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SYNOPSIS. Short pulse-labeling of log phase *Crithidia fasciculata* cells with [^{3}H]thymidine allowed the autoradiographic visualization of 2 sites of replication of kinetoplast DNA situated at the periphery of the networks and separated by 180°. Longer pulse-labeling led to the previously reported total peripheral labeling pattern. Pulse-labeled networks possess an intermediate density in ethidium bromide-CsCl equilibrium gradients between the densities characteristic of closed networks and open or linear DNA. Removal of ethidium bromide by several methods and treatment of intermediate band networks with RNase and pronase had no effect on the equilibrium rebanding pattern. Closed minicircles of *Leishmania tarentolae* are not labeled by a short pulse of intact cells with [^{3}H]thymidine. A chase of ~ 3-4 hr is required for the appearance of radioactivity in closed minicircles, a time delay which implies the existence of intermediate events between replication and eventual covalent closure of the minicircles.

Index Key Words: *Crithidia fasciculata*; *Leishmania tarentolae*; kDNA networks; minicircles; DNA replication.

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* This investigation was supported in part by Research Grant AI 09102 (to L. S.) from NIAID, U.S. Public Health Service; by Univ. of California Research Grant No. 2456 (to L. S.); and by a USPHS Biomedical Research Support Grant to the Univ. of California.

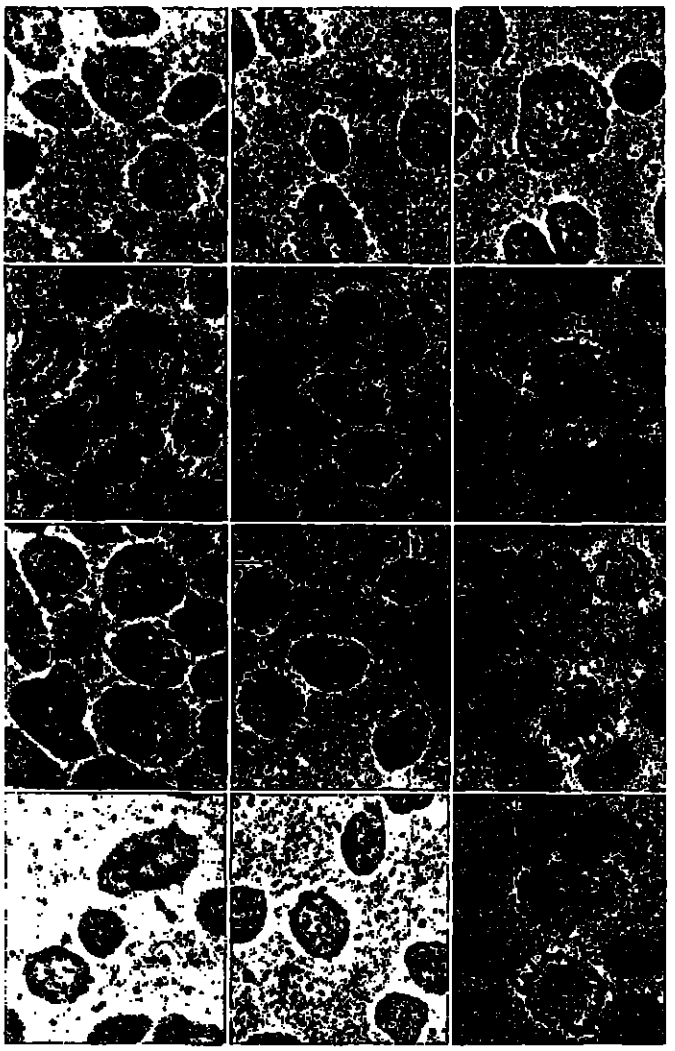

Fig. 1. Light microscope autoradiographs of kDNA networks isolated from *C. fasciculata* log-phase cells that had been pulse-labeled with [^{3}H]thymidine (51.5 C/mM, 33 μc/ml) for 1 min or 3 min at 27 C.

