SITES OF CYTOPLASMIC RIBONUCLEOPROTEIN-FILAMENT ASSEMBLY IN RELATION TO HELICAL BODY FORMATION IN AXENIC TROPHOZOITES OF *ENTAMOEBA HISTOLYTICA*

YVONNE KRESS, MURRAY WITTNER, and
ROBERT M. ROSENBAUM

From the Department of Pathology, Albert Einstein College of Medicine, New York, New York 10461

ABSTRACT

Axenic trophozoites of *Entamoeba histolytica* showed increased logarithmic growth but absence of "chromatoid" material (stacked helical arrays of ribonucleoprotein [RNP]) when grown in an all-liquid monophasic culture. Organisms grown in a liquid overlay on a semisolid slant (biphasic medium) showed slow logarithmic growth and the presence of chromatoid material. Chromatoid material accumulated in the rapidly growing trophozoites from monophasic culture during treatment with the *Vinca* alkaloid, vinblastine. Many of the glycogen-free regions of vinblastine-treated trophozoites as well as, to a lesser degree, of normal cells grown in monophasic and biphasic cultures, contained free ribosomes and randomly oriented 60 A filaments. As ribonucleoprotein assumed the packed helical configuration, areas consisting of parallel, packed filaments could be detected adjacent to and continuous with the ordered RNP arrays. This arrangement could be visualized most frequently in vinblastine-treated trophozoites grown in monophasic cultures. Depending on the tilt of the section with respect to the longitudinal axis of individual helices, 60 A filamentous material could be demonstrated associated with the RNP helices. Localization of ribonucleoprotein precursors was followed by means of high resolution radioautography with uridine-^{3}H and cytidine-^{3}H. With a short (30-min) pulse, label could be visualized only over the glycogen-free areas containing free ribosomes and filaments. With 60-min pulses, label could also be seen over the packed helical arrays. With 30-min pulses followed by a 60-min cold chase, label was seen chiefly over RNP helices. It is postulated that the areas containing ribosomes and filaments represent sites of assembly of the RNP helices possibly on a filament protein column. The possibility that the final helical configuration may be due to a property of this protein is suggested.

A characteristic ultrastructural feature of members of the genus *Entamoeba* is the presence of ribonucleoprotein in ordered helical arrays which form the classical "chromatoid bodies" evident in cysts and trophozoites. Since these bodies, as well as shorter helices free in the cytoplasm, appear to represent a major form of ribonucleoprotein in these amebae, their detailed structure and func-

tion in relation to the economy of the cell are of interest. In addition, accumulating evidence that many cell types are capable of producing ribosomal helices under suitable stimuli makes an understanding of how such structures are formed appropriate to situations other than that which exists normally in *Entamoeba*.

Previously, we described some features of

helical ribonucleoprotein structures in axenically grown and bacterized trophozoites of *E. histolytica*, stressing the association with filamentous material. Such filaments were detected in cytoplasmic RNP helices following their digestion with RNase, and also were present inside vacuoles where they appeared to be derived from helices. Recently we observed that trophozoites grown in monophasic medium showed few chromatoid bodies, and that the alkaloid vinblastine caused an increase in these bodies. Therefore, the opportunity presented to study the formation of the packed RNP helices *in situ*. The present paper describes further relationships between helical RNP bodies and filaments, and demonstrates specific regions in the cytoplasm of *E. histolytica* trophozoites which appear to constitute sites for RNP helix formation. In addition, radioautographic evidence is presented for the sequential processing of uridine into ribonucleoprotein helices via free ribosomes in the cytoplasmic filamentous regions.

MATERIALS AND METHODS

Materials

For this study, trophozoites grown in both biphasic (agar slant with a liquid overlay) and all-liquid monophasic media were examined. Monophasic medium, especially, permitted examination of a large number of organisms fixed for electron microscopy, and also allowed removal of samples from the same culture flask for studies requiring various exposures to the alkaloid, vinblastine. Cell counts were done as previously described (2).

BIPHASIC MEDIUM: Strains F-22, K-9, and 301 of *E. histolytica* were maintained at 36°C in the medium described by Wittner (2). These cultures reached optimal growth every 3–4 days, and were transferred weekly.

MONOPHASIC MEDIUM: This medium is modified from that described by Diamond (3). *E. histolytica* (strain K-9) were grown in a medium consisting of Trypticase (Baltimore Biological Laboratories, Baltimore, Md.), 2.0 g; glucose, 0.5 g; L-cysteine-HCl, 0.1 g; ascorbic acid, 0.2 g; NaCl, 0.5 g; potassium phosphate, monobasic, 0.06 g; potassium phosphate, dibasic, anhydrous, 0.1 g; distilled water, 90 ml; vitamin mixture after Evans et al. (4). The final medium was adjusted to pH 7.0. Organisms were grown in 125-ml screw capped Erlenmeyer flasks at 36°C and were transferred alternately every 72 and 96 hr.

VINBLASTINE TREATMENT: Amebae were incubated with vinblastine sulfate (88.8% potency, Lot

No. 3EL22, Eli Lilly & Co., Indianapolis, Ind.) at 36°C at a final concentration of 5.4 × 10⁻⁴ M.

COLLECTION AND FIXATION OF AMEBAE: Harvesting and fixation of organisms were done as previously described (1). Chilling facilitated the release and collection of cells attached to the glass surface. Fixation took place for 60 min in 2% glutaraldehyde in Millonig's phosphate buffer (pH 7.3). Postfixation was carried out for 45 min in 1% OsO₄ in Millonig's phosphate buffer at pH 7.3. Samples containing large numbers of amebae were removed aseptically from the same flask at regular intervals during the experimental period.

ELECTRON MICROSCOPY: Sections of material embedded in Epon-Araldite were cut with a diamond knife on an LKB Ultrotome and placed on carbon-coated grids. They were stained routinely with lead citrate (18) alone for 10 min, or with a combination of lead (2 min) preceded by 5% uranyl acetate in 40% alcohol (20 min), and were examined with an RCA 3F electron microscope at 50 kv.

