CYTOPLASMIC LABEL FOLLOWING TRITIATED THYMIDINE TREATMENT OF *ALLIUM CEPA* L. ROOTS

Cytochemical and Electron Microscope Study

CATHARINE P. FUSSELL

From the Department of Botany, Columbia University, New York. Dr. Fussell's present address is The Pennsylvania State University, McKeesport Campus, McKeesport, Pennsylvania 15132

ABSTRACT

Tritiated thymidine routinely labels onion root cytoplasm during most of the cell cycle. One-third of this label could be cytochemically identified as DNA. The balance of the label was not RNA or a lipid, or attributable to labeled impurities in thymidine-^{3}H. In electron microscope radioautographs one-third of the cytoplasmic silver grains was over organelles, presumably mitochondria and plastids. The other two-thirds of the silver grains in electron micrographs was distributed widely, 41% over ground cytoplasm and 10% over cell walls–cell membranes. Snake venom phosphodiesterase (SVDase) extracted a cytoplasmic fraction not degraded by DNase, and did not appear to extract nuclear DNA. The SVDase-extractable fraction may be DNA or a thymidine 5′-phosphoryl group in an ester linkage with another hydroxylic compound. The nature of the nonextractable fraction is considered. Possibilities discussed are: (1) technical problems such as the binding of an acid-labile nuclear DNA in the cytoplasm; (2) non-DNA, such as breakdown products, and thymine compounds other than DNA; (3) DNA, not extractable because of the nature of its binding to other compounds or because it is a "core" resistant to DNase. Until the chemical nature of this nonextractable fraction is known, cytoplasmic label following thymidine-^{3}H treatment cannot necessarily be considered DNA, nor the assumption made that thymidine-^{3}H exclusively labels DNA.

INTRODUCTION

Tritiated thymidine, introduced by Taylor, Woods, and Hughes (67) for high resolution radioautography, is a specific precursor and selective label for DNA which does not label other cellular components (65). Furthermore, thymine, the commonly labeled moiety of thymidine, occurs in higher plants and animals almost exclusively in DNA (26). Therefore, tritiated thymidine would be expected to label only DNA. However, cases of cytoplasmic labeling following thymidine-^{3}H treatment were soon reported (3, 32, 45, 62, 72). The nature of this label was dubious because: (*a*) DNA was considered to occur exclusively in the nucleus (1), and (*b*) DNA extraction techniques, where tried, did not always remove cytoplasmic label (32, 72). In this same period, other techniques indicated that DNA is a constituent of mitochondria (40) and of chloroplasts (10, 27, 52). Although both cytological and biochemical papers suggested that DNA occurs in the cytoplasm, the

experimental evidence was neither decisive nor conclusive. Therefore the present author decided to try to find out whether DNA is a cytoplasmic component by using light microscope radioautography and standard, cytological extraction techniques following thymidine-^{3}H treatment, and by determining the fine structure location of cytoplasmic label with electron microscope radioautography. While these experiments were in progress, experimental evidence for DNA in mitochondria and chloroplasts appeared (15, 20, 35, 53). Thus, the view that DNA occurs only in the nucleus yielded to the concept that DNA is also a constituent of plastids and mitochondria (19, 61).

Experiments reported in this paper show, as would be expected, that onion root cells contain DNA in the cytoplasm. However, in addition, a large fraction of cytoplasmic label could not be removed by standard extraction techniques. This residual label suggests either that tritiated thymidine is not a specific label for DNA or that some cytoplasmic DNA is so bound that it is resistant to extraction by the usual methods.

MATERIALS AND METHODS

Roots from *Allium cepa* L. bulbs (onion) were the chief experimental material. Bulbs were grown at 18°C in one-half strength Hoagland's solution (24) until roots were 2–3 mm long. Bulbs were then transferred to tritiated thymidine or control solutions, for 8 hr, with fresh solutions at the end of 4 hr. Roots were washed briefly three times in one-half strength Hoagland's solution and immediately fixed. Seeds of *Vicia faba* L., *Allium cepa* L., and *Ornithogalum virens* Lindl. were germinated and their roots treated in a similar manner.

Tritiated thymidine, labeled in the methyl group, with specific activities of 6.7 c/mmole and 14.2 c/mmole and used at concentrations of 25 μc/ml and 60 μc/ml, respectively, was obtained from New England Nuclear Corporation, Boston. As a test for decomposition products, isotope samples were chromatographed on No. 50 Whatman paper in a solvent from the upper phase of ethyl acetate/water/formic acid 12:7:1 v/v/v (39).

Roots for light microscope radioautography were fixed for 2–3 hr in Carnoy's, three parts absolute alcohol to one part acetic acid. Other fixatives used for both light and electron microscope radioautography were Champy's; 2% $KMnO_4$ in veronal-acetate buffer at pH 7.2; 10% formaldehyde, postfixation in 1% OsO_4, both fixatives phosphate buffered at pH 7.4; 1% OsO_4 phosphate buffered at pH 7.4; and 7% methanol-free formaldehyde (69) phosphate buffered at pH 6.0 or 7.4 in 0.15 M sucrose at 3°C, postfixation in 2% OsO_4 (56). The methanol-free formaldehyde was a gift of the Heyden Newport Chemical Corporation, Garfield, New Jersey.

LIGHT MICROSCOPY: Roots were routinely embedded in paraffin, sectioned at 2 or 4 μ, and mounted on gelatin-coated slides for radioautography. Slides for enzyme and acid treatments were taken to water in a graded alcohol series. Following enzyme and acid treatments, slides were washed in three changes of distilled water, and taken to 70% alcohol in a graded series. Slides were filmed with Kodak Radioautographic Stripping Film AR-10, and processed according to methods described by Taylor (64). Radioautograms were exposed for 30 days.

ELECTRON MICROSCOPY: Roots were fixed, dehydrated, and embedded in Epon (37). Gold-to-silver sections were cut on a Porter-Blum microtome. Grids for radioautography were filmed with Ilford Nuclear Research Emulsion L-4, Ilford Ltd., London, England, according to Caro and van Tubergen's (7) methods. Following photographic development, grids were stained with lead citrate (50) or a saturated solution of uranyl acetate. A few Epon-embedded roots were sectioned at 1–1½ μ, mounted on gelatin-coated slides, and filmed for light microscope radioautography with Kodak AR-10 radioautographic film.

EXTRACTIONS: Enzymes were obtained from Worthington Biochemical Corporation, Freehold, New Jersey. All extractions were carried out at 37°C for 3 hr. Deoxyribonuclease (DNase) was used at concentrations of 0.1 mg/ml–0.4 mg/ml, pH 6.3 or 6.8, with 0.003 M or 0.01 M $MgSO_4$. DNase extractions for electron microscopy were done on small tissue blocks between formaldehyde and OsO_4 fixations (52). Concentrations of ribonuclease (RNase) used were 0.2 mg/ml or 0.4 mg/ml, adjusted to pH 6.5 or 7.0 with 0.1 N NaOH. Snake venom phosphodiesterase (SVDase), having approximately 5% of the activity that DNase I has on thymus DNA (Worthington Biochemical Corporation), was used at 0.5–0.6 mg/ml in NaOH/glycine buffer at pH 9.2, with 0.005 M $MgCl_2$ (14, 30). Controls for all enzyme treatments consisted of the complete extraction solution without added enzyme.

Extractions with 5% trichloroacetic acid (TCA) were done at room temperature, approximately 25°C, for 1 hr, and with boiling TCA, 80–85°C, for 15 min. Hydrolysis with 1 N HCl for 2, 10, or 30 min was carried out at 60°C.

STAINING: Slides were stained with methyl green–pyronin after radioautographic development (17), or with the Feulgen reaction prior to filming.

SCORING: Slides were coded and randomized, and cytoplasmic silver grains within a circular reticule 25 μ^2 were counted. Background was scored by counting grains over nontissue areas adjacent to each section scored. A Carl Zeiss (Jena) microscope with

10X oculars and a 90X phase oil objective was used for scoring.

Electron microscopy was carried out with a Philips 100A microscope operated at 40–60 kv. Pictures were taken on Recorder Microfilm Type 5455, Recordak Corporation, Rochester, New York, and routinely processed.

STATISTICAL ANALYSIS: The statistical analysis was very kindly done by Miss Emily Stong, Mathematical Statistics Department, Columbia University. The data were analyzed on the 7094 computer, Columbia Computer Center, under project number UT7DE01, using a program devised by Mr. G. P. H. Styan and Mr. P. C. Trenholme also of the Mathematical Statistics Department.

Data from Extraction Experiments I and II were analyzed. An analysis of covariance was done, the assumed model in both cases being:

$$Yij = \mu + \alpha i + \beta j + \gamma ixij + eij$$

where:

Yij = observed tissue count.
i = 0, 1, 2, $\cdots$, I; I = number of treatments.
j = 1, 2, $\cdots$, J; J = number of roots.
μ = true mean.
αi = true effect of the ith treatment, i = 1, $\cdots$, I.
α_0 = true effect of untreated.
βj = true effect of the jth root.
γi = background coefficient for the ith treatment.
γ_0 = background coefficient for untreated.
xij = observed background count.
eij = random error.