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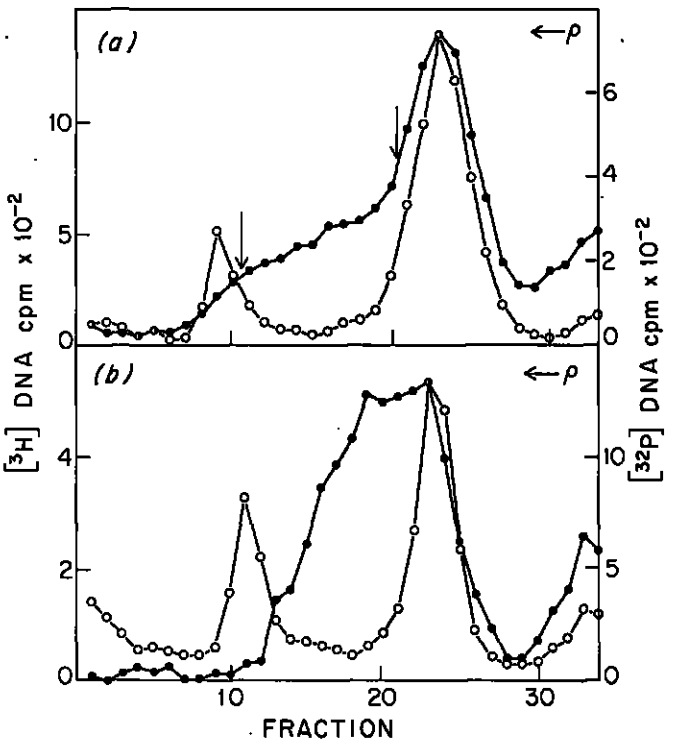

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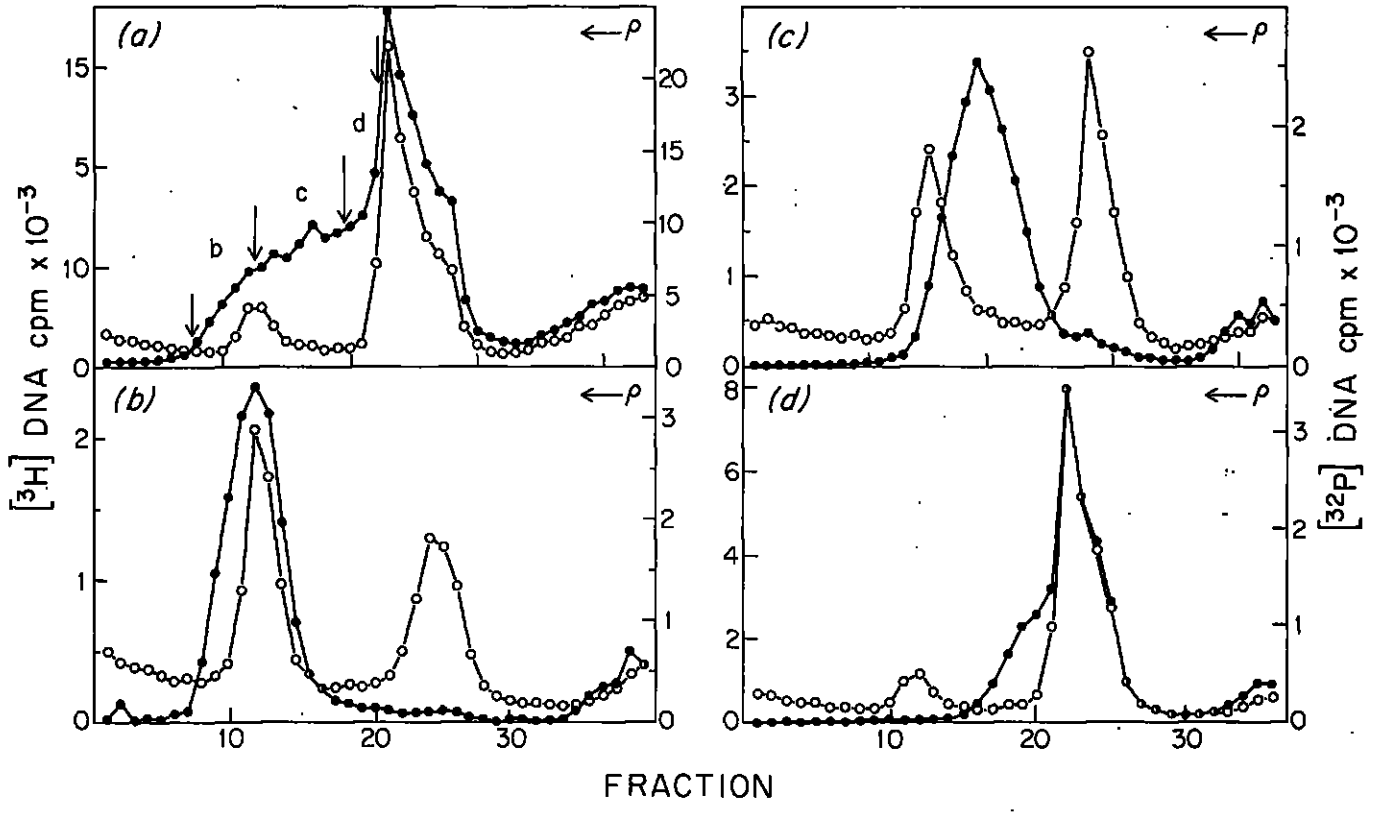