RADIOAUTOGRAPHS: Uridine-³H or cytidine-³H was employed for labeling at concentrations of 60 or 150 μCi/ml medium. Incubation generally proceeded for 30 or 60 min, whereas other experiments employed 30-min exposures to uridine-³H followed by a 30- or 60-min "cold" uridine chase in order to isolate the label (Table I). Cold chases consisted of the addition of 0.9 ml of uridine (50 mg/5.0 ml normal saline) to each 7.0 ml of medium. This con-
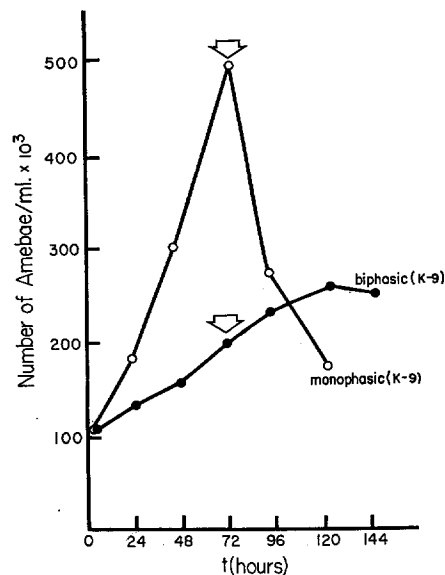

FIGURE 1 Comparative growth rates between axenic monophasic and biphasic cultures of *E. histolytica* (Strain K-9). Arrows indicate the point at which samples were taken for the observations described.
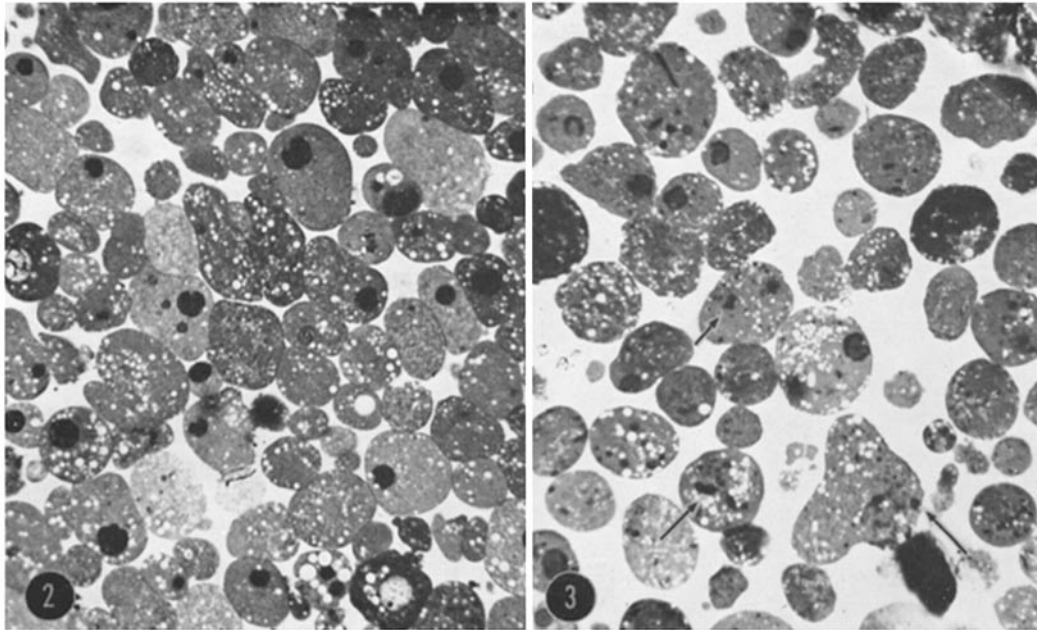
FIGURE 2   A section through a suspension of trophozoites of *E. histolytica* axenically-grown in monophasic medium. The section is stained with 0.1% toluidine blue and photographed with a yellow filter. Note variations in cytoplasmic basophilia and general absence of basophilic chromatoid material in the cytoplasm. × 680.

FIGURE 3   A section through a fixed cell block of trophozoites of *E. histolytica* exposed to 5.4 × 10⁻⁴ M vinblastine for 4 hr. Many cells show one to several basophilic chromatoid bodies in their cytoplasm (arrows). These organisms and those in Fig. 2 were taken from the same flask. × 680.

centration of uridine represented approximately a 1000-fold concentration over the uridine-³H used for the pulse. The cells were subsequently fixed and prepared for electron microscopy. Radioautographs were processed by employing the dipping technique described by Kopriwa (5) and Agfa-Gevaert Nuc 3.07 emulsion which was subsequently developed in D-19B at 18°C.

RESULTS

Trophozoites from Monophasic and Biphasic Media

Growth characteristics of amebae in monophasic and biphasic media were markedly dissimilar (Fig. 1). In biphasic medium the population density reached a maximum by 130 hr and maintained a high level for more than a week before gradually declining (1). In monophasic medium, the lag phase was so extremely short that the precise period was not determined before the time the cells began logarithmic growth. Logarithmic growth was very rapid (Fig. 1), the peak being reached by 72 hr. It declined quickly thereafter.

In monophasic medium, trophozoites usually had one nucleus but occasionally as many as seven nuclei. In general, more multinucleate cells were evident in monophasic than in biphasic cultures. In amebae grown in monophasic medium the large RNP crystals or chromatoid bodies were virtually nonexistent. Small helices were present, however, in the cytoplasm of all of the trophozoites grown in monophasic cultures. In contrast, amebae grown in biphasic medium showed numbers of large RNP bodies constituting the classical chromatoid bodies (1).

