 and SP-2 could be pooled by recording the distribution of grains in units of half-distance (HD), from the cytoplasm-cell wall interface; HD represents the distance from a radioactive line source within which one half of the developed grains should be located (HD for SP-1 is 1600 A; for SP-2, 800 A). This analysis gives meaningful information if the walls are thick enough so that they comprise several HD units; the convention adopted was to apply it to walls 3 HD units thick or more. However, the observations on all thinner walls were also included in the over-all analysis of relative concentrations of labeled material in the cell.

EXPERIMENTAL RESULTS

The results of this investigation are contained in Figs. 1–24 and in Table I.

The distribution in the cells of radioactivity absorbed as proline-^{3}H is best seen in Table I; the data show how the radioactivity was distributed as at the end of the absorption period and also after the further period of 6 days during which the tissue explants grew.

Uniform and random distribution of the developed silver grains would produce relative concentrations (Table I, column 4) of 1.0; relative concentrations significantly greater than 1.0 represent an accumulation of the radioactivity in the regions named, concentrations significantly lower than 1.0 show that the region named was avoided by the labeled substances. Systematic errors in measuring the areas of the named regions were less than 10%; this being so, the relative concentrations for all regions in Table I, except those for the mitochondria and the vesicles, can be shown by χ^2 tests to be significantly different from unity. The same tests also indicate that the shifts in relative concentration between the 8 hr labeling period and the 6 day period of subsequent growth are significant, even at the 99% confidence level, for the

plastids, the nuclei, and the nucleoli. It should be mentioned here that a pool of alcohol-soluble radioactive uncombined proline, which existed at the end of 8 hr, was the source of the larger amount of combined radioactive proline-hydroxyproline in the explants at 6 days (approximately 35,000 cpm per explant were recorded at 6 days, contrasted with almost 13,000 at 8 hr).

The conclusion is (cf. reference 19) that there was no appreciable concentration of radioactivity from proline-^{3}H in the cell walls as they existed *in situ* in the cultured carrot explants. Even the few developed silver grains that were superposed upon the cell walls originated from sites in the adjacent cytoplasm (see Fig. 1). This figure shows a concentration of developed grains in the cytoplasm which rapidly declined upon approaching the boundary of the cytoplasm and, thereafter, with distance into the wall; this is precisely what is to be expected if the cytoplasm were uniformly labeled and the cell walls were not labeled.[3]

The relative concentrations (Table I, Column 4) over the cell walls (0.55 and 0.32) were very significantly below 1.0 yet they were not zero. Since Fig. 1 shows that this was due to scattered radiation from adjacent radioactive cytoplasm, the apparent shift as between the 8-hr and 6-day samples merely reflects the fact that the cell walls of the later samples tended to be thicker and therefore less affected throughout by such scatter.

The predominance of grains over the cytoplasm and the virtual absence of grains over the cell walls may be seen especially in Figs. 7, 10, 11, and 19 (SP-1) and in Figs. 4, 8, 13, 14, 17, 18, and 20 (SP-2). The same results appear whether the radioactivity is sought in relatively old walls (Figs. 4, 7, 8, 11, 13, 19, and 20) or in walls of recently divided or even dividing cells (Figs. 9, 14, 17, and 18), and they are equally apparent whether cells were examined at the end of the absorption period (Figs. 2, and 17–20) or after the more extended period of subsequent growth (Figs. 3, 4, 7–11, 13, and 14) or after the use of proline-^{3}H or proline-^{14}C (cf. Figs. 7, 8, and 22). Neither the surface of newly forming walls (Figs. 17–19) nor the presence of adjacent plasmodesmata within walls (Figs. 10, and 17–19) was associated with a concentration of radioactivity above the very minimal scatter which was even encountered over vacuoles. The concentration of radioactivity absorbed from proline in the protoplasm (84 to 91%) and its virtual absence from the cell walls is, therefore, amply demonstrated in this material.

The presence of the radioactivity predominantly in the cytoplasm may again be seen in Figs. 6, 9, and 12 which show it even in cytoplasmic strands which were not in contact with the cell wall. Among the cytoplasmic inclusions, plastids showed the greatest tendency to accumulate radioactivity from proline; this was especially so after the period of growth during which plastids had developed while they were in contact with proline-^{3}H in the cytoplasm (cf. Figs. 3, 5, and 14). At the other extreme, vesiculate inclusions, such as those produced by Golgi bodies, were notably free of radioactivity in the form of combined proline-hydroxyproline (cf. Figs. 4, 6, and 14) and, similarly, mitochondria failed to concentrate the radioactivity from proline.

The data of Table I, Column 4 show that the greatest relative concentration of absorbed radioactivity appeared first (i.e. at 8 hr) in the nucleus and even more so in the nucleolus. With time (i.e. after 6 days) the relative concentration in these areas had declined significantly. The local concen-

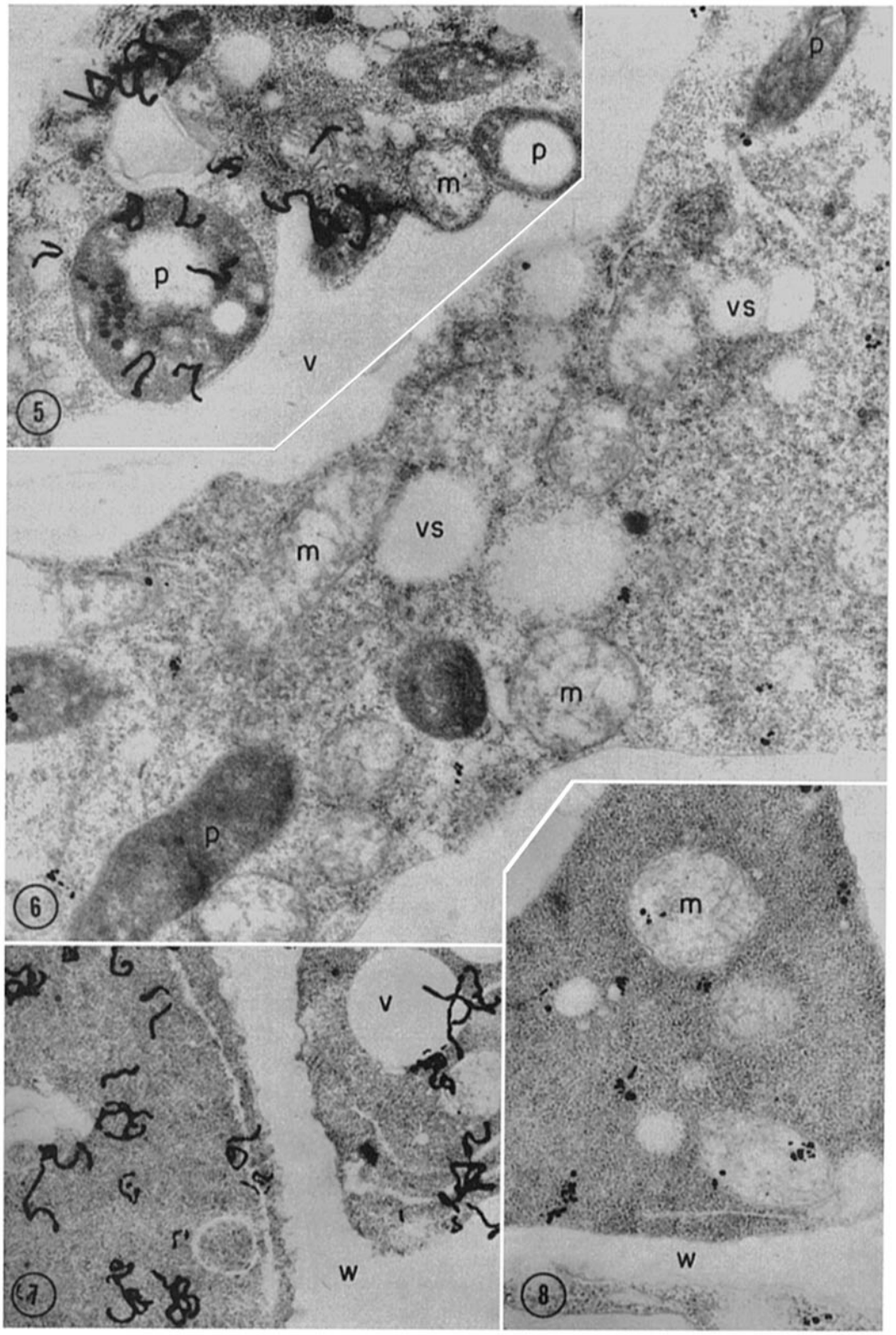
FIGURES 5–8 The distribution of radioactivity in the cytoplasm of cultured carrot cells sampled 11 days after inoculation and 6 days after they had absorbed proline-^{3}H during an 8 hr period.