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In the experiment shown in Fig. 3, three separate regions were recovered from a preparative gradient, and the DNAs were then rebanded in ethidium bromide-CsCl. The equilibrium positions remained unchanged. The lower and intermediate bands (Fig. 3b, c) were recovered, divided into 2 parts, one of which was treated with RNase A plus T_1 and then with pronase. Both DNA preparations were then subjected to band sedimentation in neutral sucrose gradients. The DNA sedimented as a single homogeneous peak in each case. There was no effect of the RNase and pronase treatments on the shape of the peak or on the $S_{20,w}$ value, and no radioactive DNA was released from the networks. These results imply that neither RNA nor protein

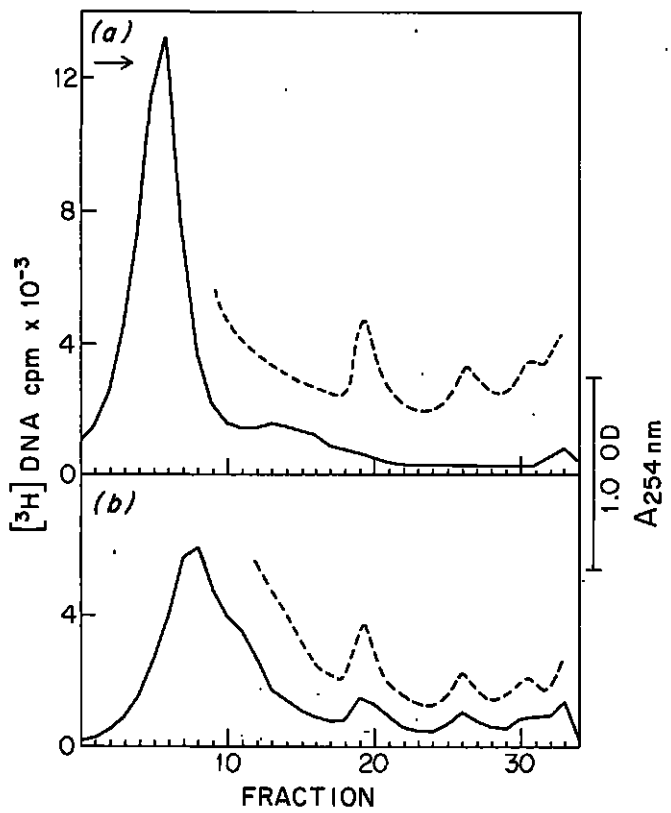

Fig. 4. Band sedimentation profiles in alkaline sucrose gradients of sonicated *L. tarentolae* kDNA isolated from cells pulse-labeled with [^{3}H]thymidine and chased in unlabeled medium. (a) 130 ml of log-phase cells (98 × 10^6 cells/ml) were pulse-labeled for 30 min at 27 C with 38 μc/ml [^{3}H]thymidine in NeoYE-199+ medium, and