Trail Marking Substance of the Texas Leaf-Cutting Ant: Source and Potency

Abstract. *The trail-marking substance of* Atta texana *(Buckley) is formed in the true poison gland and deposited by the sting. This attractant is initially produced by teneral workers. The substance obtained from mature, large workers is the most potent.*

Trail-following behavior is highly developed in the Texas leaf-cutting ant, *Atta texana* (Buckley). This ant marks its trail with a substance that is highly persistent, stable, and perceptible to ants even when it is in minute quantities. It is produced by the true poison gland and deposited by the sting, which is not used for stinging; thus its defense mechanism is used for purposes of communication.

Study of the behavior of a laboratory colony of ants reveals the method of trail marking. When a new supply of green leaves is placed near the nest, scouting workers examine it, and as they return to the nest without forage material, they touch their abdomens to the ground regularly at 2 or 3 mm intervals. Presently, other workers follow this trail, begin cutting leaves, and carry sections to the nest. They mark the trail frequently while carrying the forage, but when returning to the source of supply the marking is sporadic.

In the field, well-defined surface trails may extend several hundred feet to plants being cut. Ants may travel the same trail for months. Trails are used for foraging, never for removal of detritus as in certain other species of *Atta*. Workers forage when surface temperatures of the trail are between 52° and 85°F. Thus, trail activity usually occurs during the day in winter and at night in summer. Activity continues on wet trails, even during light rain, because the odoriferous substance is insoluble in water. A heavy rain disrupts the following of a trail. When a wet or dry trail is broken—as with a scratch of a finger or small stick—traffic is disorganized until the ants replace the scent.

The true poison gland secretes the substance that accumulates in the poison sac. Contents of the Dufour's gland produce no response although this gland is the source of the substance excreted for trail-marking by the myrmicine ants, *Solenopsis* spp. (*1*) and *Pheidole fallax* Mayr (*2*). In *Atta texana*, poison sacs were absent in larvae, empty in pupae, about half full in teneral workers, and turgid in mature workers.

The trail-marking substance is a clear, viscous liquid, soluble in methylene chloride and carbon tetrachloride. It forms a milky-white suspension in acetone, alcohol, or water. When a poison sac is crushed on a glass slide, its contents quickly solidify into a hard, shiny, amorphous material resembling clear fingernail polish.

Artificial trails were made by crushing a single poison sac on the tip of a matchstick and drawing the stick across a sheet of paper. Workers, males, and virgin queens readily followed this path. Crushed heads, thoraxes, abdomens, or contents of the hindgut failed to elicit similar behavior.

We then conducted bioassays to evaluate the potency of the substance from two teneral workers and six mature workers of different sizes. Each poison sac was crushed in 1 ml of carbon tetrachloride and shaken thoroughly. We applied 0.1 ml of the solution to a piece of paper, and described a circle with a diameter of 6 inches. Two lots of ten minor workers (medium and large workers were too excitable for tests) were released in the circle at different times. The number that traveled at least 6 inches on the artificial trail in a period of 5 minutes was used as an index of the potency of the contents of the sac. The lowest concentration that workers could detect was determined by serial dilutions.

Partially full sacs of teneral workers gave a moderate to strong response, but an even stronger reaction was obtained from the turgid sacs of mature workers (Table 1). Sacs from the biggest mature workers tended to have the highest potency. Sacs from pupae produced no response.

Contents of poison sacs crushed on matchsticks or in carbon tetrachloride and kept at room temperature had high potency after 5 months. Contents of poison sacs held at −12°C for 5 months were fully potent. The contents did not freeze. Poison sacs remained intact in dead workers after other abdominal parts had decomposed to a soupy mass; the contents of the sacs were fully potent.

Table 1. Potency of odoriferous substance deposited by teneral workers and older workers of various sizes. The response is computed from two replications of ten workers each, and the number in the table represents the number of workers following the trail over a distance of 6 inches or more. The contents of one poison sac were taken up in 1 ml of CCl_4 and serially diluted (by powers of 10) in CCl_4.