FIGURE 5 Portion of parietal cytoplasm showing a concentration of developed grains over plastids. (SP-1). × 21,000.

FIGURE 6 Longitudinal section through an intravacuolar strand where the radioactivity is localized in the granular ground cytoplasm. (SP-2). × 24,000.

FIGURE 7 Heavily labeled, granular ground cytoplasm adjacent to a cell wall that is unlabeled. (SP-1). × 18,000.

FIGURE 8 The radioactivity in this section is chiefly centered in the densely granular cytoplasm. (SP-2). × 33,000.


p
m
p
v
5
p
vs
vs
m
m
p
6
v
w
7
m
w
8

tration of radioactivity from absorbed and combined proline-^{3}H in the nucleus may also be seen in Figs. 20 and 21, and from proline-^{14}C in Figs. 23 and 24.

Thus, all the evidence obtained by studying the distribution of radioactivity present as combined proline and as the hydroxyproline derived from it is consistent with the following picture. The entering exogenous proline goes first to the nucleus and is even more concentrated in the nucleolus. Thereafter it is to be found distributed throughout the ground cytoplasm where, in these growing cells, there are abundant cytoplasmic granules (8). The total radioactivity fixed in the cytoplasm can increase with time at the expense of the soluble pool. However, Table I shows that the concentration in the plastids increases more rapidly than that in the cytoplasm as the plastids develop into chloroplasts (cf. reference 9). The distribution of developed grains seen in the preparations is consistent with the radioactivity in the cytoplasm being uniformly distributed up to the cell wall, but not extending beyond it (cf. Fig. 1).

Lest there be any doubt that the radioactivity supplied in the form of proline-^{3}H was present in the cell in any form other than combined proline and the hydroxyproline derived from it, the same procedures were carried out with generally labeled proline-^{14}C. As shown in Figs. 22–24, the evidence is again unequivocal, for there was no significant concentration of developed grains over the cell walls (cf. Fig. 22). The grains were distributed again generally throughout the ground cytoplasm (Figs. 22–24) and were also more concentrated in the nucleus and the nucleolus, especially at the end of the absorption period.

DISCUSSION

The facts observed and presented above need to be discussed from two points of view: first, their bearing upon a current idea that there is a special hydroxyproline-containing cell wall protein, which has been termed "extensin" (10, 11) and, secondly, their implications for the role of the combined hydroxyproline and hydroxyproline-rich moiety which has been shown to be prominent in cells which undergo growth induction and are capable of morphogenesis (15, 21).

In so far as cultured carrot explants and cells are concerned, the conclusions are as follows.

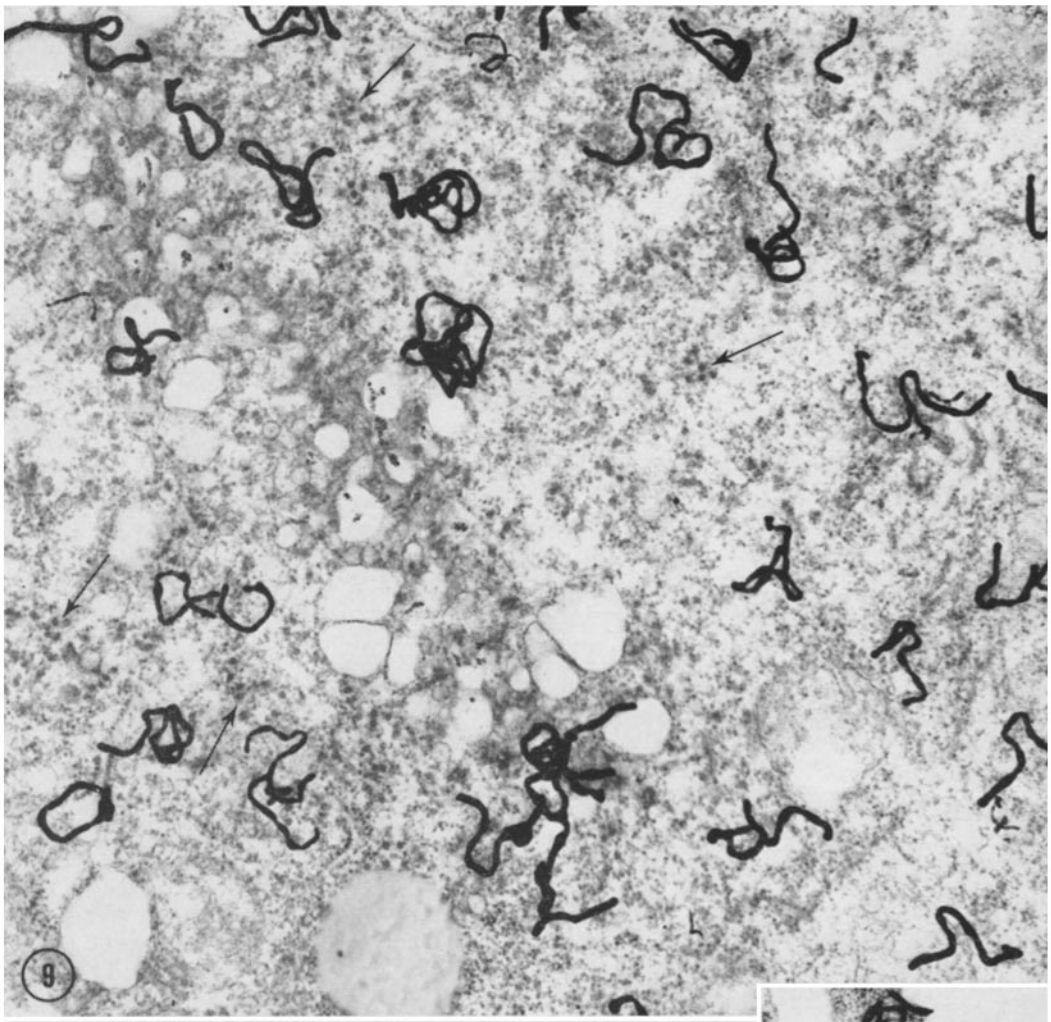
The evidence of high-resolution radioautography combined with observations on the fine structure of these cells is not consistent with the view that the proline, which undoubtedly enters into a stable structural protein moiety, where it is converted to hydroxyproline, remains largely embodied in the plant cell wall. The wall *in situ* is virtually free of radioactivity from proline even though the radioactivity is prominent elsewhere in the cells. If there is any thought that attempted localization of combined proline and its derived hydroxyproline by the use of the specifically labeled tritium source is unsound, this doubt should be dispelled because identical conclusions follow from the use of generally labeled ^{14}C sources. Therefore, the recent conclusions of Lamport (10) that over 90% of the combined proline-hydroxyproline is in the cell wall, that the measurement of combined hydroxyproline is tantamount to measuring cell wall protein, and that there is a characteristic cell wall protein ("extensin") are all inconsistent with the data here reported from the study of cell wall *in situ* and the conclusions which are drawn from them. Equally suspect is the concept that algal cells contain built-in protein as an integral and very prominent part of their cell walls (cf. reference 24).