VINBLASTINE TREATMENT: In cells grown in monophasic medium, chromatoid bodies appeared after treatment with vinblastine (5.4 × 10⁻⁴ M) for up to 6 hr (Figs. 2 and 3). In some cells chromatoid material appeared within 30
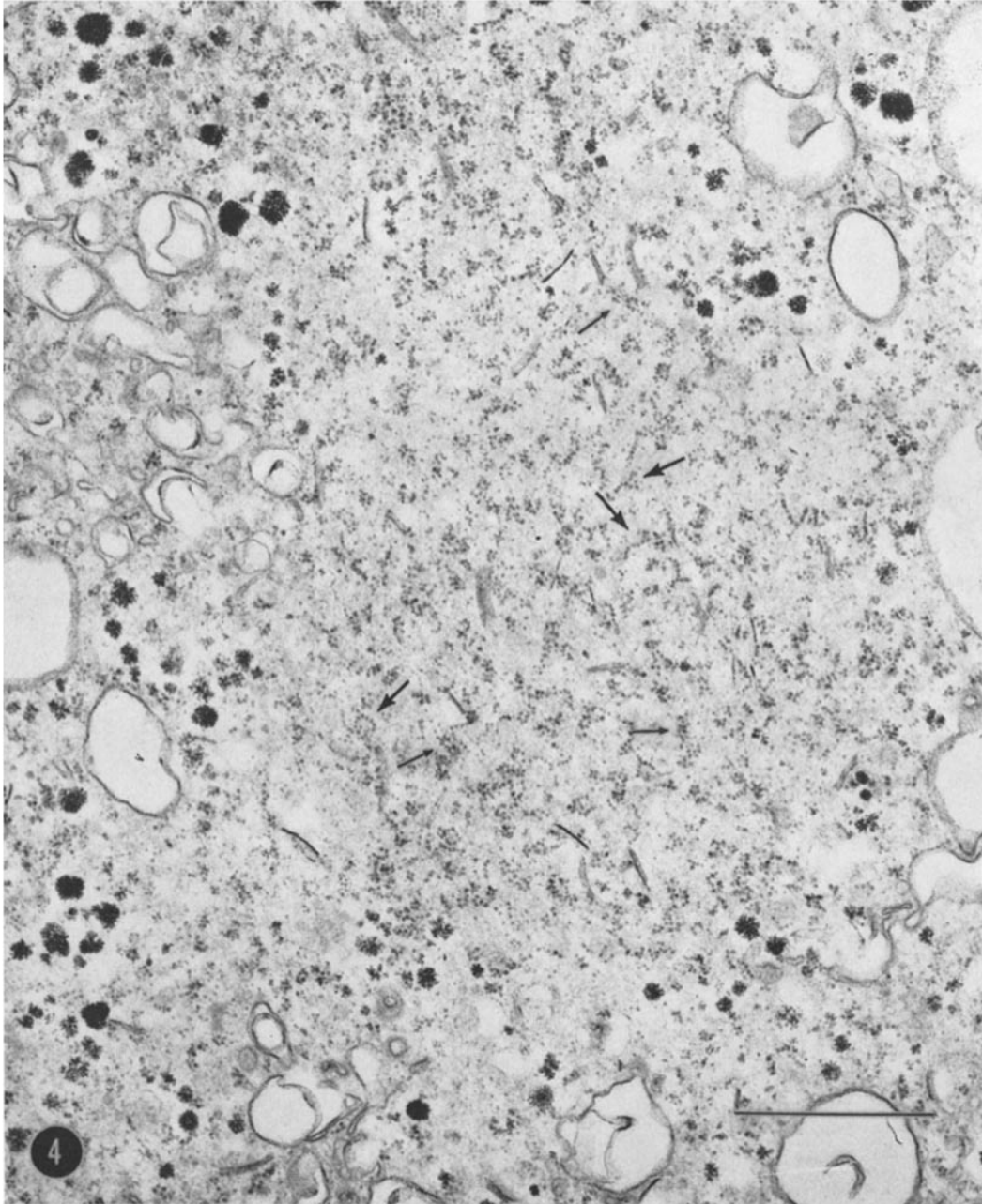
FIGURE 4 A ribonucleoprotein-filament organizing center in the cytoplasm of an axenically-grown tropho-zoite of *E. histolytica* exposed to vinblastine (5.4×10^{-4} M) for 3 hr. In addition to small helical RNP fragments (small arrows), numerous free ribosomes are scattered throughout the area. Some polyribo-somal-like aggregates can be seen (large arrows). Scale, 1 μ. $\times$ 28,000.
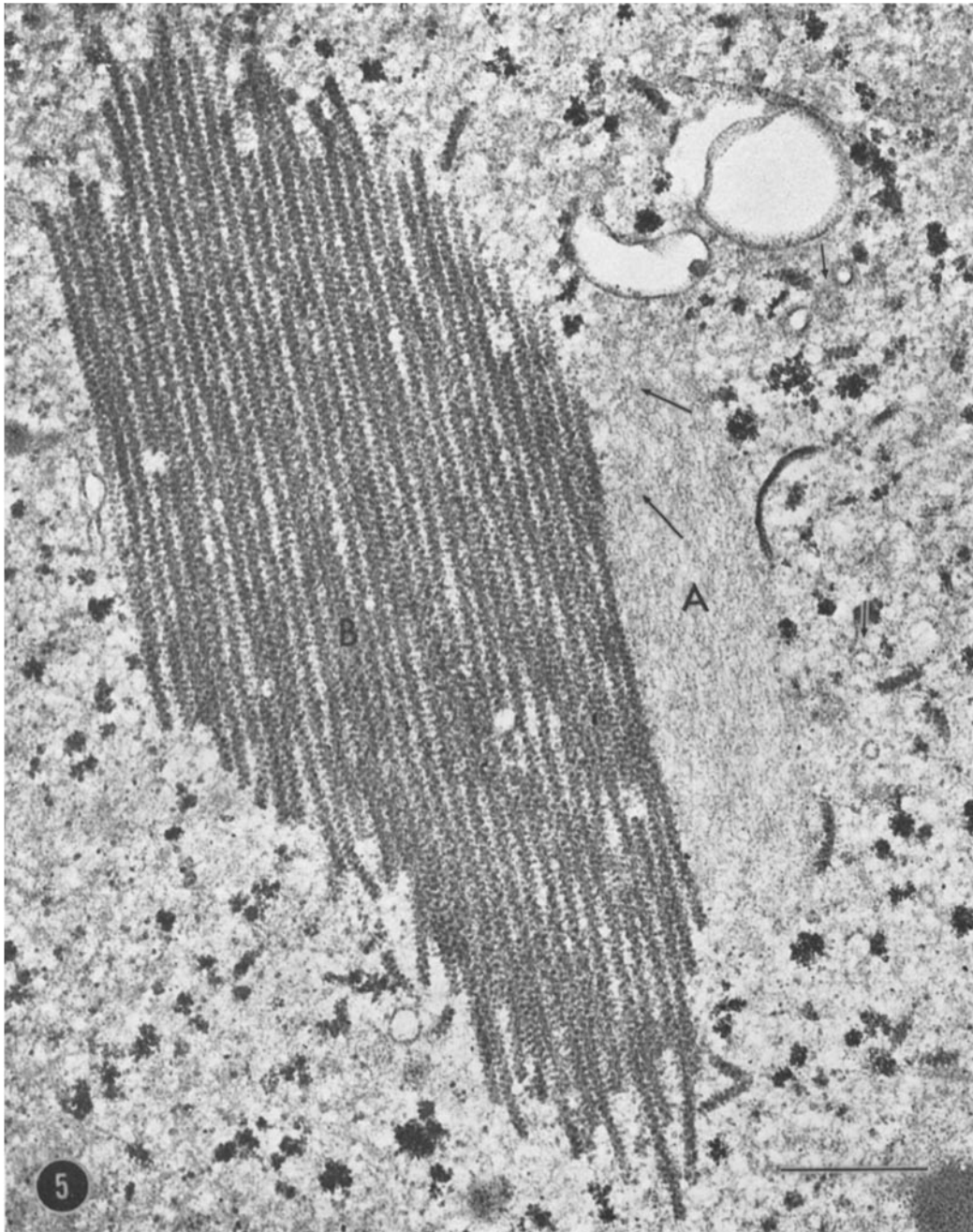