The random errors were assumed to be independently normally distributed, with means zero and common variance. Least square estimates were obtained for the parameters. Using Scheffé's S-method, a form of the F test, each treatment was compared with the untreated condition and the equality of the background coefficients was tested.

RESULTS

Cytoplasmic Labeling Following Thymidine-^{3}H Treatment

For a test of whether onion root cytoplasm could be labeled, sections from roots treated with thymidine-^{3}H for 8 hr, with fresh solutions at the end of the 4th hour, were prepared as described. Radioautograms showed that the cytoplasm of all cells, dividing and nondividing, was labeled (Figs. 2 and 4). Grain counts over the cytoplasm of interphase cells with labeled and with unlabeled nuclei were essentially similar. For instance, the average number of cytoplasmic grains per area scored was 17.9 and 17.8, respectively, in one experiment and 14.5 and 13.0 in another. The amount of cytoplasmic label is small compared with that of nuclear label, for in cells with solid black nuclei the cytoplasm is only moderately labeled (Fig. 2). Cytoplasm of onion roots treated with thymidine-^{3}H for 2 hr also was labeled in all stages of the mitotic cycle. The cytoplasm of *Vicia*, and *Ornithogalum* roots treated with thymidine-^{3}H was also labeled. In all experiments control roots, i.e. roots not treated with isotope, were also filmed. Grain counts over control sections were at background levels.

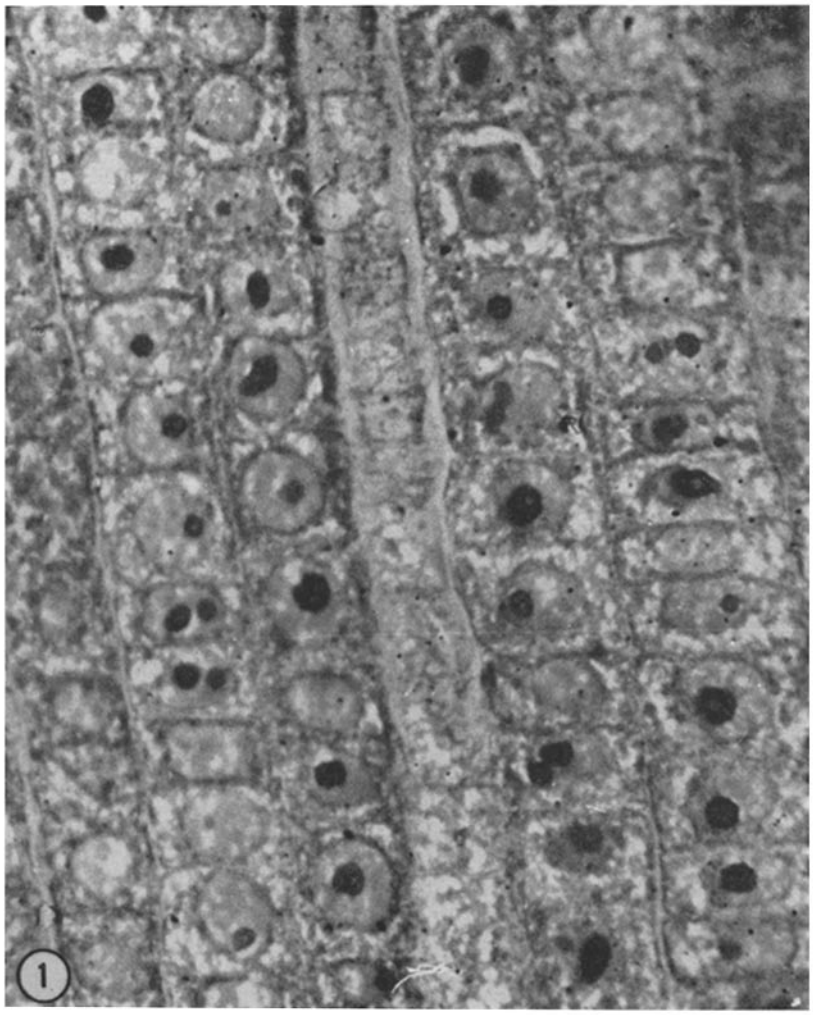
From the results of these experiments it was concluded that cytoplasmic labeling of *Allium*, *Vicia*, and *Ornithogalum* root tip cells is a consequence of thymidine-^{3}H treatment just as nuclear labeling is. However, the pattern of cytoplasmic label differs from that of nuclear label, in that it occurs over most of the cell cycle.

Cytochemical Identification of Cytoplasmic Label

With the finding that cytoplasm is routinely labeled following thymidine-^{3}H treatment, the question arises whether the label does represent DNA. As a test of this, routine enzyme and acid extractions for nucleic acids were carried out as described.

EXTRACTION EXPERIMENT I: Ten thymidine-^{3}H–treated roots, five from one bulb and five from a second; and two nonradioactive control roots (Fig. 1), one fixed just prior to isotope treatment, the other 8 hr later, were used. Serial sections were cut for each root and adjacent sections were mounted on 12 slides. 12 slides, one for each of the 12 roots, were treated as follows: unextracted (Fig. 2); 1 N HCl for 2, 10, and 30 min; DNase, pH 6.3, 0.1 mg/ml, 0.003 M $MsSO_4$ (Fig. 3), and DNase controls; cold TCA/DNase, and cold TCA/DNase controls (Fig. 4) (cold TCA/DNase-treated slides were placed in 5% TCA at 3°C for 5 min before and after DNase treatment); RNase and RNase controls; room temperature TCA; and boiling TCA.

Initially six thymidine-^{3}H–treated roots, three from each bulb, and the two nonradioactive control roots were scored. In all experiments, cells in the same general area of the root were scored. Grains over 50 cytoplasmic fields per slide, 10 fields per section, five sections per slide, and over



FIGURES 1–4 Light microscope radioautographs of onion root tip sections from Extraction Experiment I. Thymidine-^{3}H treatment for 8 hr, with fresh isotope solutions after 4 hr. Figs. 2 and 2, × 3300. Figs. 3 and 4, × 3200.

FIGURE 1 Control, not treated with thymidine-^{3}H.

25 background fields per slide, were counted. The average tissue and background grain counts for each root, for all treatments, were statistically analyzed by Miss Stong. Table I gives average grain counts for two control roots and for the six thymidine-^{3}H–treated roots for each treatment; and also F values for each treatment compared with the value for the unextracted condition. Grain counts following treatment with 1 N HCl for 2 and 30 min, DNase, cold TCA/DNase, cold TCA/DNase controls, RNase controls, and room temperature TCA were statistically different from those of the unextracted condition. Slides from the remaining four thymidine-^{3}H–treated roots of this experiment were scored later. However, 20 tissue areas, 10 per section instead of 50, were counted.

Grain counts for each treatment of a single root as a *per cent* of the grain count for the unextracted condition for that root were calculated. The average per cent decrease or increase for each treatment is given in Table II. Average

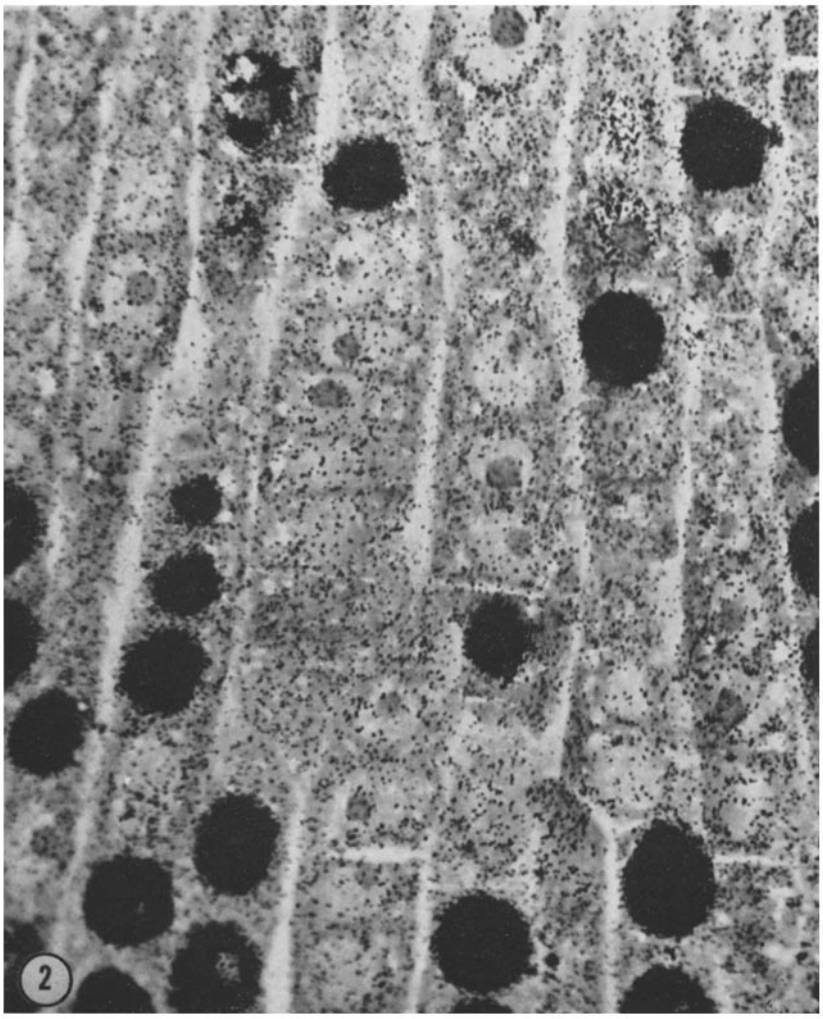


FIGURE 2 Unextracted, thymidine-^{3}H treated.