It is difficult to locate complex substances precisely in living cells, in cytologically meaningful ways, by the study and fractionation of homog-

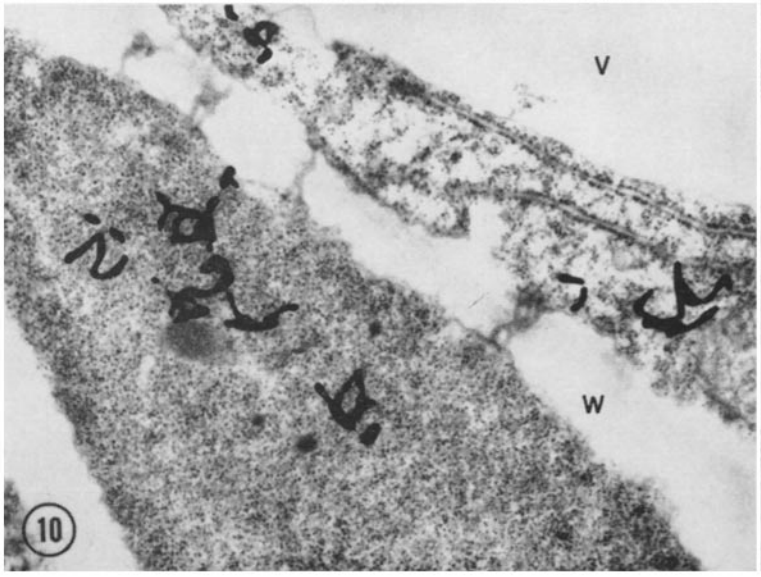
FIGURES 9–11 Localization of radioactivity in carrot tissue 6 days after it was exposed to proline-^{3}H during a period of 8 hr.

FIGURE 9 Section through the achromatic apparatus, seen in polar view, of a telophase mitotic figure. Note particularly the general absence of labeling in the cell plate of the phragmoplast which is visible as a diagonal row of grouped vesicles. The grains are, however, localized amongst the spindle-tubules (arrows) that are shown mostly in cross section. (SP-1). × 30,000.

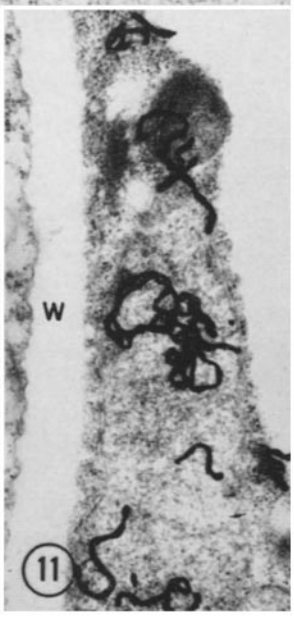
FIGURES 10 and 11 The radioactivity in these sections appears as developed grains in the densely granular cytoplasm but not in the cell walls or vacuoles. Figs. 10 and 11, (SP-1). Fig. 10, × 45,000; Fig. 11, × 26,000.