FIGURE 5 Trophozoite treated with vinblastine for 6 hr. In this crystal, which is interpreted as in process of formation, an area of parallel filaments (A) appears continuous with parallel-arranged RNP helices (B). Free ribosomes (arrows) appear among the filaments and free in the adjacent cytoplasm. Short ribosomal chains are evident in the cytoplasm. Scale, 0.5 μ. × 43,800.
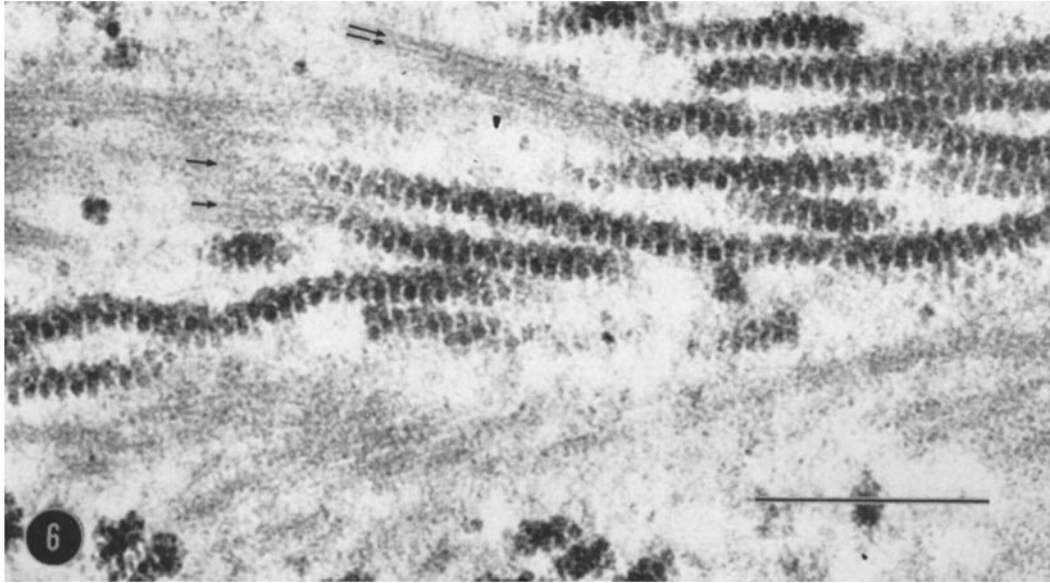
FIGURE 6 Parallel filaments (double arrows) can be seen extending from the center of ordered RNP arrays. In the case shown, several pairs of filaments can be seen which clearly belong to a helix whose body dips away from the plane of section beneath an overlying RNP helix. Filamentous material (single arrows) is evident. Free ribosomes may be seen scattered about the filaments. Scale, 1.0 μ. × 31,000.