cytoplasmic grain counts *decreased* more than 10% on sections treated with 1 N HCl for 30 min, DNase, and cold TCA/DNase, and *increased* more than 10% for the treatments 1 N HCl for 2 min, cold TCA/DNase controls, and room temperature TCA. As a check of the increased cytoplasmic grains observed in cold TCA/DNase controls, and in room temperature TCA treatment, these two treatments were repeated on four thymidine-^{3}H–treated roots and one control root. The results, summarized in Table III, confirm the initial finding that these two treatments increase cytoplasmic grain counts in thymidine-^{3}H–labeled tissue.

Slides hydrolyzed with 1 N HCl were Feulgen stained prior to filming. Nuclei in sections treated with 1 N HCl for 2 and 10 min were Feulgen positive, but nuclei in sections treated with 1 N HCl for 30 min were colorless. All other slides were stained with methyl green–pyronin. Nuclei in sections treated with DNase, cold TCA/DNase, and boiling TCA did not stain with methyl green, while nuclei in the treatments of room temperature TCA and cold TCA/DNase controls were faded. In all other treatments the nuclei were well stained with methyl green.

Nuclei in unextracted sections were very heavily labeled, often solid black (Fig. 2). The same level of nuclear labeling occurred in all other treatments except 1 N HCl for 30 min, DNase (Fig. 3), cold TCA/DNase, and boiling TCA. In the latter four

FIGURE 3 DNase extraction for 3 hr at pH 6.3, 0.1 mg/ml enzyme, 0.003 mg $MgSO_4$. Thymidine-^3H-treated root.

cases, the label was largely but never completely removed from nuclei. The parallel between the absence of Feulgen or methyl green nuclear stain and the removal of almost all nuclear grains in the treatments 1 N HCl for 30 minutes, DNase, cold TCA/DNase, and boiling TCA is double indication that these enzyme and acid extractions were effective in removing nuclear DNA in this experiment.

In summary, the results indicate that cytoplasmic label does not represent RNA, for neither RNase nor 1 N HCl for 10 min reduced the cytoplasmic grain count by statistically significant amounts compared with the unextracted condition. DNase and cold TCA/DNase treatments extracted 20 and 25% of the cytoplasmic label, respectively, statistically significant reductions (Table I); this label is considered to be DNA fractions. However, contrary to expectations, approximately three-fourths of the cytoplasmic label was not removed by DNase, and boiling TCA did not reduce the cytoplasmic grain count by a statistically significant amount over that of the unextracted condition. Treatments with 1 N HCl for 2 min, cold TCA/DNase control solution, and room temperature TCA increased the cytoplasmic

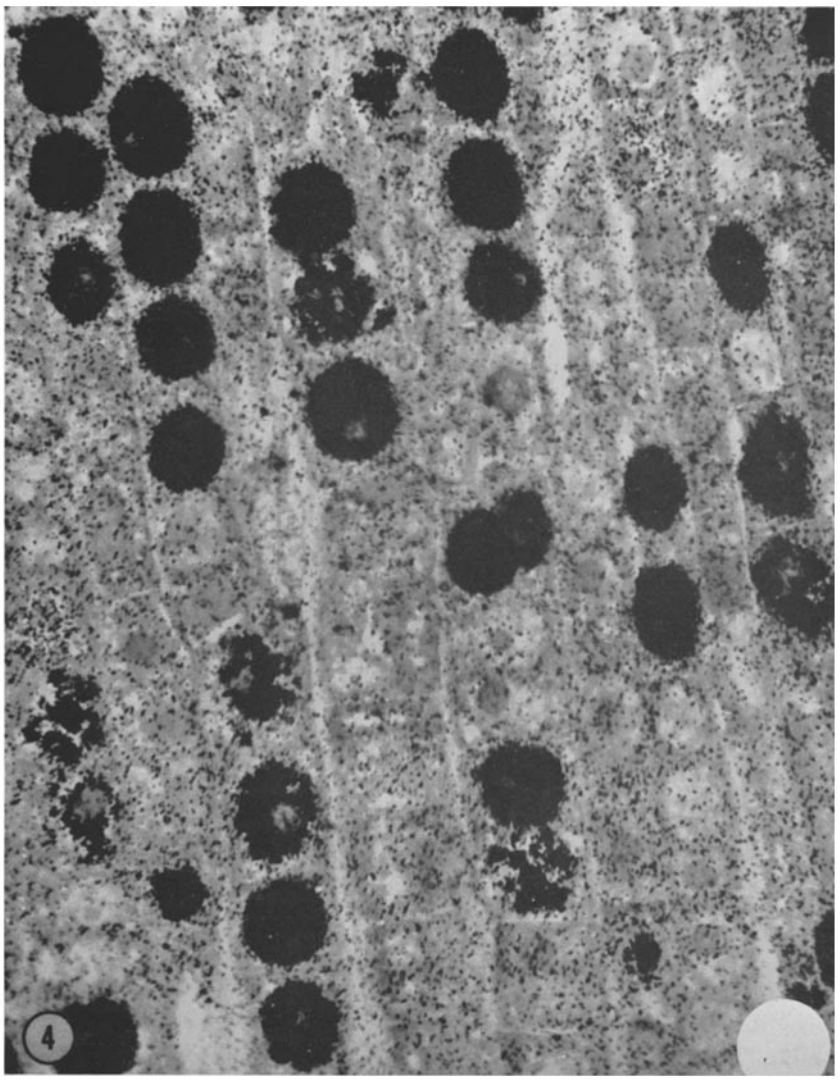


FIGURE 4 DNase/Cold TCA controls of a thymidine-^{3}H–treated root. Sections treated with 5% cold TCA for 5 min before and after 3-hr DNase control treatment.

grain counts by statistically significant amounts (Table I).

EXTRACTION EXPERIMENT II: It was puzzling to find that 75% of the cytoplasmic label was not susceptible to DNA extraction methods, if it is assumed that most label following thymidine-^{3}H treatment represents DNA (11, 65), and that DNA extraction methods are as effective on cytoplasmic as on nuclear DNA. The results suggested that either one or both assumptions are incorrect, and that the unextracted fraction is not DNA, or is DNA resistant to pancreatic DNase, and/or that the enzyme concentration used was inadequate. For the purpose of exploring whether the nonextractable cytoplasmic label might be DNA but resistant to pancreatic DNase, two other deoxyribonucleases were tried: acid DNase from spleen, an endonuclease degrading DNA to oligonucleotides with 3′-monoesterified phosphate end groups, and snake venom phosphodiesterase (SVDase), an exonuclease giving deoxyribonucleoside-5′-phosphates (13). Preliminary experiments with acid DNase at three concentrations, and before or after pancreatic DNase, did not

TABLE I

Extraction Experiment I. Average Cytoplasmic and Background Grain Counts F Values for Each Treatment Compared with Untreated

Treatment	Controls	Thymidine-^{3}H treated			
	Average net grains	Average total tissue grains	Average background grains	Average net tissue grains	F values
Unextracted	0.06	18.77	0.83	17.94	—
1 N HCl—2 min	0.12	20.63	0.95	19.68	2.541*
1 N HCl—10 min	0.32^n	19.20	1.07	18.13	0.014
1 N HCl—30 min	0.18	16.34	0.76	15.58	4.881‡
DNase	0.06	14.47	0.62	13.85	13.810‡
DNase controls	0.03^n	17.25	0.71	16.54	1.652
Cold TCA/DNase	0.08	14.01	0.78	13.23	19.675‡
Cold TCA/DNase controls	0.07^n	23.20	0.87	22.33	16.934‡
RNase	0.27^n	17.83	0.53	17.30	0.269
RNase controls	0.01^n	17.02	0.75	16.27	2.436*
Room temperature TCA	0.06	22.45	2.40	20.05	3.949‡
Boiling TCA	0.02	18.77	2.13	16.64	0.429

Two control roots and six thymidine-^{3}H-treated roots. For each treatment of each root, 50 tissue areas and 25 background areas were scored.

^{n} Negative value.

* Significant at the 2.5% level.

‡ Significant at the 0.5% level.