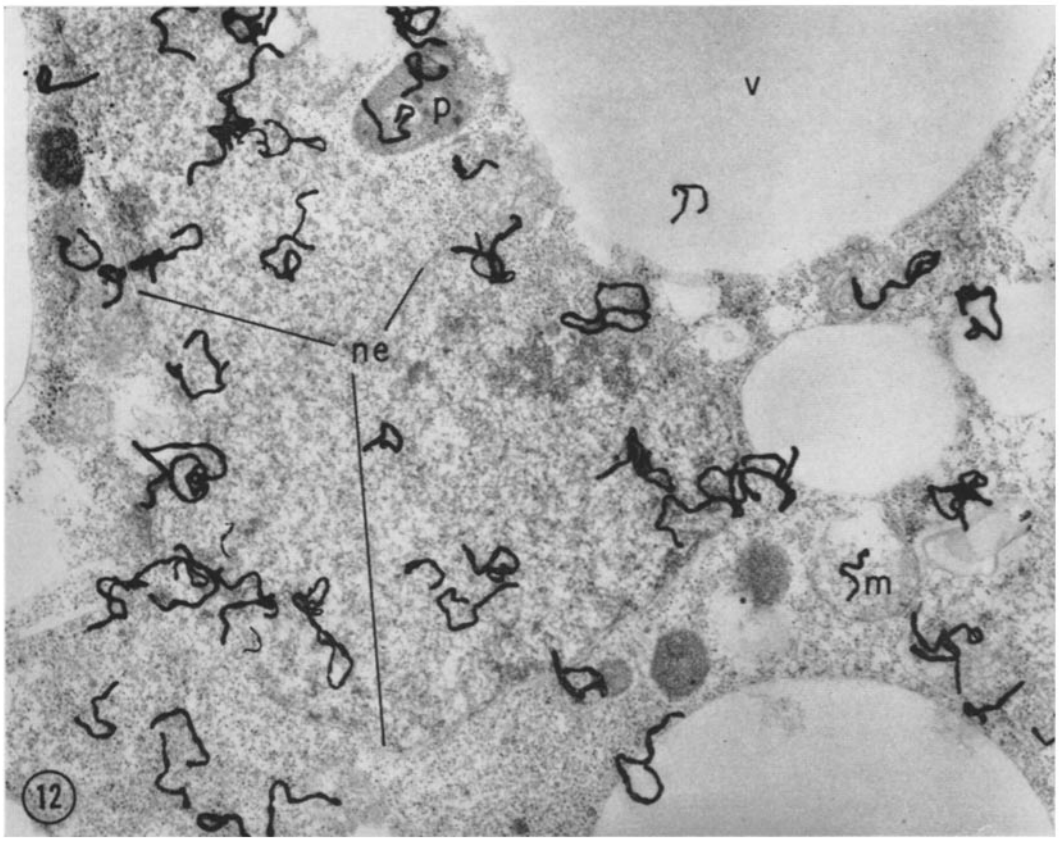
9



10



11



FIGURES 12 and 13 The distribution of radioactivity in the nucleus and cytoplasm of carrot cells following 6 days of culture after incubation in a medium containing proline-^{3}H for 8 hr.

FIGURE 12 The radioactivity is here distributed between the nucleus and its contiguous cytoplasm in this section which also passed through an intravacuolar strand. (SP-1). × 25,000.

enates made from a crop in bulk. Once the organization of living cells is destroyed chemical changes occur by the proximity of mutually reactive substances which, in the intact cell, may exist in separate compartments. Moreover, in a dense crop, many cells may become senescent and undergo changes as part of a sort of biochemical differentiation. The combined use of radioautography and electron microscopy can locate these substances as they are fixed in positions which they occupy in vivo. Moreover, the statistical interpretation of the distributed radioactive sites as revealed by developed silver grains now permits a distinction to be made between accumulations of radioactive sites near to, as compared to within, a boundary surface layer (cf. Fig. 1).

In another investigation in this laboratory, use has been made of more biochemical methods applied to homogenates of cells which have been subjected to fractionation and extraction by standard procedures. The full detail of this work will be published elsewhere. As in former work, so in this more recent study there is a preponderance of the combined proline-hydroxyproline in the large particles, fragments, and cell debris which are obtained at relatively low centrifugal speeds. All the indications are that this material is of high molecular weight and is difficult to remove from the cellular debris and to purify.

Earlier work (12, 17) had, however, shown that there were, in the more soluble fractions from 100,000 *g* centrifugations, several electrophoretic-

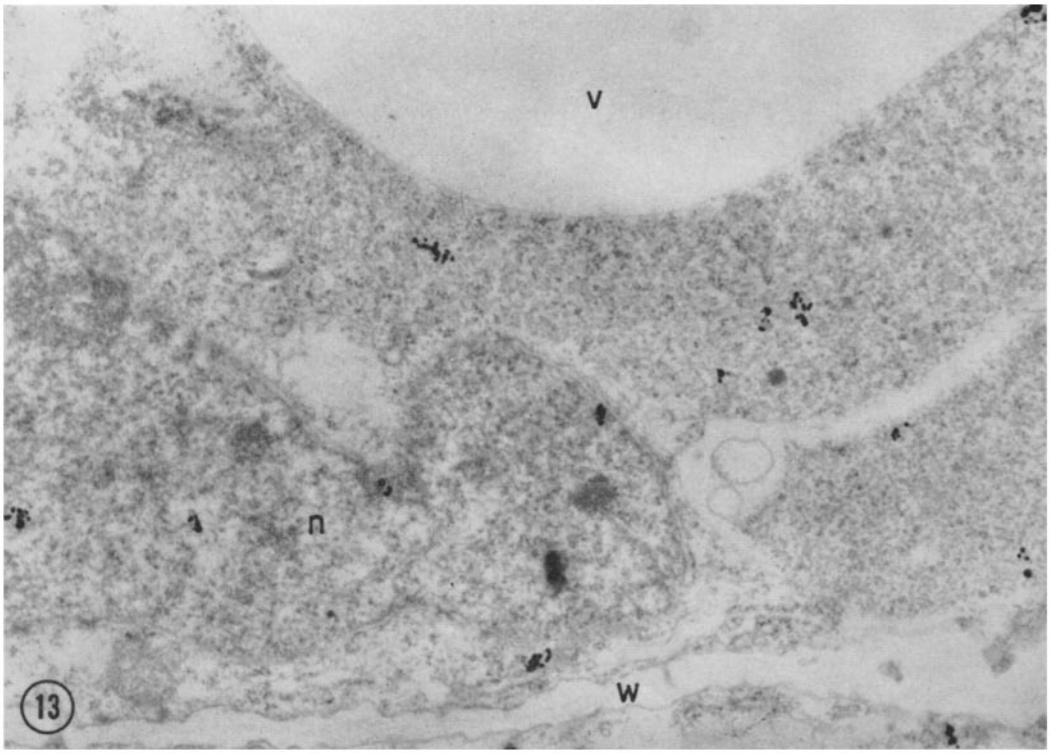
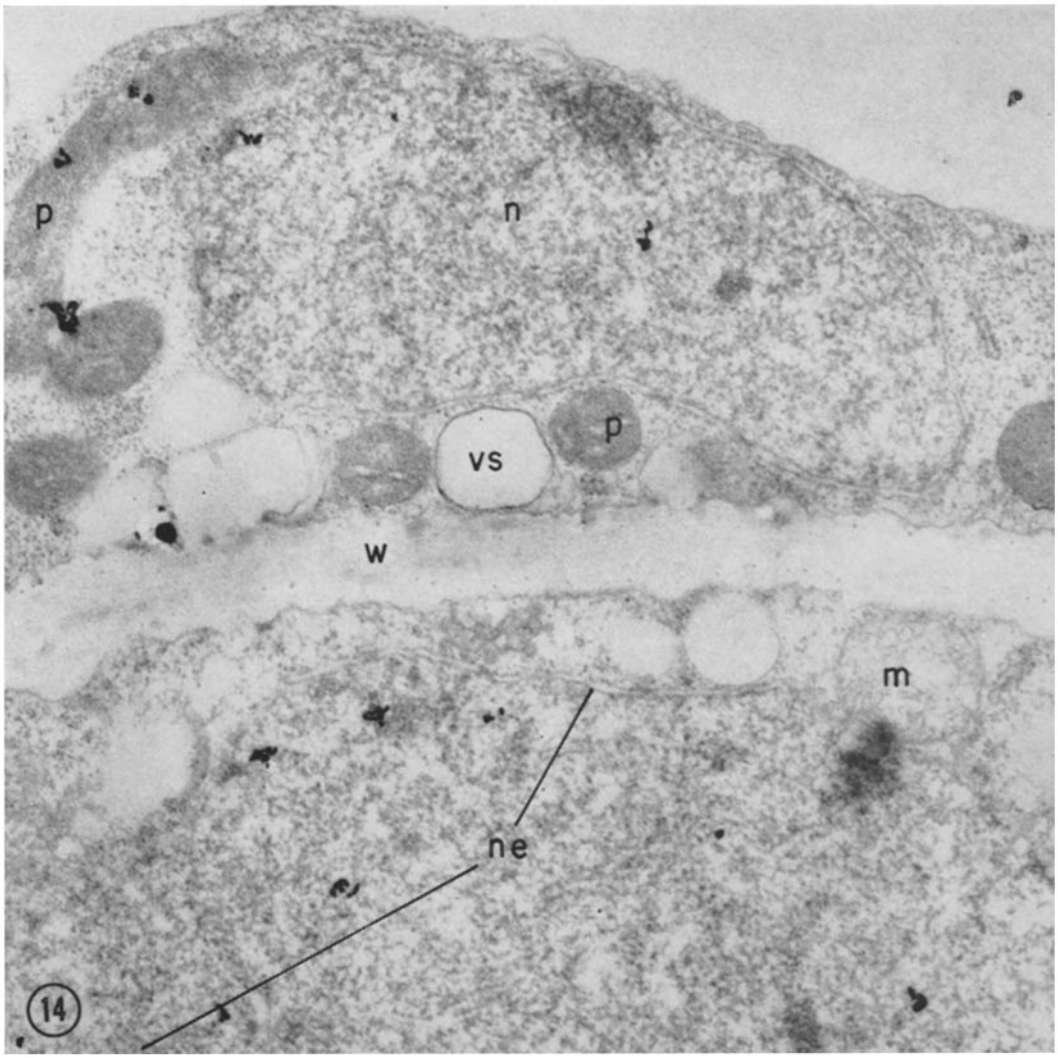