Worker length (mm)	Poison sac (length × width) (mm)	Response					
		1	10	10^2	10^3	10^4	10^5
Teneral workers (sacs partially full)							
5.0	0.30 × 0.20	13	1	1	2	0	0
5.0	.35 × .25	9	5	0	1	0	0
Mature workers (sacs turgid)							
2.2	.10 × .10	17	1	0	0	0	0
2.3	.10 × .05	16	1	0	0	0	0
5.0	.50 × .40	20	18	6	0	0	0
6.0	.60 × .40	19	18	3	0	2	0
8.0	.70 × .60	14	10	12	0	1	0
9.0	.80 × .55	17	14	9	1	0	0

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Myoglobin: Inherited Structural Variation in Man

Abstract. *Ultrafiltrates of human myoglobin from 200 individuals were examined by starch-gel electrophoresis. Only two variants, $Mb^{Aberdeen}$ and $Mb^{Annapolis}$, of the commonly occurring myoglobin Mb^{+} were encountered. Each variant is apparently inherited as an autosomal codominant. Heterozygotes for either mutation lack apparent muscular disease. Combined electrophoresis and chromatography of $Mb^{Annapolis}$ indicate a loss of arginine at a position near the C-terminal portion of the molecule.*

Although considerable genetic variation occurs among the hemoglobins of man and other species, no such variation has been reported for myoglobin. We have discovered two examples of inherited structural variation of human myoglobin. The detection and characterization of additional myoglobin mutants

may be useful in assigning function to various portions of a molecule whose protein fine structure is particularly well delineated.

Samples of myoglobin were prepared from skeletal muscle obtained at autopsy (*1*) or at diagnostic muscle biopsy (*2*) and usually stored, until needed, at −15°C. Storage for several months produced no obvious alteration in the electrophoretic properties of myoglobin.

Aqueous muscle extracts were prepared at −1°C by grinding muscle with three volumes of water in a Servall homogenizer at 50,000 rev/min for 1 minute. Homogenates were centrifuged at 40,000*g* at 4°C for 30 minutes. Supernatant fluids were placed in bags made from No. 8 Visking cellulose tubing (*3*) and subjected to ultrafiltration with water vapor pressure at 2° to 5°C for several hours (*4*). Under these conditions, myoglobin, molecular weight (MW) 18,000, readily passes through the cellulose bag while both hemoglobin, MW 67,000, and an added sample of ovalbumin, MW 45,000, are retained within the bag. The ultrafiltrate, after dialysis against water, has a 410 mμ/280 mμ absorption ratio of 2.40 to 2.60 indicating that myglobin constitutes approximately half of the large proteins in the ultrafiltrate (*5*). Recovery of ultrafiltered myoglobin approaches 2.5 mg per g of wet weight muscle provided the bag contents are subjected to multiple additions of water and continued ultrafiltration for 24 to 36 hours. Preparation by ultrafiltration has the advantages of simplicity and gentle treatment. The concentration of ultrafiltered myoglobin is easily increased by secondary ultrafiltration in collodion bags which have a porosity less than 5 mμ (*6*) and retain myoglobin.

Screening for structural variants was accomplished by vertical starch-gel electrophoresis (*7*) of undialyzed, unconcentrated ultrafiltrates of myoglobin containing 0.2 to 0.75 mg/ml. Sharp resolution of the myoglobin components is provided by electrophoresis (15 to 20 hr at 4°C, 4.6 v/cm). Gels were buffered with EBT [0.001*M* EDTA, 0.025*M* boric acid, and 0.045*M* tris buffer at *p*H 8.5 (*8*)]. Lesser resolution is obtained with borate or discontinuous buffers. The products of electrophoretic analyses were developed by both benzidine and amido black stains.

Starch-gel electrophoretic patterns from normal and exceptional subjects are shown in Fig. 1. Reactions of the heme group can alter mobility (*9*); to avoid confusion, all specimens were converted to cyanmetmyoglobin. Cyanmetmyoglobin from most subjects contains one major component, shown in Fig. 1, slots No. 3 and No. 5. Several more rapidly migrating minor components (*9*) can usually be seen in these preparations and as many as seven minor components can be observed in concentrated specimens. Migration is not dependent upon the method of preparation since myoglobin obtained by other means (*10*) has mobility identical to that obtained with ultrafiltration. Moreover, the source of material has no influence on mobility. In particular, the migration of myoglobin derived from six specimens of adult cardiac muscle and from the skeletal muscle of six newborn infants is identical (Fig. 1) to that of myoglobin from adult skeletal muscle. Therefore, the commonly occurring major electrophoretic component is designated Mb^{+}.