TABLE II

Extraction Experiment I. Average Per cent Loss or Gain of Cytoplasmic Grains*

1 N HCl—2 min	10.03
1 N HCl—10 min	2.21
1 N HCl—30 min	12.90*
DNase	19.94*
DNase controls	5.13*
Cold TCA/DNase	24.96*
Cold TCA/DNase controls	22.81
RNase	1.91*
RNase controls	7.40
Room temperature TCA	11.64
Boiling TCA	4.48*

10 thymidine-^{3}H-treated roots.

TABLE III

Extraction Experiment I. Repeat of Cold TCA/DNase Control and Room TCA Extractions

Average cytoplasmic grain counts

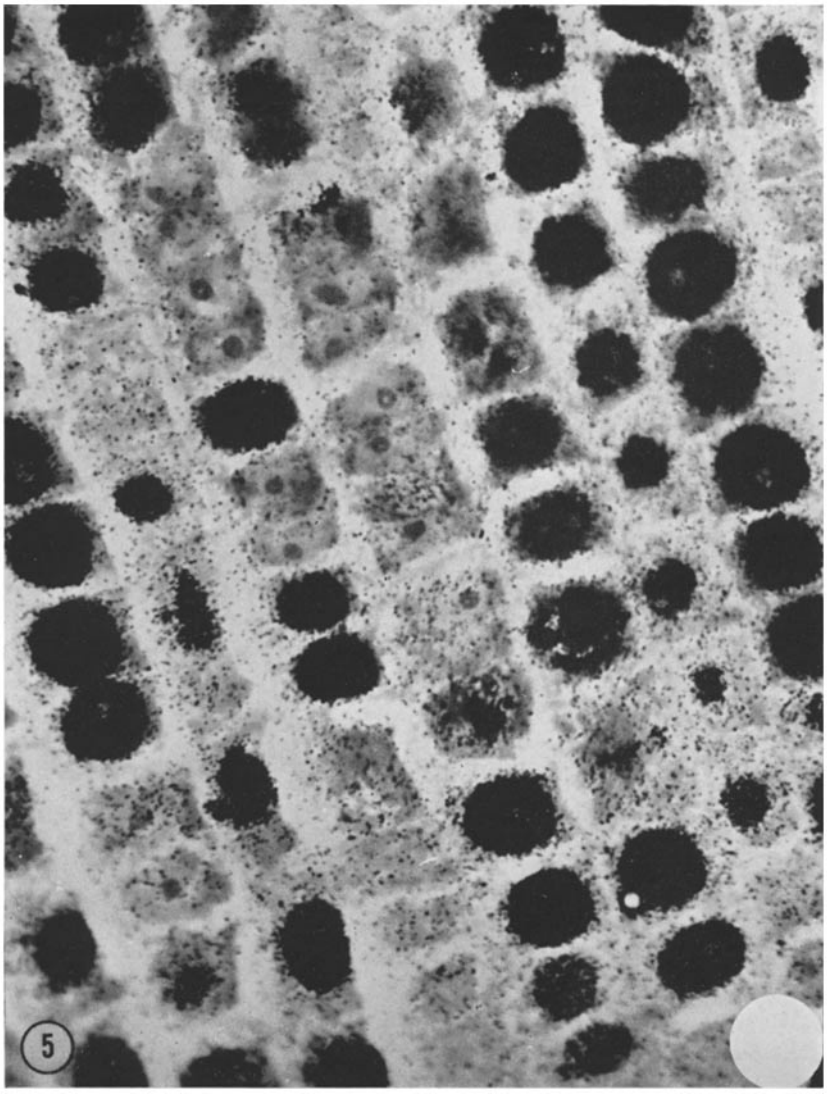
	Unextracted	Cold TCA/DNase control	Room temperature TCA
Controls			
Tissue	0.55	0.20	0.30
Background	0.35	0.20	0.65
Net	0.20	—	0.35^n
Thymidine-^{3}H			
Tissue	9.86	13.20	14.23
Background	1.00	0.70	3.01
Net	8.86	12.50	11.21

One control root and four thymidine-^{3}H-treated roots; 20 tissue and 10 background areas scored for each treatment of each root.

^{n} Negative value.

affect the cytoplasmic grain level and, consequently, was not further used. However, SVDase at several concentrations reduced cytoplasmic label. Double enzyme extractions, pancreatic DNase followed by SVDase, reduced the cytoplasmic grain count by roughly the amount removed by each enzyme acting alone.

On the basis of the preliminary tests, a more extensive experiment was done to characterize further the cytoplasmic label. Ten thymidine-^{3}H-treated roots and one control root from the lot treated for Extraction Experiment I were used. SVDase singly and in combination with DNase was tried, and RNase and boiling TCA extractions were repeated as a check of the previous results.



FIGURES 5–8 Light microscope radioautograms of onion root tip sections treated with thymidine-^{3}H for 8 hr, with fresh isotope solutions after 4 hr. Fig. 5 × 3200. Figs. 6 and 7 ; × 3300. Fig. 8 × 3800.

FIGURE 5 Extraction Experiment II. SVDase treatment for 3 hr at pH 9.2, 0.5 mg/ml enzyme, 0.005 M $MgCl_2$. Note that enzyme treatment does not extract nuclear label.

In the DNase extractions (0.3 mg/ml of enzyme, 0.01 M $MgSO_4$, pH 6.8) higher enzyme and Mg^{++} concentrations were used than previously. Four of the roots were treated with boiling alcohol/ether for extracting lipids. Ten slides were prepared for each of the 11 roots, as described above. One slide from each root was treated as follows: unextracted, DNase, SVDase (Fig. 5), DNase followed by SVDase (Fig. 6), RNase, controls for each enzyme extraction, and boiling TCA.

Grains over 20 tissue areas, 10 per section, and over 10 background areas were counted (Table IV). The statistical analysis run by Miss Stong was done for only six roots because some SVDase sections were lost during extraction. The F values for each treatment compared with that for the

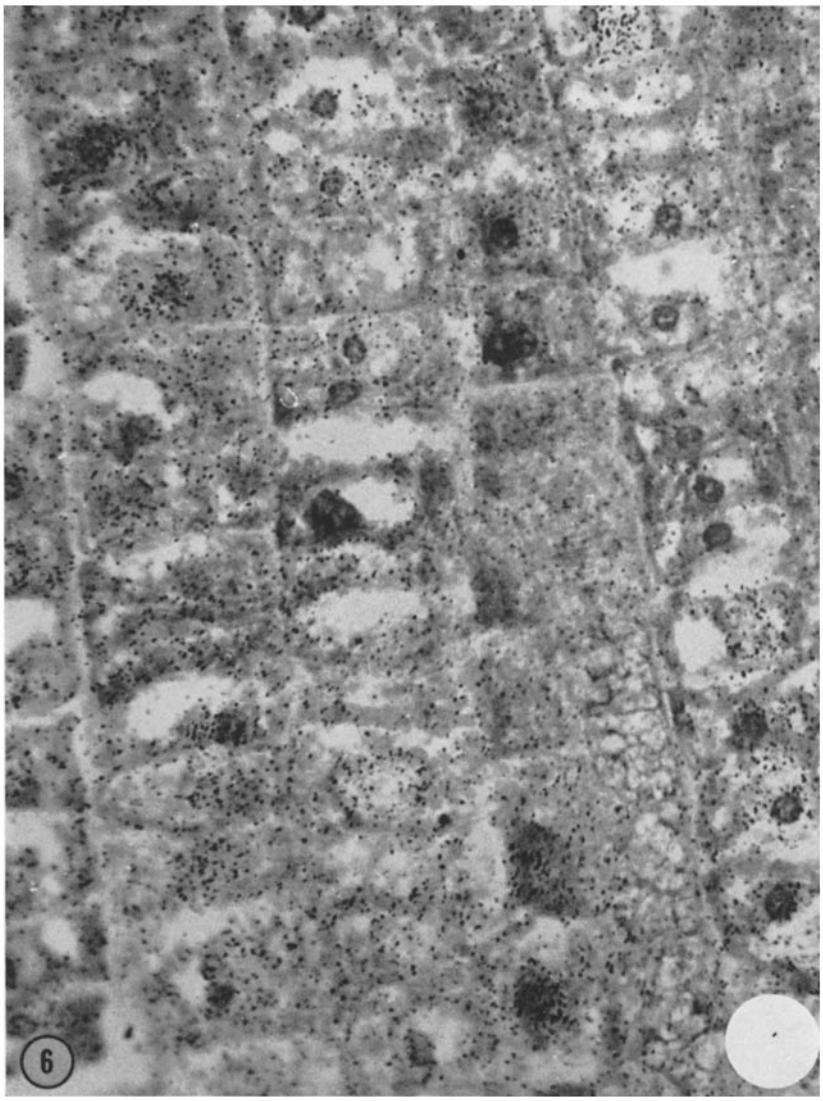


FIGURE 6 Extraction Experiment II. DNase/SVDase-treated sections. This sequence of enzyme treatments gave greatest reduction of cytoplasmic grains.

unextracted condition are given in Table IV. SVDase and DNase/SVDase extractions reduced grain counts by statistically significant amounts. Neither boiling TCA nor RNase reduced grain counts, confirming the results of Extraction Experiment I. The reduced cytoplasmic grain count after DNase treatment was not significantly different from the count of the unextracted condition, although the difference approached significance. This can, perhaps, be explained by the fact that the grain count for one root was unaccountably higher than the count for its unextracted condition, possibly biasing the statistical results. DNase removed an average of 16% of the cytoplasmic grains or, disregarding the one anomalous root, 23%. SVDase removed 25% and DNase/SVDase removed 45% of the cytoplasmic grains, confirming the preliminary experimental findings that SVDase reduced cytoplasmic label, and that the effects of DNase followed by SVDase are roughly additive.