FIGURE 13 In this section through a nucleus and its surrounding parietal cytoplasm, the radioactivity is localized as in Fig. 12. (SP-2). × 26,000.

ally separable moieties with different proline-hydroxyproline ratios as well as more electrophoretically immobile materials. In the most recent work the most uniform preparations, with the highest content of hydroxyproline in relation to proline, have been obtained after procedures of extraction and sucrose density gradient separations etc., which also isolated the nucleic acids. Although the proline-hydroxyproline containing moiety, which also contains a range of protein amino acids, has proved to be separable from the associated nucleic acids, all the indications are that it consists of a proteinaceous material so similar to nucleic acids in its solubility and other properties that it is extracted along with these substances and, in fact, may exist in homogenates in loose chemical combinations with them. It would be entirely plausible if the more mobile forms of combined proline-hydroxyproline should turn out to be those that are produced in the nucleolus and, subsequently, enter the cytoplasm and are there combined, progressively with time (12), into larger and less mobile complexes.

An observation of some potential significance was made by treating the total extracted soluble (i.e. 100,000 *g* supernatant) protein with glutaraldehyde. This device simulates the fixation procedure for electron microscopy and, significantly, the glutaraldehyde here caused the formation of fibrous material which also contained the products of radioactive proline. It is as if the cytoplasm of the cells contained molecules which are retained in place due to glutaraldehyde fixation but which, when extracted from fresh tissue, are not sedimented even at 100,000 *g*.

A sequence of studies in this laboratory (18, 23) has dealt with the incorporation, without hydroxylation, of proline-^{14}C into particulate cell-free preparations. These studies point to the need for some part of the cellular machinery which is specifically associated with the stable proline-containing proteinaceous entity here in question and with its conversion to hydroxyproline. This feature,



FIGURES 14–16 The presence of radioactivity in nuclei of growing carrot cells 6 days after the tissue was exposed to proline-^{3}H.

FIGURE 14 Daughter nuclei with an unlabeled intervening cell wall. The developed silver grains are localized over regions containing granules. (SP-2). × 42,000.

missing in the isolated purified particulate preparation, could well be supplied by the role of the nucleolus in the nuclei of actively growing and potentially dividing cells as indicated by the early concentration there of radioactivity from proline. The growth factors, in coconut milk and other sources, which promote cell division in the cultured cells also promote the synthesis of the hydroxyproline-rich moiety, even as they render the system containing abundant newly synthesized ribonucleic acids (20) functional in synthesis, growth and cell division.

Implication of the nucleolus with the biogenesis of the hydroxyproline-rich protein moiety immediately suggests that it is involved with nucleic acids and with ribosomes. The purest preparations, i.e. membrane-free, carrot root ribosomes, have clearly shown that their ribosomal protein

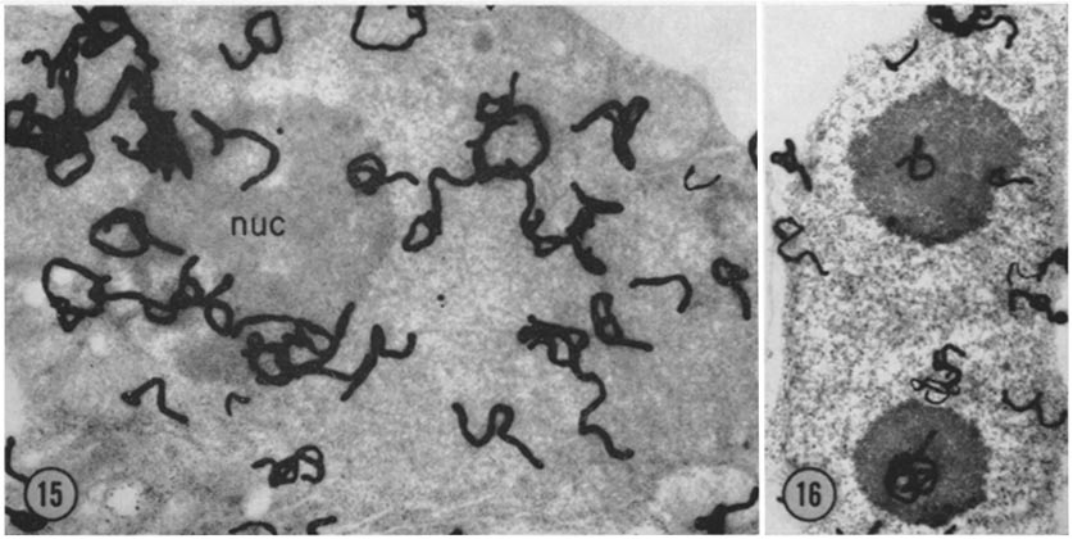


FIGURE 15 The developed silver grains over this section of a nucleus are here distributed over the nucleolus and the nucleoplasm. (SP-1). × 22,000.

FIGURE 16 Section through a nucleus, with two nucleoli, the radioactivity being generally distributed. (SP-1). × 16,000.

does *not* itself contain hydroxyproline in quantity (unpublished observations). The following speculation, however, is entertained as much as a basis for further work as for its inherent probability.