Two subjects with major myoglobin variants were encountered among 200 individuals. One variant specimen contained Mb^{+} and an additional major component that migrated to the cathode from the origin (Fig. 1, slot No. 2). This component, representing approximately one-quarter of all myoglobin, is designated $Mb^{Aberdeen}$ after the place of origin of the propositus. The propositus was a stillborn, anencephalic, premature white male who had no evidence of primary muscular or cardiac disease. Muscle biopsies indicated that the father has only Mb^{+} whereas the mother has both Mb^{+} and $Mb^{Aberdeen}$. The mother is 30 years old and well.

The other variant specimen contained Mb^{+} and a rapidly moving major component designated $Mb^{Annapolis}$ (Fig. 1, slot No. 4). The propositus was a Negro boy, 8 years old, who died from congenital hydrocephalus and who gave no evidence of primary muscular or cardiac disease. The muscle of the father has only Mb^{+} whereas that of the mother has both components, Mb^{+} and $Mb^{Annapolis}$. The mother is 37 years old and free of evident muscular and cardiac disease. All subjects are homozygous for normal adult hemoglobin *A*.

The possibility that one or both variants represented increased concentration of a normally present minor component was excluded by the results of electrophoresis of concentrates from variants and from Mb^{+}. In each instance, the migration of variant myoglobin differs from that of any minor component. In addition, each variant has a unique minor component which migrates more rapidly to the anode than the major variant.

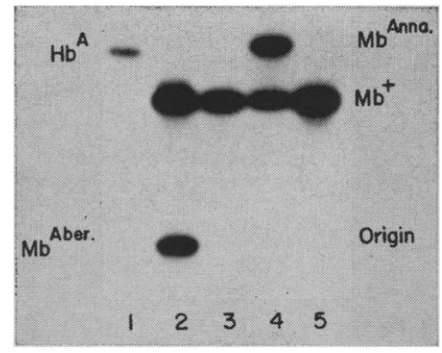

Fig. 1. Benzidine stain of starch-gel electrophoresis of human cyanmetmyoglobin, 0.5 to 1.0 mg/ml. *1*, hemoglobin *A*; *2*, slow $Mb^{Aberdeen}$ and Mb^{+}, propositus; *3*, Mb^{+} newborn Negro female; *4*, Mb^{+} and fast $Mb^{Annapolis}$, propositus; *5*, Mb^{+}, adult white female.

Since each variant appears as a heterozygote both in mother and son, the genetic structural locus for the major component of human myoglobin is necessarily autosomal. It is assumed, in such reasoning, that the major component is produced by only one locus. The recent suggestion that *X*-linked Duchenne muscular dystrophy results from a structural abnormality of myoglobin (*11*) thus seems unlikely. In the event of *X*-linkage of the structural locus, males would exhibit only one major component. Nonetheless, it is possible that gene duplication has provided two structural loci each productive of major components. One of these loci might be *X*-linked. The products of each locus might also have identical electrophoretic behavior and thus escape detection.

The similarity of the molecular weight of Mb^{+} and $Mb^{Annapolis}$ is suggested by identity in rate of escape from cellulose bags during ultrafiltration and by identical behavior in a system where porosity of the starch gel is varied (*12*). In this system when Mb^{+} and ovalbumin were used as markers of known molecular weight in EBT buffer, the molecular weight of $Mb^{Annapolis}$ was estimated as 15,000. This crude estimate suggests that $Mb^{Annapolis}$ is monomeric.

In the form of cyanmetmyoglobin, Mb^{+} and $Mb^{Annapolis}$ were separated by starch-block electrophoresis (*13*) in