Increased DNase and Mg^{++} concentrations had

TABLE IV

Extraction Experiment II. Average Cytoplasmic and Background Grain Counts F Values for Each Treatment Compared with Untreated

Treatment	Controls	Thymidine-^{3}H treated			
	Average net grains	Average total tissue grains	Average background grains	Average net tissue grains	F values
Unextracted	0.10	19.80	1.08	18.72	—
DNase	0.10	16.20	0.62	15.58	1.512
DNase controls	0.30	17.14	0.50	16.64	0.874
SVDase	0.35	14.29	0.63	13.66	3.177*
SVDase controls	0.35[n]	18.13	0.42	17.71	0.399
DNase/SVDase	0.15	10.38	0.55	9.83	8.407‡
DNase/SVDase controls	0.30	18.26	0.67	17.59	0.418
RNase	0.35[n]	17.71	0.43	17.28	0.572
RNase controls	0.11	18.40	0.58	17.82	0.346
Boiling TCA	0.20	17.25	0.57	16.68	0.841

One control root and six thymidine-^{3}H–treated roots. 20 tissue and 10 background areas scored for each treatment of each root.

[n] Negative value.

* Significant at the 1% level.

‡ Significant at the 0.5% level.

little or no effect on the level of cytoplasmic label, removing 23% compared with 20% in Extraction Experiment I. Therefore, the residual, nonextractable cytoplasmic grains cannot be attributed to inadequate DNase or Mg^{++} levels. Also, boiling alcohol/ether had no effect on the grain level, indicating that the labeled material is not a lipid.

All slides were stained with methyl green–pyronin. Nuclei treated with DNase, DNase/SVDase, or boiling TCA were colorless, and their nuclear label was largely removed. Nuclei in all other treatments were stained by methyl green and were well labeled. The parallel staining and labeling results indicate that DNase, DNase/SVDase, and boiling TCA removed nuclear DNA in this experiment. However, SVDase alone does not appear to extract a major fraction of nuclear label (Fig. 5), although it would be impossible to detect a fractional loss because of the high level of label initially present. Nuclei of SVDase-treated cells could be stained with the Feulgen reaction, providing a third test that SVDase does not extract a major portion of nuclear DNA from tissue sections.

Sections from several roots contained annular tracheids. In all treatments except controls, the tracheidal rings were clearly labeled (Fig. 7). Grain counts were not made, but annular ring label appeared to be largely resistant to the enzyme and acid extractions used in this experiment.

In summary, the results of Extraction Experiment II confirmed that cytoplasmic label following thymidine-^{3}H–treatment is not RNA, nor is it reduced by boiling TCA. DNase removed about 23% of the label, SVDase about one-quarter, and a combined DNase/SVDase extraction about 45%. This leaves slightly more than one-half the cytoplasmic label not extractable by any methods tried, including higher DNase and Mg^{++} concentrations, acid DNase, and lipid extraction. SVDase alone did not remove a major fraction of nuclear DNA. Annular rings of tracheids were labeled in all slides except controls.

Electron Microscope Radioautography

Preliminary experiments to test the effects, if any, of electron microscopy fixatives on the level of cytoplasm label showed that fixation in OsO_4, or in formaldehyde followed by postfixation in OsO_4, retained approximately the same level of silver grains in the cytoplasm as fixation in Carnoy's. However, $KMnO_4$ fixation reduced cytoplasmic grain counts by about one-half compared with the other three fixatives.

On the basis of these preliminary experiments, onion roots treated with thymidine-^{3}H, specific activity 14.2 c/mmole, were fixed in methanol-free formaldehyde and postfixed in OsO_4. Between the

FIGURE 7 Tracheids with labeling over the annular rings.

formaldehyde fixation and the OsO_4 postfixation, some 1-hr formaldehyde-fixed roots were treated with DNase, 0.4 mg/ml enzyme, 0.01 M $MgSO_4$ at pH 6.8, or with DNase control solution.

FINE STRUCTURE LOCATION OF CYTOPLASMIC LABEL: Electron microscope radioautographs of unextracted sections showed not only many well labeled nuclei, but also label over cytoplasm and cell walls (Figs. 9 and 10). Contrary to expectation, cytoplasmic label was located over all elements of the cytoplasm and did not appear to be restricted to any particular component or class of organelles. A count of 197 cytoplasmic grains in seven cells, i.e. four cells from one root and three cells from three other roots, was distributed as listed in Table V. 41% of the grains was over ground cytoplasm in these cells, apparently chiefly over ribosomes (Figs. 9 and 10) but possibly over oblique cuts of membranes which were not discernible. 35% of the grains was over organelles, largely unidentifiable but presumably mitochondria, promitochondria, plastids and proplastids. About 30% of the labeled organelles had more than one silver grain. Approximately

TABLE V

Cytoplasmic Label Distribution from Electron Microscope Radioautographs Following Thymidine-^3H Treatment

Cytoplasmic components		No. o grainsf	% of total	
Cell walls		12	6.1	
Plasma membranes		7	3.6	
Ground cytoplasm		80	40.6	
Vacuolar membranes		11	5.6	
Vacuolar interiors		14	7.1	
Golgi apparatus		1	0.5	
Mitochondria		7	3.6	
Organelles:				
With one grain	31			
With two grains	8			34.5
With three grains	3			
With four grains	1			
Total number of grains over organelles		60	30.3	
Storage material		4	2.0	
Unidentified long membrane		1	0.5	
Total		197		

Silver grains over the cytoplasm of seven cells.

10% of the label was over cell walls-plasma membranes. The balance, about 14%, was over other cytoplasmic components. This tabulation of cytoplasmic label showing widespread distribution (Table V) confirmed the impression obtained from examining not only a number of grids from this experiment, but also those of previous experiments in which OsO_4 fixation and 10% formaldehyde fixation followed by postfixion in OsO_4 were used. Background was checked and did not account for this distribution of cytoplasmic label.

The cytoplasm of DNase-treated sections in electron microscope radioautographs unfortunately did not retain sufficient detail to permit determination of the location of unextracted grains.

EXTRACTION EXPERIMENT III: Sections from the same blocks used for electron microscope radioautography were filmed for light microscope radioautography. Cytoplasmic grains were counted over 20 tissue areas, 10 per section, and over 10 background areas (Table VI). DNase reduced cytoplasmic grains by one-third and greatly reduced label over nuclei. Nuclei of unextracted and DNase control sections were Feulgen positive, while nuclei of DNase-treated sections

TABLE VI

Extraction Experiment III. Roots Fixed and Embedded for Electron Microscopy. Light Microscope Radioautograms

Average cytoplasmic grain counts

	Unextracted	DNase	DNase control
Controls			
Tissue	0.40	0.65	0.35
Background	0.43	0.50	0.20
Net	0.03[n](3)	0.15 (2)	0.15 (1)
Thymidine-^3H			
Tissue	9.45	6.45	9.16
Background	0.63	0.69	0.71
Net	8.82 (14)	5.76 (18)	8.45 (9)

20 tissue and 10 background areas scored for each treatment of each root.

[n] Negative value.

( ), Number of roots scored.

were Feulgen negative. Since DNase-treated sections were Feulgen negative and nuclear label was extracted, it is clear not only that the DNase hydrolyzed DNA in this experiment but also that DNase is effective on tissue blocks fixed briefly in formaldehyde.

Cell walls in all thymidine-^3H–treated sections were labeled. Label was particularly heavy in epidermal walls, and especially evident in plasmolyzed cells (Fig. 8). Control sections were not labeled above background.

In summary, cytoplasmic label in electron micrographs was distributed over all cytoplasmic areas but showed two major concentrations, 41% over ground cytoplasm and 35% over organelles. Thick sections from DNase-treated blocks filmed for light microscopy had 35% less cytoplasmic label than thick sections from unextracted or DNase control blocks. The fine structure location of the DNase-extracted fraction could not be determined. Cell walls–plasma membranes were labeled in both light and electron microscope radioautograms.