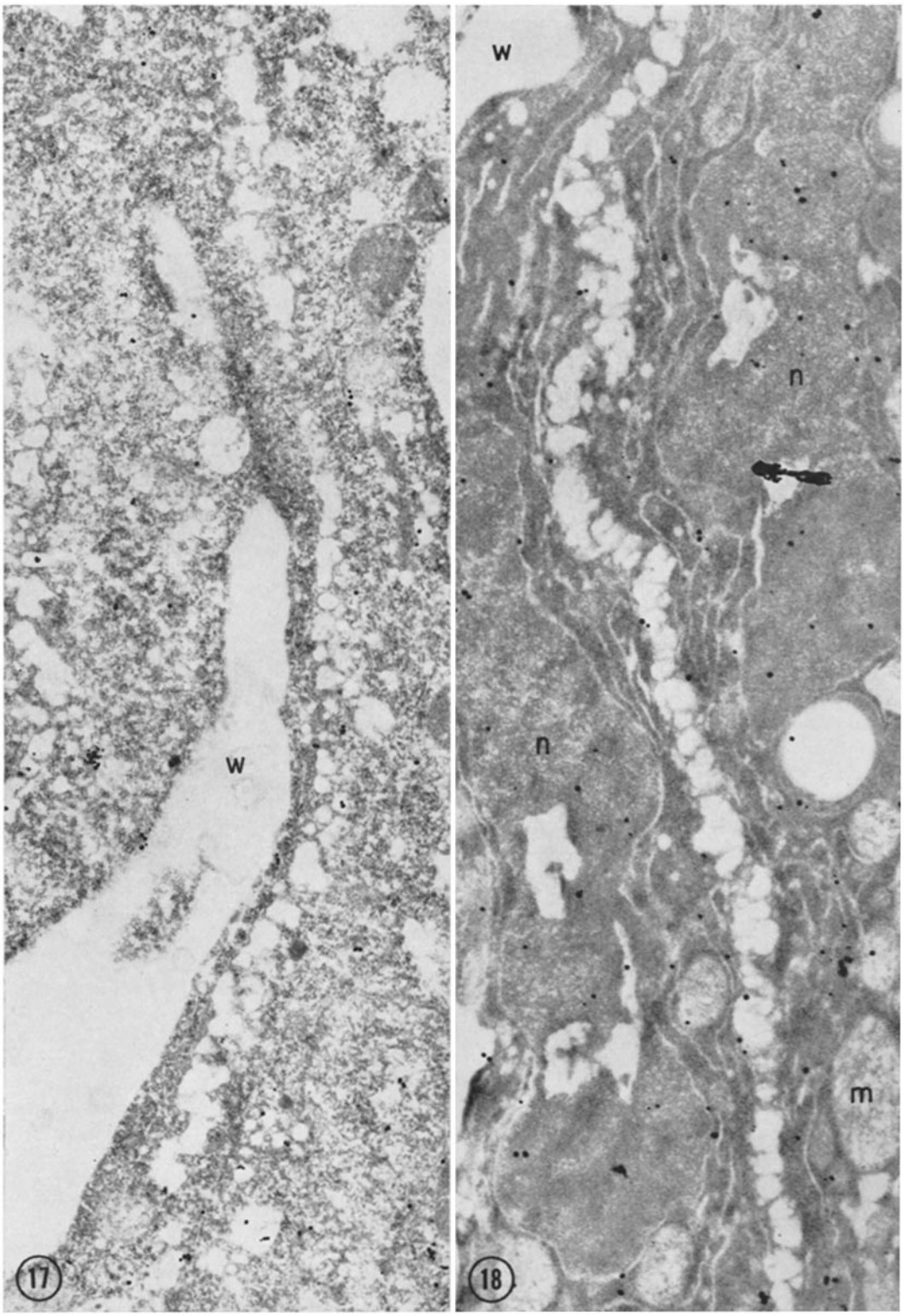
The belief, subject to later proof, now is that the hydroxyproline-rich, stable, proteinaceous moiety in growing dividing cells exists as a structural entity in their ground cytoplasm and that it exists there in a form which permits the small, regular clusters of ribosomal granules to function as units, i.e. as polysomes. This moiety may, in fact, be the missing structural entity which distinguishes the relatively inactive isolated, membrane-free, ribosomal granules from their counterparts *in situ* which obviously display levels of biosynthesis and metabolic activity which are of an entirely different order of magnitude. Only further work can test whether these, otherwise reasonable, concepts may be justified.

This work was supported by U. S. Public Health Service research grants No. GM 09609 and GM 10422 from the National Institute of General Medical Sciences, National Institutes of Health; and by a Career Development Award No. K3-NB-3738 from the NIH Division of Neurological Diseases and Blindness.

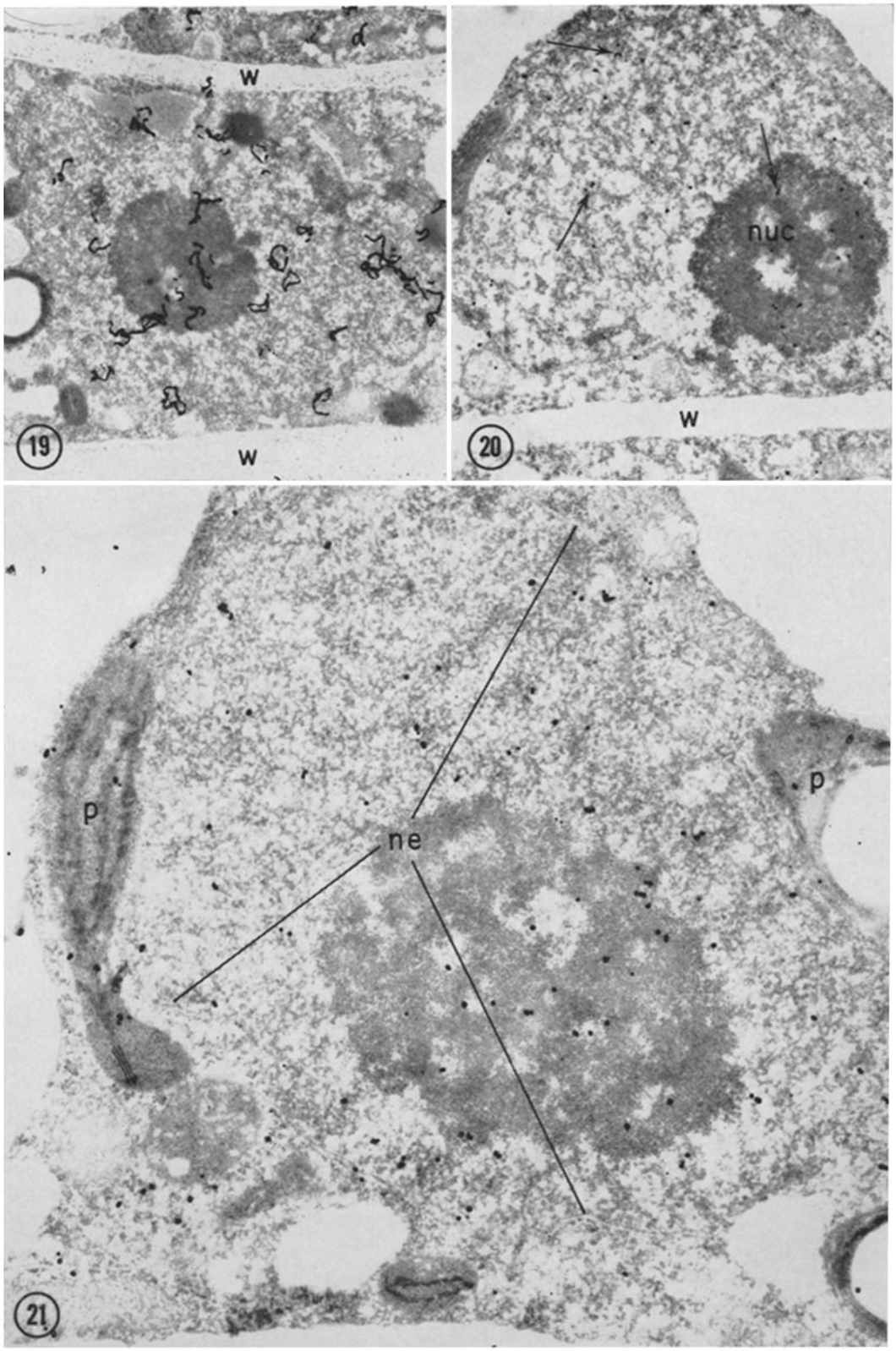
Received for publication 6 June 1968, and in revised form 23 July 1968.