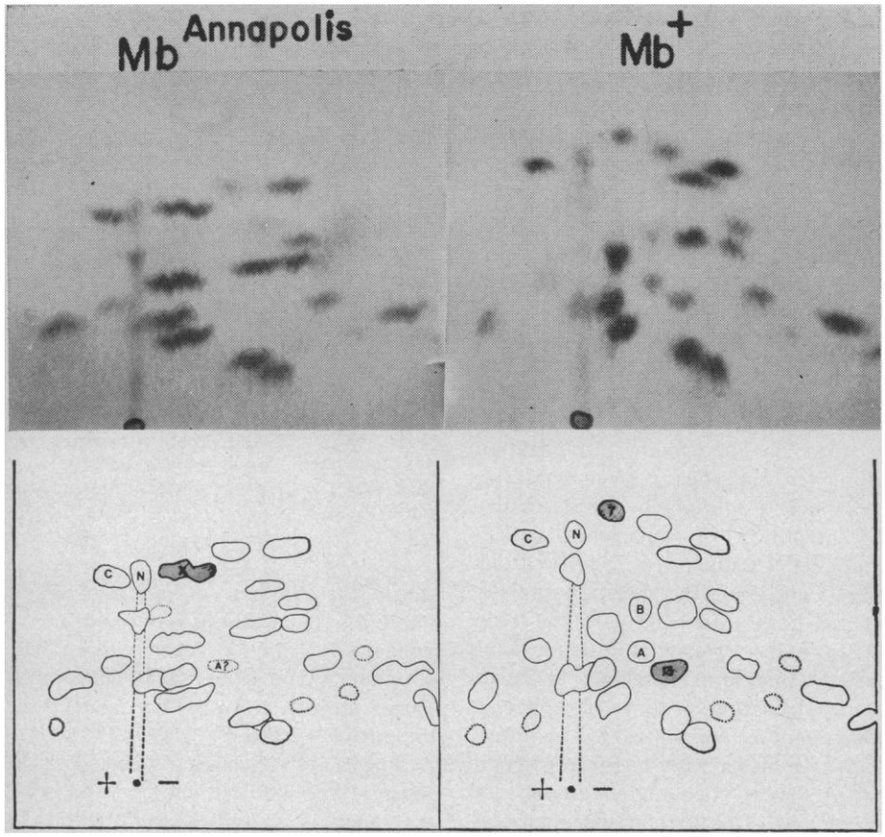

Fig. 2. Peptide patterns ("fingerprints") of tryptic digests of $Mb^{Annapolis}$ (left) and Mb^{+} (right) developed with 0.2 percent ninhydrin. Photographs above, diagrams below. Horizontal axis, electrophoresis; vertical axis, chromatography.

0.1*M* barbital buffer, *p*H 8.5, for 72 hours at 5 v/cm and 4°C. The $Mb^{Annapolis}$ continued to migrate more rapidly than Mb^{+}; however, in this system, both move more slowly than hemoglobin *A*. The similarity of the relative mobilities of Mb^{+} and $Mb^{Annapolis}$, both in starch-gel and starch-block electrophoresis, is further evidence for their similar molecular weight. Myoglobin was completely recovered from starch blocks. In the propositus 44.8 percent was Mb^{+}, 41.8 percent $Mb^{Annapolis}$, and 13.4 percent minor rapid components. In the mother 45.1 percent was Mb^{+}, 42.8 percent $Mb^{Annapolis}$, and 12.1 percent minor components. The relative proportions of minor components recovered from $Mb^{+}/Mb^{Annapolis}$ subjects are similar to those present in Mb^{+}/Mb^{+} homozygotes. Absorption spectra of the acid metmyoglobin (12μM solutions) of Mb^{+} and $Mb^{Annapolis}$ were identical in the 240 to 740 mμ range suggesting that the $Mb^{Annapolis}$ mutation does not impinge upon the heme group (*14*). The two forms also had identical cyanmetmyoglobin spectra in the 480 to 700 mμ range.

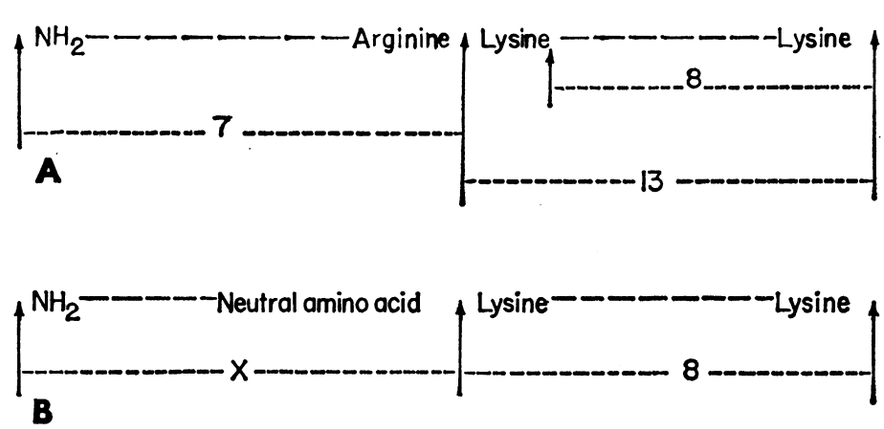

Fig. 3. Amino acid sequences in Mb^{+} and $Mb^{Annapolis}$.