were then mixed with 370 ml of unlabeled culture. KDNA was isolated, sonicated and centrifuged in alkaline sucrose gradients as described in Methods. The optical density at 254 nm was used to mark the position of the closed monomeric circles and to normalize the graphs. (b) Cells were pulse-labeled for 30 min as in (a) and were then resuspended in unlabeled medium for 4 hr. KDNA was isolated, sonicated and centrifuged as in (a).

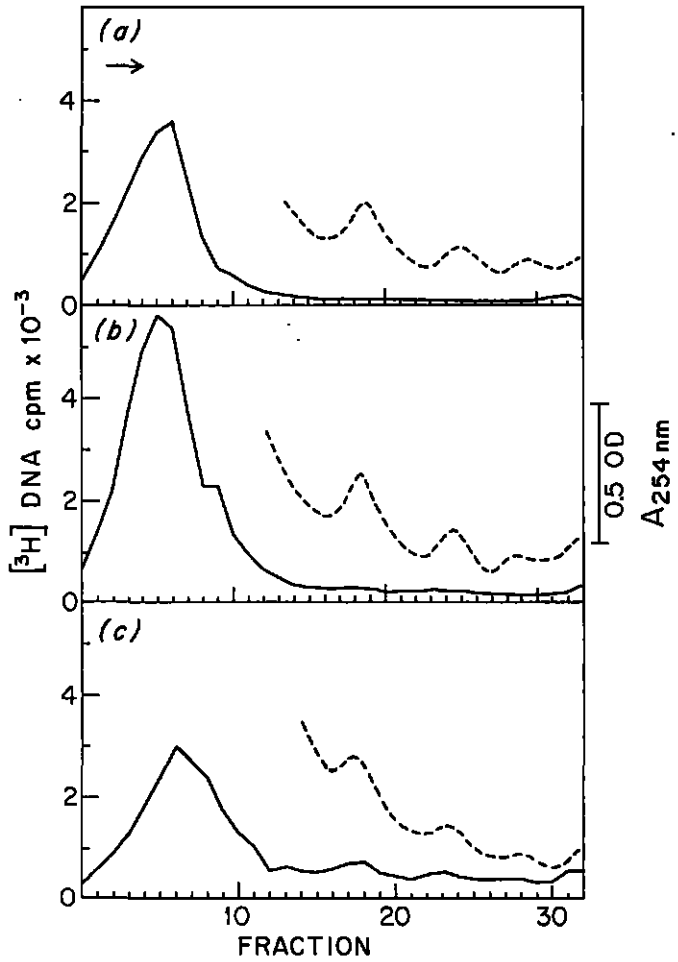

Fig. 5. Band sedimentation profiles in alkaline sucrose gradients of sonicated *L. tarentolae* kDNA isolated from cells pulse-labeled with [^{3}H]thymidine and chased in unlabeled medium. Three hundred ml of log-phase cells (29 × 10^6 cells/ml) were pulse-labeled for 30 min at 27 C with 10 mc [^{3}H]thymidine. The cells were then pelleted and washed by centrifugation and mixed with 1200 ml of unlabeled culture (29 × 10^6 cells/ml). At each time point, 400 ml of culture were harvested for isolation of kDNA. (a) Immediately after the 30 min pulse. (b) After 1 hr of chase. (c) After 3 hr of chase.

play a structural role in the composition of the pulse-labeled intermediate band.