REFERENCES

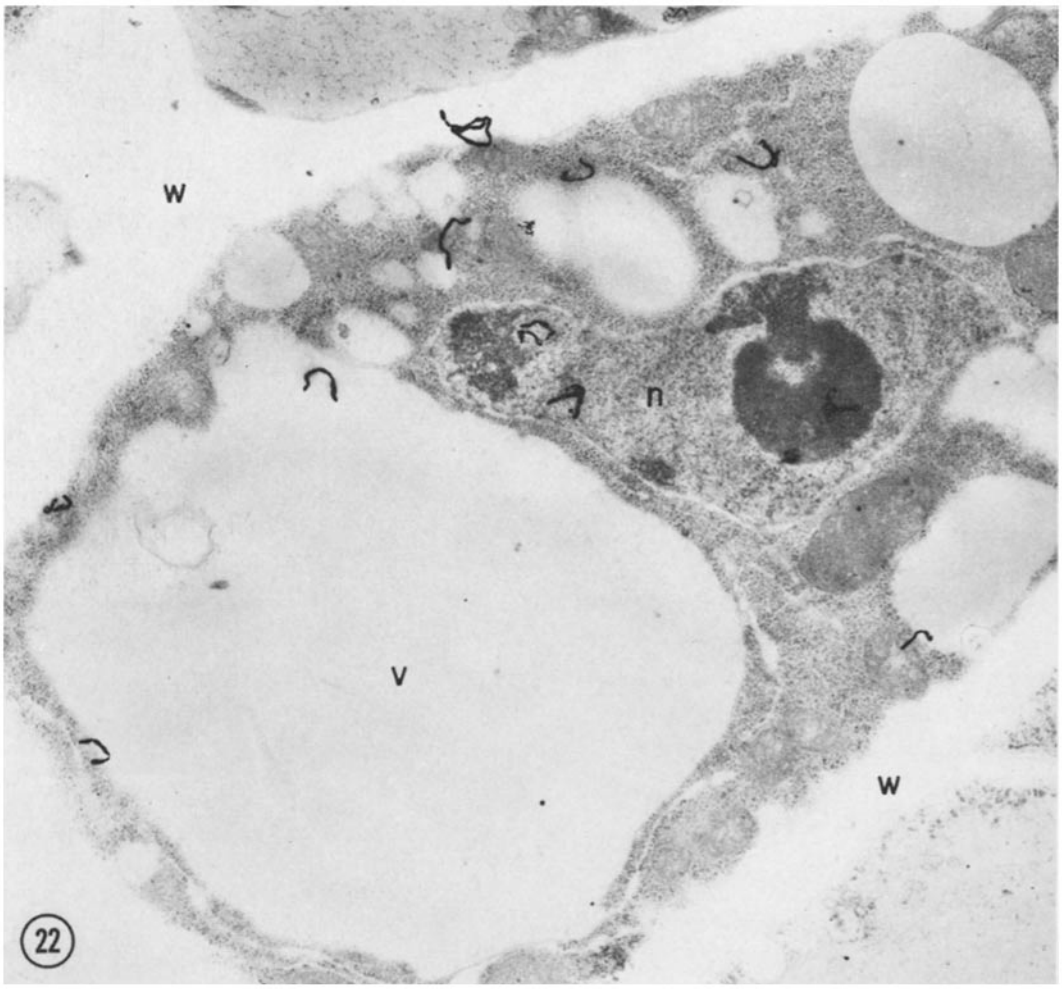
1. BACHMANN, L., and M. M. SALPETER. 1965. Autoradiography with the electron microscope A quantitative evaluation. *Lab. Invest.* **14**:1041.
2. CAPLIN, S. M., and F. C. STEWARD. 1948. Effect of coconut milk on the growth of explants from carrot root. *Science.* **108**:655.
3. CLELAND, R. 1963. Hydroxyproline as an inhibitor of auxin-induced cell elongation. *Nature.* **200**:908.
4. CLELAND, R. 1967a. Inhibition of cell elongation in *Avena* coleoptile by hydroxyproline. *Plant Physiol.* **42**:271.
5. CLELAND, R. 1967b. Inhibition of formation of protein-bound hydroxyproline by free hydroxyproline in *Avena* coleoptile. *Plant Physiol.* **42**(9):1165.
6. CLELAND, R., and A. M. KARLSNES. 1967. A possible role of hydroxyproline-containing proteins in the cessation of cell elongation. *Plant Physiol.* **42**:669.



FIGURES 17 and 18 Localization of radioactivity in cells containing developing walls from tissue that was sampled immediately after it had absorbed proline-^{3}H during a period of 8 hr. In these sections, in which the cell walls in question are to be seen as continuous rows of vesicles passing from top to bottom in the figures, the radioactivity is localized in the adjacent cytoplasm and *not* in the region of the developing cell walls. Figs. 17 and 18, (SP-2). Fig. 17, × 22,000; Fig. 18, × 24,000.