Extraction Experiment IV

Radiochromatograms showed that isotopes used in the three previous extraction experiments concontained small amounts of labeled impurities. The main contaminants were thymine and a smaller amount of an unidentified compound just back of the solvent front. As a check on whether

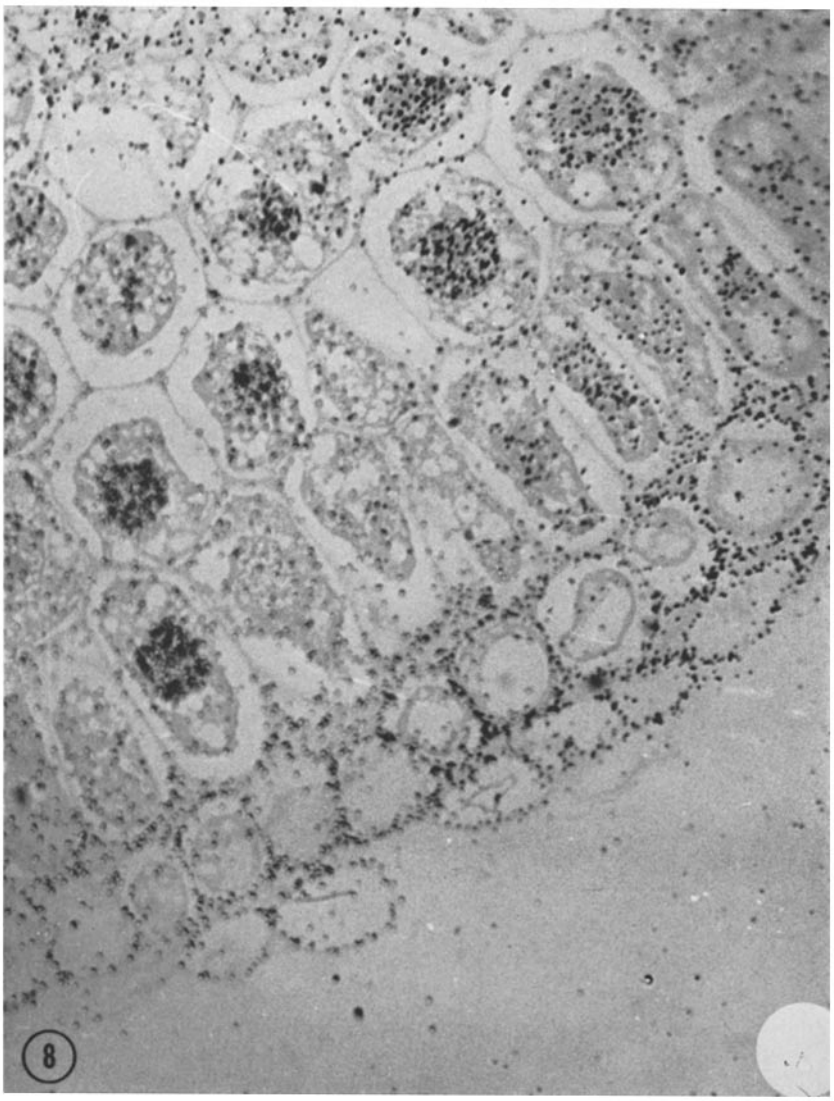
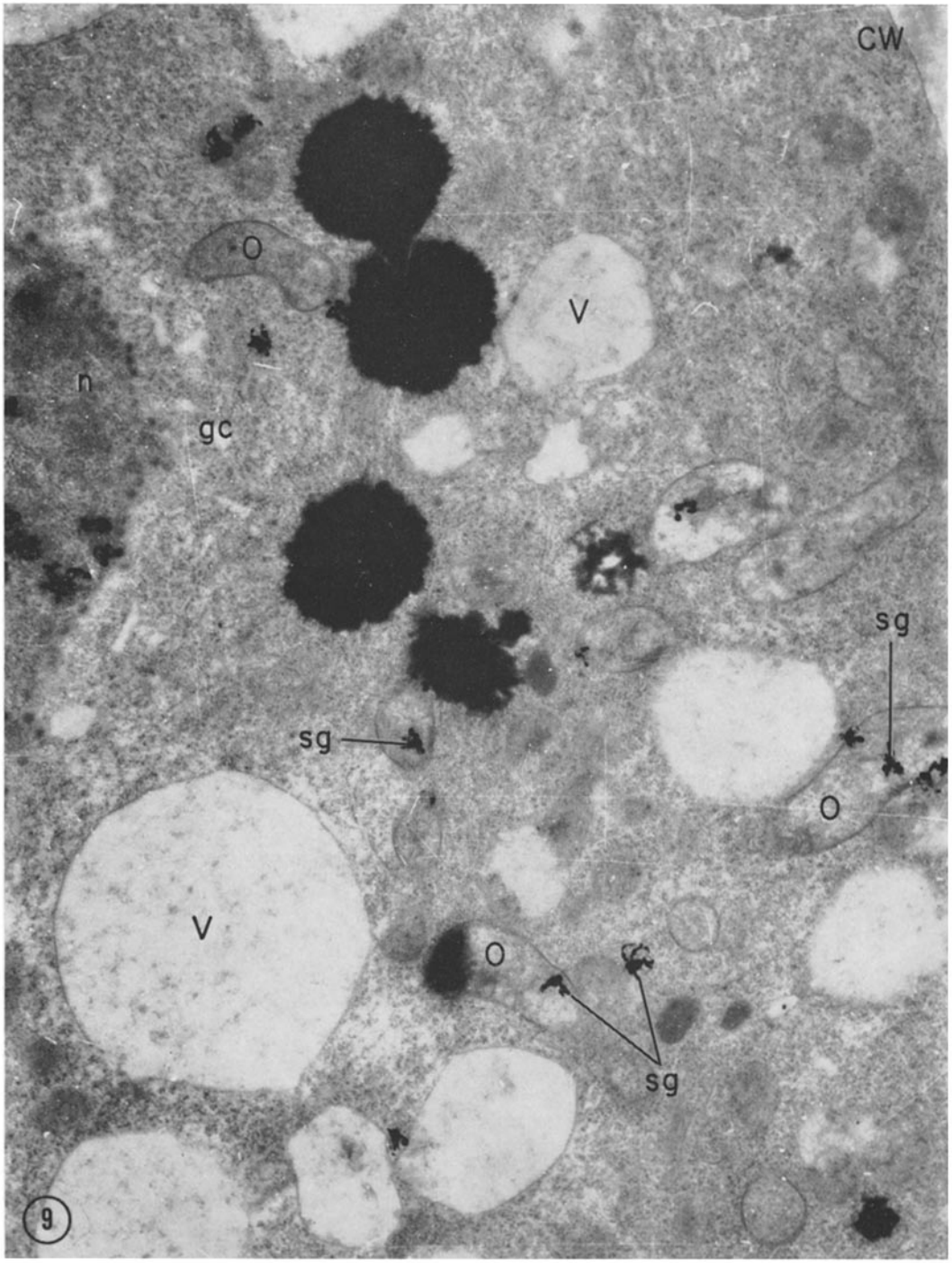


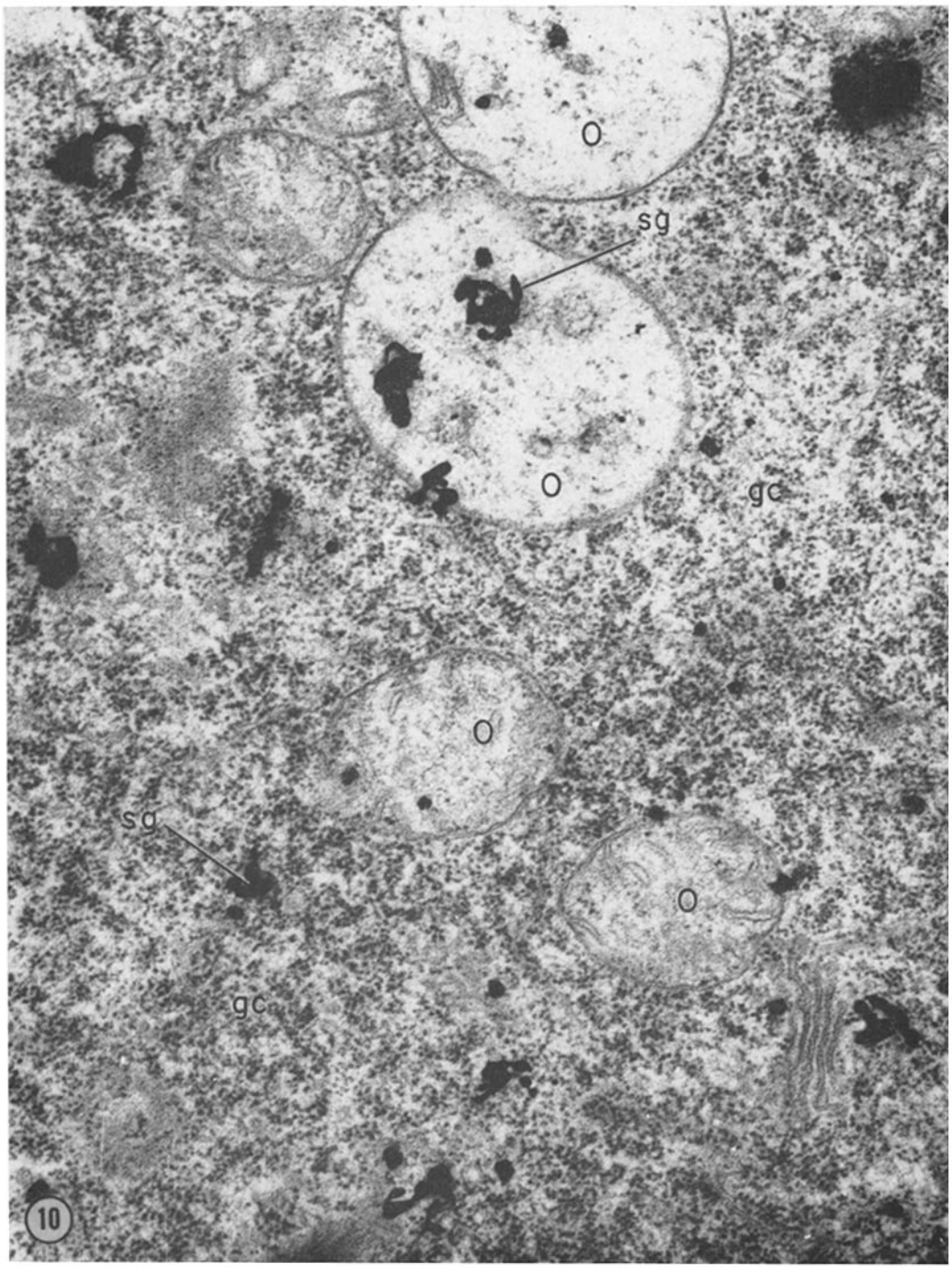
FIGURE 8 Extraction Experiment III. Cross-section of a formaldehyde/OsO_4-fixed tissue block embedded in Epon. Label over cell walls is clearly evident in this plasmolyzed section.