min after initial vinblastine exposure. Generally, these bodies were fewer in number and smaller than the chromatoid bodies which characterize cysts, but in many cases they were larger than those found in normal trophozoites grown in biphasic medium.

Ultrastructure

FREE RIBOSOME AND FILAMENTOUS AREAS: In both untreated and vinblastine-treated trophozoites many glycogen-free areas were seen in the cytoplasm In our present material, the pattern evident in many but not all glycogen-free regions during early vinblastine treatment (30 min to 3 hr) showed these regions to be composed of an amorphous, electron-opaque material interspersed with numbers of free ribosomes (Fig. 4). Further examination showed the presence of linear arrays of ribosomes in a form suggestive of polysomes, but the arrays were not in the configuration characteristic of the larger helical bodies.

With longer periods of vinblastine exposure (3 to 6 hr), many more glycogen-free regions composed of packed filaments and free ribosomes became evident. While some areas contained

densely packed filaments, others were also frequently associated with the stacked RNP helices such that both areas seemed intimately associated with each other (see, for example, Fig. 5, areas A and B). Scattered among the filaments were free, single ribosomes or chains of ribosomes (Fig. 5). Depending on the plane of section, other filamentous configurations branched out from either side of a series of parallel filamentous stalks so as to produce a bouquet or fountain-like appearance (Figs. 7, 8). All of these features could be demonstrated in cells not exposed to vinblastine, but not with the frequency evident in cells exposed to the alkaloid.

ASSOCIATION BETWEEN FILAMENTS AND RNP PARTICLES: Regions consisting of RNP helices in the packed, parallel configuration in association with parallel filaments were especially evident in vinblastine-treated trophozoites by the third hour of exposure. Higher magnification of RNP arrays showed details of their association with filaments (Fig. 6), this association most frequently taking the form of a group of parallel filaments, each about 60 A across, which emerge from the ordered RNP arrays (Fig. 6, arrows) as if to constitute a framework upon which the helical
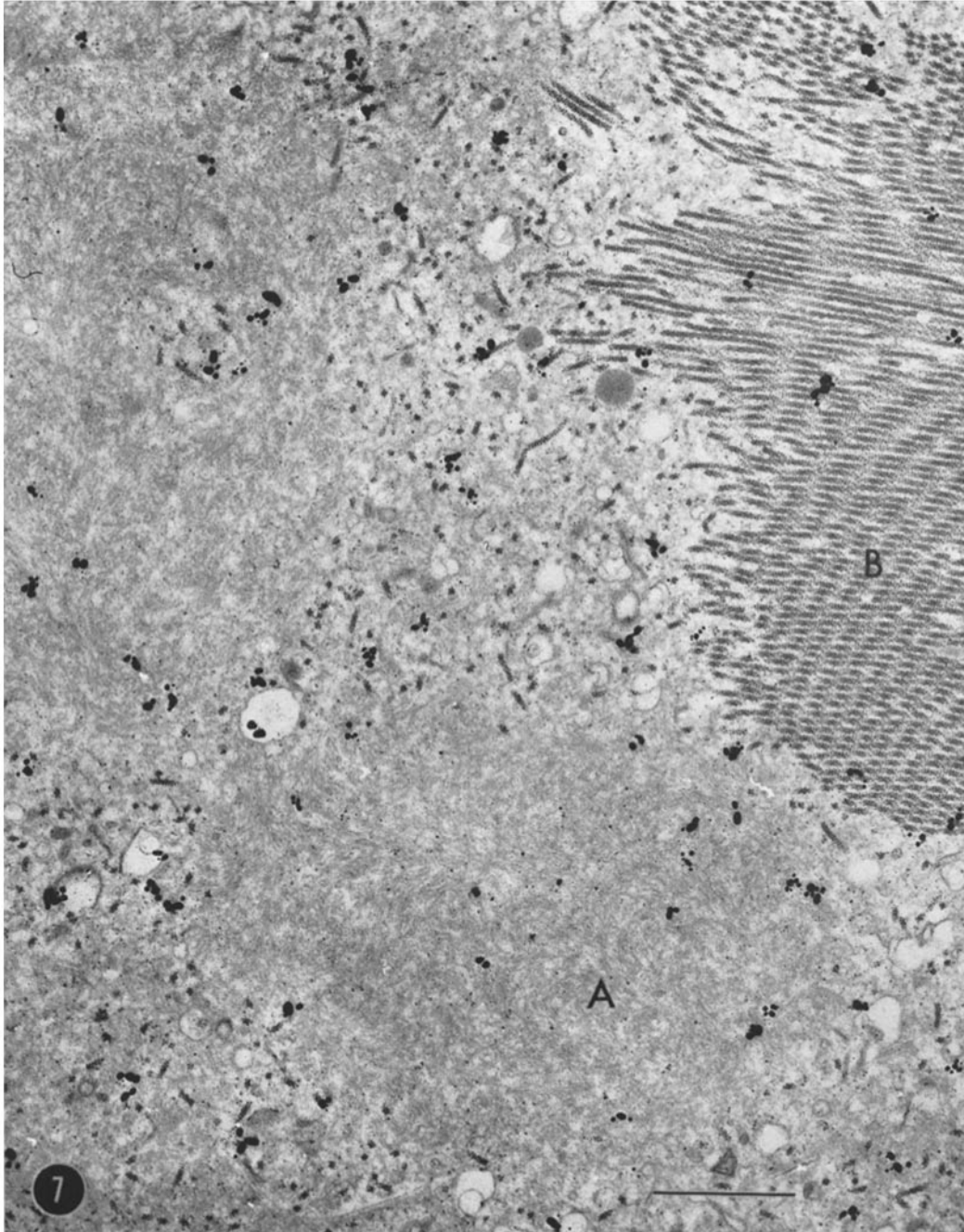
FIGURE 7 Radioautograph of an axenically-grown trophozoite of *E. histolytica* exposed to uridine-^{3}H continuously for 3 hr. In two areas composed of filaments (the lower one of which is marked *A*), radioautographic reaction can be visualized. In addition, a reaction is present over the RNP helices (*B*). Scale, 1.0 μ. $\times$ 20,400.
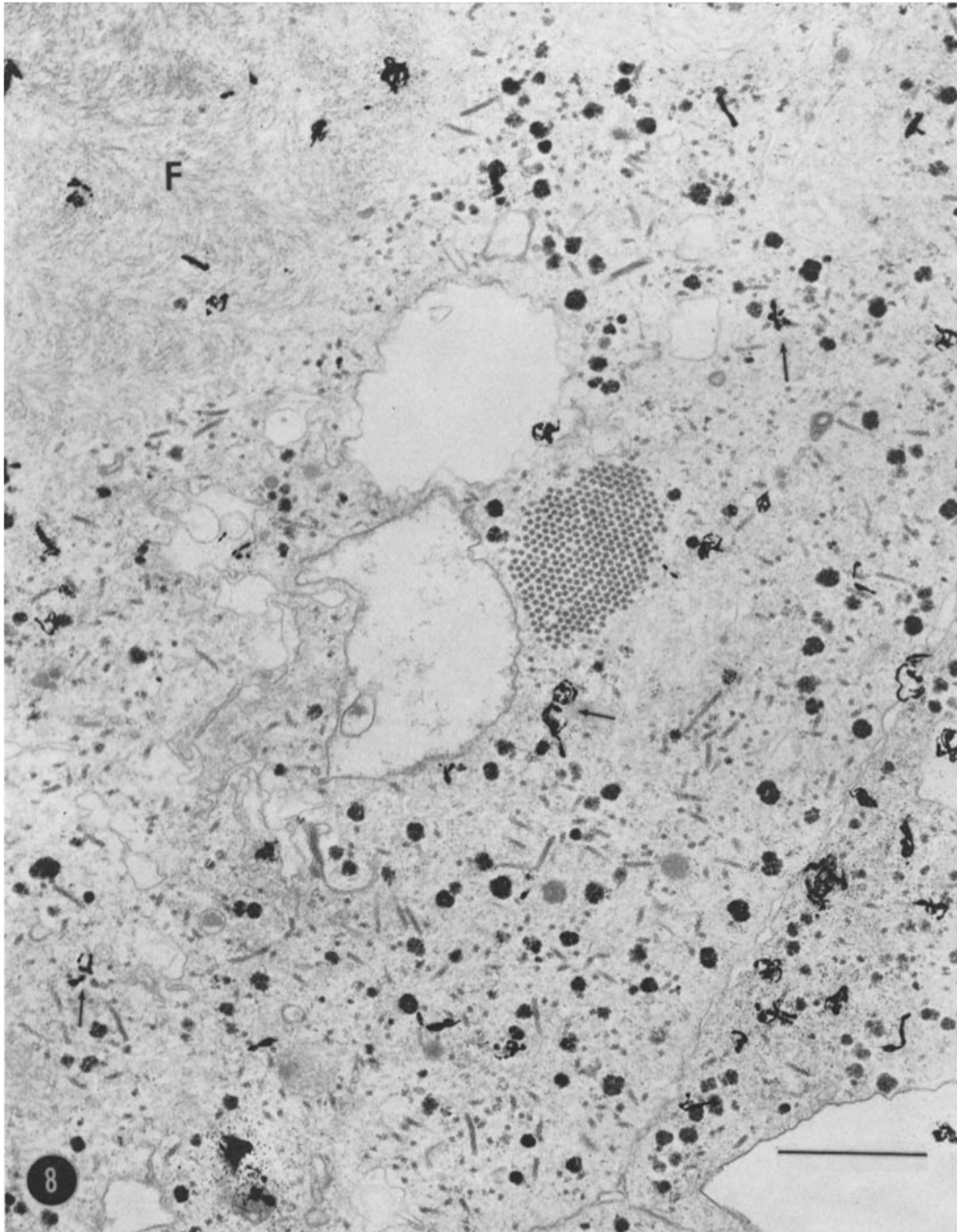
FIGURE 8   Radioautograph of an axenically-grown *E. histolytica* trophozoite exposed to uridine-^{3}H for 30 min. Label is present in regions containing filaments (*F*). A cross section of packed RNP crystals is shown unlabeled. Several sites of so-called "free" cytoplasmic label (arrows) are present. Scale, 1.0 μ. $\times$ 21,600.