Purified (*5*) $Mb^{Annapolis}$ and Mb^{+} were prepared from muscle of the propositus and his mother. Apomyoglobin was obtained from these materials (*9*); it was dissolved in water, adjusted to *p*H 8.0, heated at 90°C for 6 minutes, and thereafter digested with trypsin at *p*H 8.0. "Peptide patterns" (fingerprints) of digests were obtained by a combination of the electrophoretic procedure of Ingram (*15*) and the chromatographic solvent system containing pyridine, butanol, acetic acid, and water (*16*). One-mg digests of Mb^{+} and $Mb^{Annapolis}$ from the muscle of the propositus were "fingerprinted" simultaneously (*15*). Representative results are shown in Fig. 2. Similar peptide patterns were obtained from a 0.5-mg Mb digest of $Mb^{Annapolis}$ derived from the mother. The Mb^{+} peptide pattern from the propositus is the same as that obtained with digests from ten different Mb^{+}/Mb^{+} subjects. "Fingerprints" of Mb^{+} prepared by ultrafiltration and electrophoresis are indistinguishable from those prepared by other methods (*9*).

Although the peptide pattern of $Mb^{Annapolis}$ is imperfect, interrelationships in the position of peptides indicate that 2 Mb^{+} peptides, arbitrarily designated 7 and 13, are absent in $Mb^{Annapolis}$, and a new peptide, *X*, has appeared. Comparative electrophoresis (*17*) at *p*H 1.9 (*18*) of amino acids derived from individual peptides further suggests that these are the pertinent dissimilarities (*19*). Peptides A and B (Mb^{+}, Fig. 2) are inconstantly present in Mb^{+} "fingerprints" and their absence in $Mb^{Annapolis}$ is probably irrelevant (*20*). Differences between Mb^{+} and $Mb^{Annapolis}$ in the staining intensity of several other peptides is not believed significant. In particular, the intensity of the two peptides immediately on the right of *X* and the one just below *N* (Fig. 2) is normally variable.

Peptide 7 contains arginine whereas peptides *X* and 13 contain lysine. The qualitative amino acid composition of *X* and 7 is otherwise similar (*21*). Conversely, *X* lacks certain amino acids which are characteristic of 13. Therefore, *X* is not produced by fusion of 7 and 13. The origin of *X* and the loss

of 13 becomes understandable through homology with sperm-whale myoglobin. The amino-acid composition of peptide 7 corresponds exactly to tryptic peptide 1 from sperm-whale myoglobin and the composition of 13 to the sum of tryptic peptides 11 and 8 from sperm whale myoglobin (*22*). By homology these peptides could be arranged 7-13 and arginine is approximately 15 residues from the C-terminal portion of the molecule (*23*). Robert L. Hill (*24*) has generously provided details of sequence for human myoglobin which support this view, and, moreover, indicate that peptide 13 bears both an N-terminal and a C-terminal lysine. Thus Mb^{+} contains the sequence shown in Fig. 3*A* and $Mb^{Annapolis}$ the sequence shown in Fig. 3*B*. Peptide 8, present both in Mb^{+} and $Mb^{Annapolis}$, has qualitative amino acid composition which is identical to 13. The substitution of a neutral amino acid for arginine in peptide *X* is sufficient to account for the increased electrophoretic mobility of the $Mb^{Annapolis}$ molecule.

The functional importance of myoglobin mutants is uncertain since we do not know what effect $Mb^{Aberdeen}$ or $Mb^{Annapolis}$ might produce in homozygotes. It is possible that homozygotes for these and other mutants may exhibit muscular disease (*25*).