these impurities, which accumulated during storage of only several months, accounted for some or all of the nonextractable cytoplasmic label, highly purified thymidine-^{3}H, chromatographically checked, was used. Radioautograms of six thymidine-^{3}H–treated roots, three from one bulb, three from another, and one control root were prepared. Treatments were: unextracted; DNase, pH 6.8, 0.3 mg/ml, 0.01 M $MgSO_4$; and DNase controls. 20 tissue areas and 20 background areas per slide were scored (Table VII). Cytoplasmic label in unextracted and in DNase controls was essentially the same, while DNase reduced the cytoplasmic grain count by approximately 35% (Table VII).

The amount of cytoplasmic label extracted by DNase in four different experiments varied between 20 and 38%. The per cent of cytoplasmic label extracted from roots treated with very pure thymidine-^{3}H in Extraction Experiment IV (Table VII) was 35%. DNase extractions on roots treated with thymidine-^{3}H containing impurities reduced



FIGURES 9 and 10 Electron microscope radioautographs of thymidine-^{3}H–labeled onion roots. *cw*, cell wall. *gc*, ground cytoplasm. *n*, nucleus. *O*, organelle. *sg*, silver grains. *v*, vacuole. × 50,000 and 70,000, respectively.



For legend, see p. 278.

TABLE VII

Extraction Experiment IV. DNase extraction of Roots Labeled with Highly Purified Thymidine-^3H
Average cytoplasmic grain counts

	Unextracted	DNase	DNase controls
Controls			
Tissue	0.30	0.35	0.20
Background	0.30	0.35	0.15
Net	—	—	0.05
Thymidine-^3H			
Tissue	21.00	13.83	21.08
Background	0.65	0.42	0.36
Net	20.35	13.41	20.72

One control root and six thymidine-^{3}H-treated roots; 20 tissue areas and 20 background areas scored for each treatment of each root.

cytoplasmic label by 20 and 25% in Extraction Experiment I (Table I); by 38% in Preliminary Extraction Experiment II; by 23% in Extraction Experiment II (Table IV); and by 35% in Extraction Experiment III (Table VI). Thus, it is concluded that radioactive impurities in thymidine-^{3}H did not account for substantial amounts, if any, of cytoplasmic label.

DISCUSSION

The results of these experiments are consistent with the interpretation that DNA is a cytoplasmic component of onion root cells and that some DNA is located in mitochondria and plastids. The evidence is the following: (1) cytoplasm was routinely labeled by thymidine-^{3}H, which is a specific label for DNA (67) and which supposedly does not label other cellular components (65); (2) DNase extractions reduced cytoplasmic label by statistically significant amounts; and (3) mitochondria and plastids were labeled in electron microscope radioautographs.

DNase extraction of cytoplasmic label has been reported in *Amoeba* (45, 48), *Tetrahymena* (55, 58), *Vicia faba* roots and *Lilium longiflorum* buds (63), *Physarum polycephalum* (22), and Swiss chard leaves (61). The implication in these papers is that all label was removed; however, no supporting quantitative data are given. DNase alone failed to reduce cytoplasmic label in *Agapanthus* anthers (32), in *Zea mays* chloroplasts (33), or in *Drosophilia* oöplasm (41). In contrast, Steffensen and Sheridan (57) extracted approximately 89% of label over chloroplasts of the alga *Dictyota*, according to calculations made from their data. Bell (4) reports grain counts following DNase extraction of fern egg cells, but, the data are not sufficient to calculate the per cent of cytoplasmic label extracted although apparently a considerable amount of label was removed. Four DNase extractions of onion roots (Tables I, IV, VI, and VII) consistently reduced cytoplasmic label 20–38%, indicating that at least this fraction of the label represents DNA. These are the only results which show a major fraction of cytoplasmic label susceptible to DNase and a second larger fraction of label resistant to DNase. The reason why the reported DNase extractions have given such a spectrum of results for removal of cytoplasmic label—from 0 to 100%—is not known.

From the light microscope radioautographs, several other properties of cytoplasmic labeling were noted. First, the amount of cytoplasmic label is very small compared with the amount of nuclear label (Fig. 2). Calculations of the distribution of label between cytoplasm and nuclei in meristematic cells have shown that 5% of the total cellular label after an 8-hr thymidine-^{3}H treatment is in the cytoplasm (18). Thus, when thymidine-^{3}H radioautographs show lightly labeled nuclei, very little, if any, cytoplasmic label would be evident. This undoubtedly is one reason why cytoplasmic label has not been consistently observed in radioautographs.

A second feature noted was that, in contrast to nuclear labeling which is discontinuous and generally occurs in the middle third of interphase (64), cytoplasmic labeling is a fairly continuous process during most of the cell cycle. 2-hr thymidine-^{3}H treatment labeled the cytoplasm of all onion root cells. Van't Hof (68) has reported that onions grown at 23°C have a mitotic cycle of 17.4 hr. If 20 hr is taken as a rough approximation of the mitotic cycle of roots grown at 18°C, a 2-hr isotope treatment would have made label available for 10% of the cell cycle. However, any discontinuity in labeling for 2 hr or less would not have been detected.

In electron microscope radioautographs, silver grains were widely distributed in the cytoplasm (Table V) and not confined to one or two organelles as anticipated. Nevertheless, it is reasonable to assume that silver grains over mitochondria and plastids represent DNA. In the first place, DNase extractions show that onion roots contain cytoplasmic DNA (Tables I and IV). Secondly, al-

though the evidence is circumstantial, it was found that sections from the same blocks filmed for light and electron microscopy gave identical results. DNase extracted 35% of the cytoplasmic label in the light microscope sections (Table VI), while 35% of the silver grains in electron micrographs was over organelles (Table V). Whether these fractions are one and the same could not be determined. Also, reports that mitochondrial DNA and plastic DNA are sometimes refractory to DNase degradation suggests that caution should be observed in interpreting the results. For instance, Kirk (31) reported that it is harder to extract chloroplast DNA with $HClO_4$ than is nuclear DNA, and that only 42% of the diphenylamine-positive material could be extracted by DNase. Nass et al. (42) found that DNase digested only 20–35% of mitochondrial DNA even after complete disruption.

Biochemically, DNA has been identified in mitochondria of *Neurospora* (35), yeast (54), chick heart and liver (49), *Phaseolus aureus*, *Brassica rapa*, and onion bulbs (59); and DNA has been found associated with chloroplasts and plastids (15, 20. 53, 61)

The results obtained from DNase extraction experiments on onion roots (Tables I, IV, VI and VII) and from electron microscope radioautographs, together with the results from biochemical studies reported in the literature, support the conclusion that onion root cytoplasm contains DNA and that DNA is a constituent of mitochondria and plastids.

Another feature revealed by electron microscope radioautographs was that 41% of the cytoplasmic label was over ground cytoplasm. Wolstenholme and Plaut (70) found that 49% of cytoplasmic label in *Amoeba* was not associated with any recognizable structural entity, which the present author interprets to mean ground cytoplasm and ribosomes. Cytoplasmic label in *Drosophila* egg cytoplasm has been shown to have the following distribution: 36% over mitochondria, 28% within 0.25 μ of mitochondria, and the remaining 36% not associated with any organelle (41). Thus, three different studies on three different organisms report that 36–49% of the cytoplasmic label is over ground cytoplasm. These remarkably similar results suggest that ground cytoplasm is regularly labeled following thymidine-^{3}H treatments.