Localization	Total no. sites counted among 25 cells	Grain count			
		30 min Continuous label	60 min Continuous label	30 min Label + 30 min chase	30 min Label + 60 min chase
Filamentous regions	125	472 (63%)	379 (49%)	346 (55%)	91 (19%)
RNP bodies (unpacked and packed arrays)	97	0	85 (11%)	87 (14%)	323 (69%)
"Free" cytoplasmic label	*	282 (37%)	307 (40%)	193 (31%)	56 (12%)
Total grains counted		754 (100%)	771 (100%)	626 (100%)	470 (100%)

* Represents remaining cytoplasmic label not associated with filaments or RNP material but possibly representing label of randomly dispersed, free ribosomal material. The values given represent all that label in each of the 25 cells not confined to filamentous regions or the RNP bodies.

RNP arrays are situated. The degree to which such a relationship could be visualized depended on the tilt of the individual helix with respect to the plane of section. Fig. 6 illustrates such apparent flexibility of these helical RNP arrays, especially when the more random, loosely packed configuration is encountered.

RADIOAUTOGRAPHY: Label was confined to three clearly definable areas in the cytoplasm: (a) the RNP helices in their several forms (short, unpacked helices and packed crystalline helical arrays), (b) areas of cytoplasm containing "free" or unbound ribosomal material either with or (c) without the presence of filaments. For localization of label within these sites, a series of sections prepared for radioautography was used. The total number of grains in 25 sectioned trophozoites was counted, and the number of grains clearly associated with each of the above three regions in the same section was determined. Such counts were used to obtain an indication of the amount of label identifiable with these regions under four different conditions (Table I) of labeling. 3 hr of continuous exposure to uridine-^{3}H resulted in the presence of label in filamentous regions as well as in areas occupied by both packed and single helical arrays (Fig. 7). With only a 30-min pulse of uridine-^{3}H, label was present over filamentous regions and over cytoplasm containing free ribosomes; no activity could be detected over packed helical ribonucleoprotein (Fig. 8). Experiments employing a 30-min pulse of uridine-^{3}H followed

by a 60-min uridine "chase" showed label localized primarily in RNP arrays, with little activity being present in filamentous areas or elsewhere in the cytoplasm (Fig. 9). Counts of labeled cytoplasmic sites under four conditions of exposure to uridine-^{3}H are presented in Table I. With 30-min continuous incubation, 63% of the total grain count in sections of 25 individual cells appeared localized in filamentous regions. No label was visualized in RNP bodies in these sections. Uridine-^{3}H appeared in helical RNP bodies after a 60-min pulse or a 30-min pulse followed by a 30-min cold uridine chase, although activity was still high in the filamentous regions. With a 30-min pulse followed by a 60-min cold uridine chase, 69% of the total grains were concentrated in the short helices and compact helical arrays constituting the chromatoid bodies (Table I).