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References and Notes

1. Seventy-four individuals were Negro and 109 white.
2. Four subjects were Negro and 13 white. Individuals with primary muscular disease were excluded.
3. Supplied by Visking Company, 6733 W. 65 St., Chicago 38, Ill.
4. As many as 20 specimens can be simultaneously ultrafiltered in the apparatus described by E. A. Peterson and H. A. Sober, in *Methods in Enzymology*, S. P. Colowick and N. O. Kaplan, Eds. (Academic Press, New York, 1962), vol. 5, p. 25.
5. Acid metmyoglobin, when homogeneous as judged by electrophoresis and in the analytical ultracentrifuge, has an absorbance maximum at 410 mμ (molar extinction coefficient is 15.4 × 10^4) and a 410 mμ/280 mμ absorbance ratio of approximately 4.60 to 5.00.
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19. Individual peptides from "fingerprints" of Mb^{+} and $Mb^{Annapolis}$ were eluted with distilled 6*N* HCl and thereafter hydrolyzed at 100°C for 18 hr. The identity of peptides containing the free amino and free carboxyl portions of myoglobin, indicated as *N* and *C* respectively in Fig. 2, is based on amino acid composition.
20. Peptides A and B were absent in two of five individual Mb^{+} specimens. In a sixth specimen these peptides were present in one tryptic digest but absent in another, suggesting origin through partial tryptic digestion.
21. Quantitative amino-acid analysis of peptides 7, 13, and *X* is in progress.
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Chromosomal and Nucleolar RNA Synthesis in Root Tips during Mitosis

Abstract. *Comparative rates of RNA synthesis in chromatin and nucleolar fractions during mitosis in root-tip cells of* Allium *and* Nigella *were studied by pulse-labeling of cells with tritiated cytidine. Although the rate of RNA synthesis decreases in the condensing chromosomes during prophase, it remains normal in the nucleolar fraction as long as nucleoli are maintained. RNA synthesis stops in mitotic cells lacking distinct nucleoli. In the late telophase or very early interphase cells, RNA synthesis resumes at a faster rate in the pronucleolar bodies than in the chromatin.*

Recent autoradiographic studies with a pulse-labeling procedure revealed that nuclear RNA synthesis decreases at the start of mitosis and is practically absent from late prophases through early telophases; it resumes in late telophase cells (*1*, *2*, *3*, *4*). In those studies, no attempt was made to distinguish between RNA synthesis in the two principal nuclear components, that is chromatin and nucleoli. In this report, however, a quantitative study of RNA synthesis in these components through the mitotic cycle is made.

Primary roots of *Nigella arvensis* (fennel-flower) and the secondary roots of *Allium cepa* (onion) were exposed to tritiated (H^3) cytidine (50 to 100 μc/ml; specific activity 1 to 1.9 c/m*M*) for 3 to 6 minutes. Roots were fixed in a mixture of acetic acid and alcohol, squashed [after pectinase digestion (*5*)], or cut into sections 2 to 5 μ in thickness after having been embedded in paraffin. Stripping-film autoradiographs were made and stained with methyl-green-pyronin through the processed film.

With a film-exposure time of 1 to 2 weeks, practically all the radioactivity in the cell was confined to RNA, as determined by a standard ribonuclease-digestion test. Silver-grain counts were made over chromatin and nucleoli of interphase and mitotic nuclei. Chromatin and nucleolar areas of some nuclei were determined on camera lucida drawings. After counting, some of the preparations were stained by the nucleolar silver-staining procedure (*6*) to check certain nucleoli which were not clearly stained with pyronin. This method is very sensitive for detecting minute amounts of nucleolar material: the same interphase nucleoli of root-tip cells of *Allium* are clearly stained by azure B (a nucleic acid stain) and silver (a non-RNA stain) (Fig. 1, *A*, *B*); however, the silver-staining procedure is more sensitive than azure B in revealing the presence of pronucleolar bodies in telophase cells. Prophase and telophase cells were subdivided into "early" and "late" stages on the basis of chromosome condensation and their dispersion in the nucleus. In general, early prophases contain distinct nucleoli while such nucleoli are absent in late prophases (Fig. 1, *C*, *D*). In early telophase cells, chromosomes are covered with numerous pronucleolar granules; these granules fuse into bodies of different sizes in late telophase cells (*6*).

In root-tip cells of both *Nigella* and *Allium* that were exposed to H^3-cyti-