An apparent exception is Bell and Mühlethaler's (5) electron microscope radioautographic study on fern egg cells. They reported that cytoplasmic label, considered DNA, was almost exclusively over mitochondria and proplastids; label over ground cytoplasm was virtually absent. Quite likely the explanation of these results is technical. Bell and Mühlethaler (5) used as a fixative $KMnO_4$ which does not fix ribosomes (36, 47) and which oxidizes thymine to urea (28). Consequently, ribosomes with any associated radioactivity would have been extracted during fixation and dehydration. This interpretation is also supported by observations noted during preliminary fixation experiments with onion roots. In thymidine-^{3}H–treated onion roots, the cytoplasmic label of roots fixed in $KMnO_4$ was about one-half the cytoplasmic label of roots fixed in Carnoy's, formaldehyde, or OsO_4. Therefore, Bell and Mühlethaler's (5) report that cytoplasmic label from thymidine-^{3}H occurs mainly over organelles probably is a consequence of fixation in $KMnO_4$. Indirectly, their results are an indication that thymidine-^{3}H radioactivity is associated with ribosomes.

Although DNA was identified in onion root cytoplasm, the surprising findings were that only one-third of the cytoplasmic label could be characterized cytochemically as DNA, and that label in electron micrographs was distributed over all cytoplasmic areas, not just over organelles. Attempts to characterize the large DNase-resistant fraction in light microscope radioautograms by standard cytochemical extractions were fruitless. Cytochemically, this fraction is not RNA, for neither RNase nor 1 N HCl hydrolysis for 10 min removed statistically significant amounts of label (Tables I and IV); nor is it a lipid, for extraction with boiling alcohol-ether did not affect the level of label; nor can its persistence be attributed to inadequate amounts of DNase, for increased enzyme and Mg^{++} concentrations did not affect the per cent of label extracted; nor can it be accounted for by decomposition products in the radioactive compounds. The nature of the SVDase-extracted fraction is uncertain. This fraction could be DNA, in which case slightly less than one-half the cytoplasmic label would be DNA, or it could be any nucleoside 5′-phosphoryl group in an ester linkage with another hydroxylic compound (30). The persistent and stubborn fact obtained from four DNase extractions and from other enzyme and acid extractions is that one-half to three-fourths of the cytoplasmic label derived from thymidine-^{3}H could not be extracted

from onion roots by standard cytochemical methods. These results together with the electron microscope radioautographic observations suggest (*1*) that the premise (11, 65) that thymidine-^{3}H is an exclusive label for DNA is incorrect; (*2*) that DNA occurs in cytoplasmic components not previously detected; (*3*) technical problems; and (*4*) some combination of the three foregoing suggestions.

Nonextractable cytoplasmic label may reflect technical difficulties. A curious finding was that certain extractions—room temperature TCA for 1 hr, 1 N HCl hydrolysis at 60°C for 2 min, and brief treatments with cold 5% TCA before and after DNase control solutions—increased cytoplasmic grain counts by statistically significant amounts over that of the untreated condition (Tables I and III). At least two explanations are possible. The first is that these three acid treatments extract to some extent a major cytoplasmic constituent, perhaps RNA (60), which occurs in high concentrations in meristematic cells (8); (also see electron micrographs, Figs. 9 and 10, for heavy concentrations of ribosomes). Removal of a cytoplasmic constituent could uncover tritium-labeled components allowing radioautographic film to register the beta rays instead of their being absorbed in overlying cytoplasm. Reduction in self-absorption of tritium radiation has a significant effect on radioautographic grain counts (46).

The second explanation is that an acid-labile nuclear fraction is extracted by the three treatments and that some of it binds to the cytoplasm. Woods (71) found that Feulgen hydrolysis removed two DNA fractions, one very labile to acid treatment, the other more stable. A DNA fraction in lily anthers that has greater lability during early stages of DNA synthesis than later has been reported by Taylor and McMaster (66). DNA in solution will bind to cytoplasm and to nuclei of Carnoy's-fixed tissue sections extracted with DNase, RNase, or hot TCA (12, 34) and to unextracted tissue (12). Thus, the increased cytoplasmic grain counts observed may well be an acid-labile nuclear fraction bound to the cytoplasm. Some portion of the nonextractable cytoplasmic label may be such a fraction.

During fixation, all low molecular weight and acid-soluble substances supposedly are removed, leaving only macromolecular components (44). However, a small fraction, including radioactive precursors of DNA and thymidine breakdown products, could have been trapped in precipitating proteins or in the cell walls. Incomplete removal of low molecular weight radioactive substances during fixation could contribute to cytoplasmic and cell wall labeling.

There are at least two general ways in which thymidine-^{3}H may not be an exclusive label for DNA. One way would be the incorporation of breakdown products of thymidine into other compounds. Although the catabolic pathway of thymidine is known (13), almost no work has been done on the fate of breakdown products of thymidine in the cell. Takats and Smellie (63) concluded, from radioautographic and extraction experiments on *Lilium longiflorum* and *Vicia faba*, that the breakdown products of thymidine-^{3}H do not sufficiently account for the bulk of the cytoplasmic label observed, although a very small amount of the label could represent breakdown products. Cytochemically, Lima-de-Faria and Moses (33) found that neither DNase nor RNase extracted chloroplast label in *Zea mays* leaves, but that hot TCA and trypsin reduced the label by almost one-quarter. Their interpretation was that part of the tritium is closely associated with protein. An exploratory protein extraction experiment on onion roots was tried with trypsin, α-chymotrypsin and pepsin according to the procedures of Kaufmann, Gay, and McDonald (29). However, all three proteases so markedly distorted the cytoplasm that no realistic scoring was possible in this system. No further attempts at protein extraction were made.

A second way in which thymidine-^{3}H may not be specific for DNA would be the presence of thymidine or thymine compounds other than DNA in cells. Such compounds have not been found in higher plants or animals, although thymidine diphosphosugars (43) are common components of bacteria. These compounds may well occur in higher organisms as well, especially plants, because thymidine diphosphosugars are involved in sugar transformations and as glycosyl donors (6, 21) of cell wall constituents. Thymidine diphosphosugars, if present in higher organisms, would be labeled by thymidine-^{3}H since nucleoside diphosphate sugars are synthesized from nucleoside triphosphates (21). The labeled cytoplasmic fraction which was extracted by SVDase and which appeared to be resistant to DNase may possibly represent thymidine diphosphosugars

because SVDase hydrolyzes the diester linkage of thymidine diphosphosugars.

Another possible source of non-DNA thymine label is transfer RNA (tRNA). One molecule of ribothymidine has been found in alanine tRNA, and this base is quite likely present in all tRNA (25). If tRNA containing labeled thymidine were fixed along with the ribosomes, it could account for cytoplasmic label associated with ribosomes.

In summary, the nonextractable cytoplasmic label in part may include (*1*) a small amount of breakdown product (63), perhaps some of which is associated with proteins (33); (*2*) thymidine diphosphosugars; (*3*) other non-DNA thymine-containing molecules such as tRNA.

On the other hand, the nonextractable cytoplasmic label could be DNA not extractable by common methods. Nonextraction is not proof against DNA. Chargaff (9) found that DNase does not completely degrade isolated calf-thymus DNA but leaves a resistant residue of small oligonucleotides (core). A naturally occurring DNA/RNA complex isolated from *Chlorella* was not broken down by either DNase or RNase (51). DNA of intact T-2 virus is not affected by DNase, but DNA isolated from T-2 virus is affected by DNase (23). Cytochemically, Muckenthaler and Mahowald (41) found that label in *Drosophila* ooplasm could be extracted by DNase only after treatment of the sections for removal of protein. DNA-protein associations, presumably nonnuclear, have been reported for yeast lactic dehydrogenase (2, 38) and for avidin (16). Thus, the nonextractable cytoplasmic label may be DNA, bound or combined with other molecules in such a way as to be resistant to DNase.

Various ideas have been presented to account for the large amount of nonextractable cytoplasmic label. But plausible as they may be, the fact remains that the chemical nature of this fraction is unknown. If the label is largely in situ, then the questions are: Is this fraction, in whole or in part, DNA? Is thymidine-^{3}H a completely specific and exclusive label for DNA, or are its breakdown products incorporated into other molecules, or is thymidine or thymine utilized in the synthesis of non-DNA thymine compounds? If the label observed is in DNA, then DNA is present in heretofore unexpected places, i.e. ground cytoplasm, and cell walls–cell membranes. If the label is not in DNA, then the results of experiments on thymidine-^{3}H treatment need careful interpretation. In order to answer these questions, thymidine-^{3}H–labeled components need to be identified biochemically and all radioactivity from administered thymidine-^{3}H needs to be accounted for. Until such information is in hand, the assumption that thymidine-^{3}H is a completely selective label for DNA and is not utilized in the metabolism of other cellular components (65) is dubious; and furthermore, silver grains in radioautographs cannot unequivocally be equated with DNA.

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