DISCUSSION

The observations presented here associate cytoplasmic filamentous material with the formation of ordered helical ribosomal arrays constituting the chromatoid bodies in trophozoites of *E. histolytica*. Earlier observations (1) on RNase digestion of trophozoites embedded in water-soluble methacrylate suggested the presence of such filaments, the existence of which could be consistent with the postulated "core" material in equivalent helical crystals of *E. invadens* (6) as well as HeLa cells (7). In addition, the present study identifies cytoplasmic filamentous areas
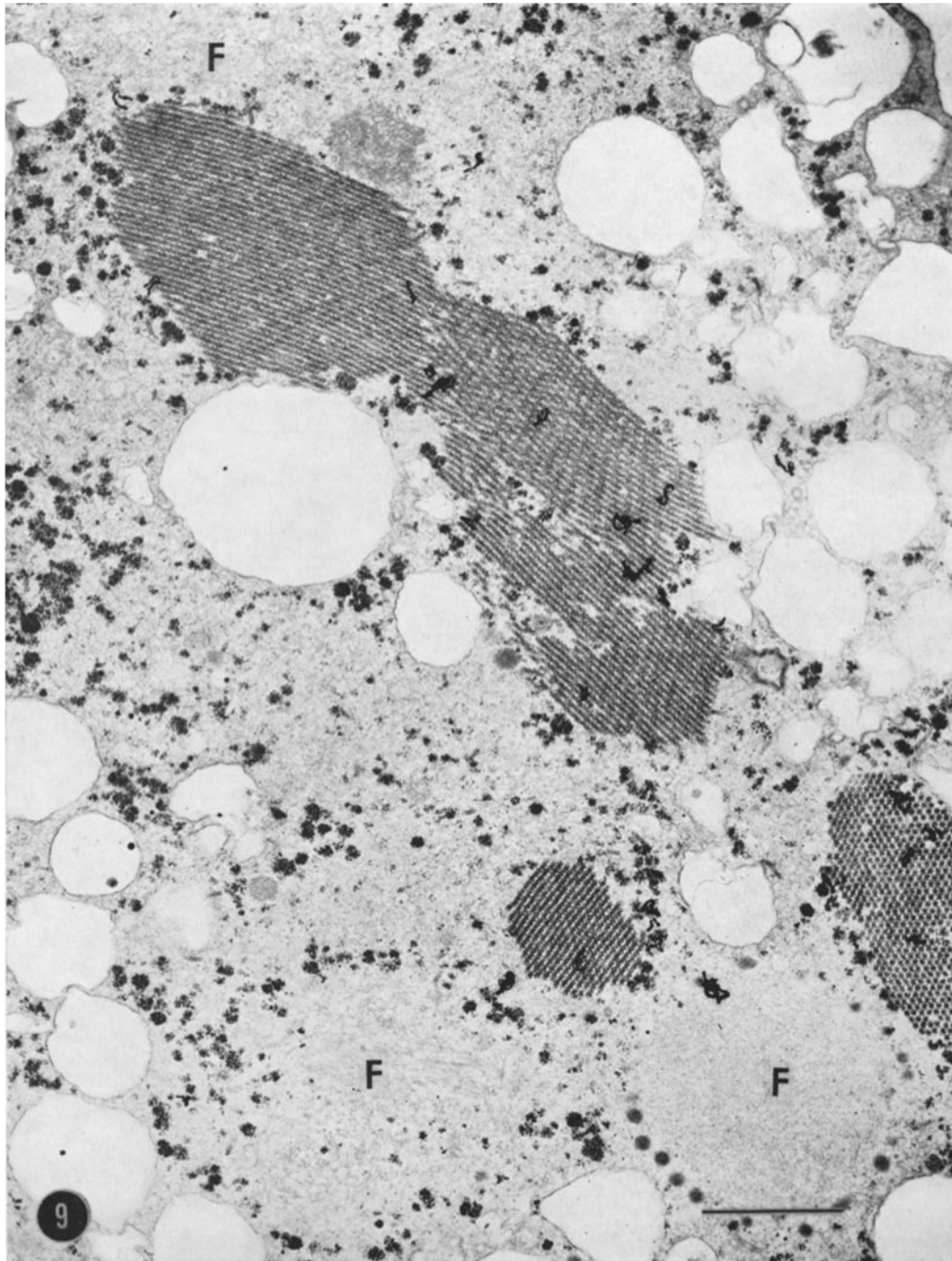
FIGURE 9   Radioautograph of an axenically-grown *E. histolytica* trophozoite exposed to a uridine-^{3}H pulse for 30 min followed by an unlabeled uridine chase for 60 min. Several filamentous regions (*F*) show absence of label whereas label is concentrated in packed helical configurations. Scale, 1.0 μ. $\times$ 20,400.

with helical RNP assembly by the use of radio-autography to trace the processing of a ribo-nucleoprotein precursor such as uridine. On the basis of these observations, we regard the filamentous regions as representing sites of ribonucleo-protein-filament assembly, the ultimate product being the helical RNP body. Evidence pointing to their role in the formation of helical RNP bodies comes from the fact that the initial (30-min) appearance of uridine-³H associated with free ribosomes is localized in the filamentous regions and the fact that with such short pulses the fully formed, packed RNP helical arrays show no label. Longer, continuous exposure to labeled precursors leads to the appearance of label in RNP components of helical material, especially packed helical arrays. After 30 min of pulse labeling with uridine-³H followed by a 60-min cold uridine chase, label was found predominantly in helical RNP bodies. These observations suggest that an average time of transfer from cytoplasmic filamentous regions to RNP helices is 60 min.