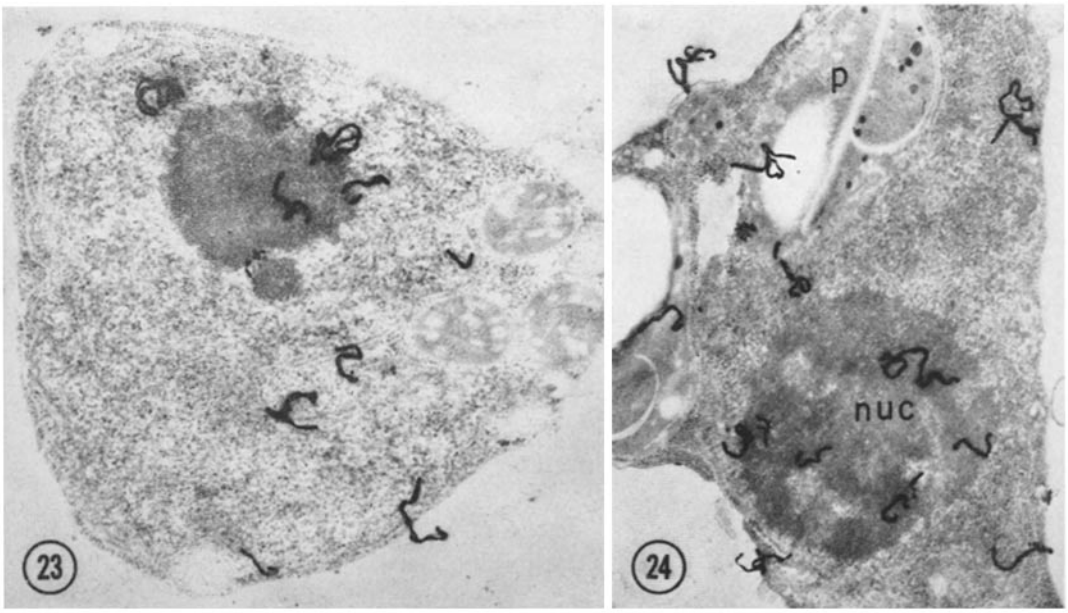
FIGURES 19–21 Radioactivity in nuclei in cells from tissue that was sampled immediately after it was exposed to and absorbed proline-^{3}H for 8 hr. The radioactivity in these sections is chiefly concentrated in both the nucleoli and the nucleoplasm with some labeling of the granular elements of the surrounding cytoplasm. (Note arrows which indicate typical developed grains.) Fig. 19, (SP-1); Figs. 20 and 21, (SP-2). Fig. 19, × 11,000; Fig. 20, × 20,000; Fig. 21, × 29,000.



FIGURES 22–24 Localization of radioactivity in cultured carrot tissue after it had absorbed proline-^{14}C during a 24 hr period. (SP-1).

FIGURE 22 A section through a cell from explanted tissue that was fixed 11 days after inoculation and 5 days after the 24 hr period of contact with the labeled proline. × 12,000.

7. HOLLEMAN, J. 1967. Direct incorporation of hydroxyproline into protein of sycamore cells incubated at growth-inhibitory levels of hydroxyproline. *Proc. Natl. Acad. Sci. U. S.* **57**(1):50.
8. ISRAEL, H. W., and F. C. STEWARD. 1966. The fine structure of quiescent and growing carrot cells: Its relation to growth induction. *Ann. Botany, London.* **30**:63.
9. ISRAEL, H. W., and F. C. STEWARD. 1967. The fine structure and development of plastids in cultured cells of *Daucas carota*. *Ann. Botany, London,* **31**:1.
10. LAMPORT, D. T. A. 1965. The protein component of primary cell walls. *Advan. Botan. Res.* **2**:151.
11. LAMPORT, D. T. A. 1967. Hydroxyproline-o-glycosidic linkage of the plant cell wall glycoprotein extensin. *Nature.* **216**:1322.
12. LYNDON, R. F., and F. C. STEWARD. 1963. The incorporation of ^{14}C-proline into the proteins of growing cells. I. Evidence of synthesis in different proteins and cellular components. *J. Exptl. Bot.* **14**:42.
13. POLLARD, J. K., and F. C. STEWARD. 1959. The use of C^{14}-proline by growing cells: Its con-



FIGURES 23 and 24 Portions of intravacuolar strands, shown in cross section, containing the nuclei of cells from tissue that was sampled 6 days after inoculation and at the end of a 24 hr period of contact with the labeled proline. Fig. 23, × 13,000; Fig. 24, × 12,000.

version to protein and to hydroxyproline. *J. Exptl. Bot.* **10**:17.

14. SALPETER, M. M. 1966. General area of autoradiography at the electron microscope level. *In* Methods in Cell Physiology. D. Prescott, editor. Academic Press, Inc., New York. **2**:229.
15. STEWARD, F. C. 1958. Growth and organized development of cultured cells. III. Interpretations of the growth from free cell to carrot plant. *Am. J. Bot.* **45**:709.
16 STEWARD, F. C., and S. M. CAPLIN. 1951. A tissue culture from potato tuber: The synergistic action of 2,4-D and of coconut milk. *Science.* **113**:518.
17. STEWARD, F. C., and L. O. CHANG. 1963. The incorporation of ^{14}C-proline into the proteins of growing cells. II. A note on evidence from cultured carrot explants by acrylamide gel electrophoresis. *J. Exptl. Bot.* **14**:379.
18. STEWARD, F. C., R. D. HOLSTEN, and A. D. KRIKORIAN. 1965. Does protein synthesis occur in aseptic cell free systems? *Plant Physiol.* **40** (Suppl.) :v.
19. STEWARD, F. C., H. W. ISRAEL, and M. M. SALPETER. 1967. The labeling of carrot cells with H^3-proline: Is there a cell wall protein? *Proc. Natl. Acad. Sci. U. S.* **58**:541.
20. STEWARD, F. C., M. O. MAPES, A. E. KENT, and R. D. HOLSTEN. 1964. Growth and development of cultured plant cells. *Science.* **143**:20.
21. STEWARD, F. C., and J. K. POLLARD. 1958. ^{14}C-proline and hydroxyproline in the protein metabolism of plants: An episode in the relation of metabolism to cell growth and morphogenesis. *Nature.* **182**:828.
22. STEWARD, F. C., J. K. POLLARD, A. A. PATCHETT, and B. WITKOP. 1958. The effects of selected nitrogen compounds on the growth of plant tissue cultures. *Biochim. Biophys. Acta.* **28**:308.
23. SUTCLIFFE, J. F., E. G. BOLLARD, and F. C. STEWARD. 1960. The incorporation of ^{14}C into the protein of particles isolated from plant cells. *J. Exptl. Bot.* **11**:151.
24. THOMPSON, E. W., and R. D. PRESTON. 1967. Proteins in the cell walls of some green algae. *Nature.* **213**:684.
25. THOMPSON, J. F., and F. C. STEWARD. 1952. The analysis of the alcohol-insoluble nitrogen of plants by quantitative procedures based on paper chromatography. I. The analysis of certain pure proteins. II. The composition of the alcohol-soluble and insoluble fractions of the potato tuber. *J. Exptl. Bot.* **3**:170.