Our initial observations on *E. histolytica* with the light microscope showed that axenic trophozoites grown in monophasic medium had few, if any, chromatoid bodies; and with the electron microscope, correspondingly few packed helical RNP bodies were demonstrable. Trophozoites grown in biphasic medium, on the other hand, often possessed numbers of packed RNP crystals. The relative lack of large, packed RNP arrays associated with rapid growth of cells in monophasic medium suggests that there may be an inverse relationship between the growth rate of these cultures and aggregates of ordered RNP arrays. This is reminiscent of older concepts of the nature of chromatoid bodies (1) whereby the appearance of large chromatoid bars in "resting" cysts had been regarded as due to accumulation of "storage material".

The means by which incubation of monophasic medium-grown amebae with vinblastine resulted in the formation of large numbers of crystalline ribosomal arrays are not clear, although it is tempting to consider a direct effect of vinblastine on the formation of core filaments for the RNP helices. This effect would be similar to the action of vinblastine on microtubular protein, which also can take a helical configuration (8, 9, 12, 13, 14). It is of interest to note that vinblastine also produces an association of ribosomes and stalks of

filamentous protein in L-cells (10) and in *E. coli* (11).

In addition to being present in members of the genus *Entamoeba*, helical ribosomal arrays have now been described from a variety of differentiating vertebrate cell types also possessing more conventionally structured ribonucleoprotein (16, 17). These include plant cells (19), bacteria (11), cultured cells (7, 10), and adult mammalian cells (20). In some cases such helical RNP configurations are considered to represent polyribosomes (7, 10, 15), but the means whereby such helices are formed and maintained remain obscure. Thus far, members of the genus *Entamoeba* appear to be the only cell types which normally and persistently maintain RNP helices.

REFERENCES

1. ROSENBAUM, R. M., and M. WITTNER. 1970. Ultrastructure of bacterized and axenic trophozoites of *Entamoeba histolytica* with particular reference to helical bodies. *J. Cell Biol.* 45:367.
2. WITTNER, M. 1968. Growth characteristics of axenic strains of *Entamoeba histolytica*, Schaudinn 1903. *J. Protozool.* 15:403.
3. DIAMOND, L. 1968. Techniques of axenic cultivation of *Entamoeba histolytica* Schaudinn, 1903 and *E. histolytica*-like amebae. *J. Parasitol.* 54:1047.
4. EVANS, V. J., J. C. BRYANT, N. C. FIORAMONTI, W. McQUILKIN, K. K. SANFORD, and W. R. EARLE. 1956. Studies of nutrient media for tissue culture cells *in vitro*. I. A protein-free chemically defined medium for cultivation of Strain L cells. *Cancer Res.* 16:77.
5. KOPRIWA, B. M. 1967. A semiautomatic instrument for a radioautographic coating technique. *J. Histochem. Cytochem.* 14:923.
6. MORGAN, R. S., and B. G. UZMAN. 1966. Nature of the packing of ribosomes within chromatoid bodies. *Science (Washington).* 152:214.
7. WEISS, P., and N. B. GROVER. 1968. Helical array of polyribosomes. *Proc. Nat. Acad. Sci. U.S.A.* 59:763.
8. BENSCH, K. G., R. MARANTZ, H. WISNIEWSKI, and M. L. SHELANSKI. 1969. *Vinca* alkaloid

induced formation of ordered structures from microtubule protein and from brain extracts. *Science (Washington)*. **165**:495.

9. BENSCH, K. G., and S. E. MALAWISTA. 1969. Microtubular crystals in mammalian cells. *J. Cell Biol.* **40**:95.

10. KRISHAN, A., and D. HSU. 1969. Observations on the association of helical polyribosomes and filaments with vincristine-induced crystals in Earle's L-cell fibroblasts. *J. Cell Biol.* **43**:553.

11. KINGSBURY, E. W., and H. VOELZ. 1969. Induction of helical arrays of ribosomes by Vinblastine sulfate in *Escherichia coli*. *Science (Washington)*. **166**:768.

12. MARANTZ, R., M. VENTILLA, and M. SHELANSKI. 1969. Vinblastine induced precipitation of microtubule protein. *Science (Washington)*. **165**:498.

13. MARANTZ, R., and M. L. SHELANSKI. 1970. Structure of microtubular crystals induced by vinblastine *in vitro*. *J. Cell Biol.* **44**:234.

14. WISNIEWSKI, H., M. L. SHELANSKI, and R. D. TERRY. 1968. Effects of mitotic spindle inhibitors on neurotubules and neurofilaments in anterior horn cells. *J. Cell Biol.* **38**:224.

15. DJACZENKO, W., A. BENEDETTO, and R. FEZZI. 1970. Formation of helical polyribosomes in poliovirus-infected cells of the 37 RC line. *J. Cell Biol.* **45**:173.

16. BEHNKE, O. 1963. Helical arrangement of ribosomes in the cytoplasm of differentiating cells of the small intestine of the rat foetus. *Exp. Cell Res.* **30**:597.

17. WADDINGTON, C. H., and M. M. PERRY. 1963. Helical arrangement of ribosomes in differentiating muscle fibers. *Exp. Cell Res.* **30**:599.

18. REYNOLDS, E. S. 1963. The role of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.

19. WOODING, F. B. P. 1968. Ribosome helices in mature cells. *J. Ultrastruct. Res.* **24**:157.

20. MONNERON, A. 1969. Experimental induction of helical polysomes in adult rat liver. *Lab. Invest.* **20**:178.