In order to examine the rate of appearance of the [^{3}H]thymidine pulse label in covalently closed minicircles, we performed a pulse-chase experiment with log-phase *L. tarentolae* cells. This species was selected due to the availability of effective methods to isolate closed monomeric minicircles from networks (7, 10). Cells were pulse-labeled with [^{3}H]thymidine for 30 min and were then washed by centrifugation and resuspended in unlabeled medium at 27 C for varying periods. Equal aliquots of the culture were removed onto filter discs and the acid-insoluble radioactivity was measured to confirm the existence of a true chase. The radioactivity/unit vol was constant throughout the chase. Although this method only provides a measurement of total cell incorporation, the kDNA represents such a large portion of the total cell DNA that continued incorporation into kDNA from a sequestered isotope pool would have been clearly apparent.

To measure the specific activity of covalently closed monomeric minicircles, at each chase time an aliquot of cells was removed and kDNA networks were isolated, sonicated, and subjected to band sedimentation in alkaline sucrose gradients. Both the optical density at 254 nm and the acid-insoluble radioactivity were measured. The results of 2 separate experiments are shown in Figs. 4 and 5. It is clear that the specific activity of closed minicircles does not increase significantly until after 3 to 4 hr of chase. This is a striking delay, since Brack et al. (1) calculated that only a few seconds are required for the complete replication of the larger 0.45 μm minicircles from *Trypanosoma cruzi*. This time delay for the appearance of radioactivity in closed minicircles in *L. tarentolae* after an *in vivo* pulse with [^{3}H]thymidine implies the existence of intermediate events between replication and final closure of the circular molecules. It is entirely consistent with our working hypothesis that extensive recombination occurs after replication but, of course, does not prove this hypothesis.

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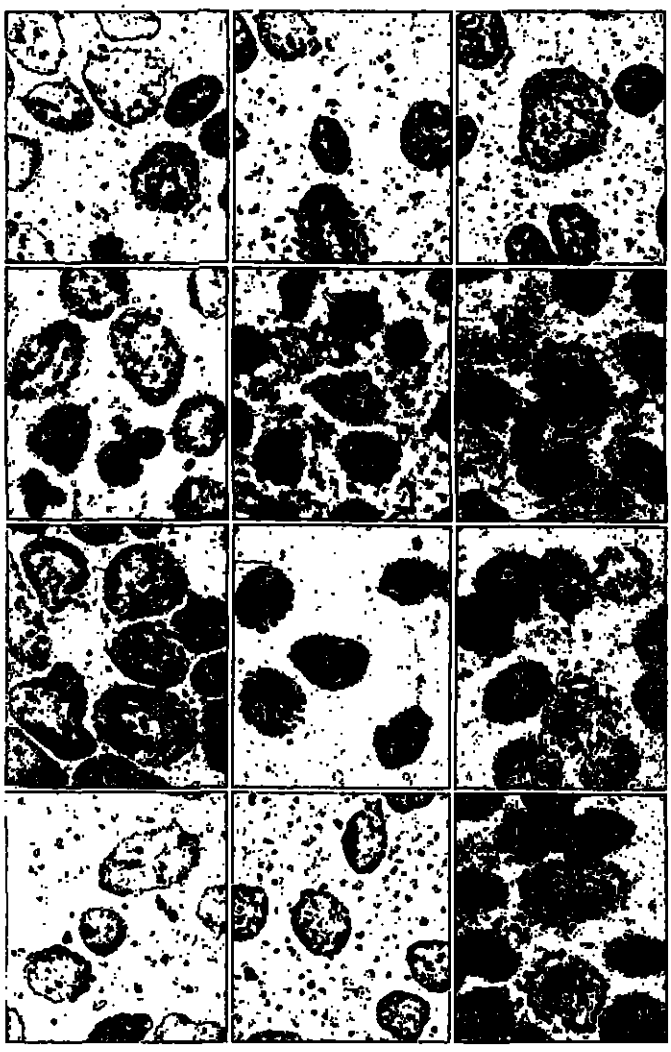

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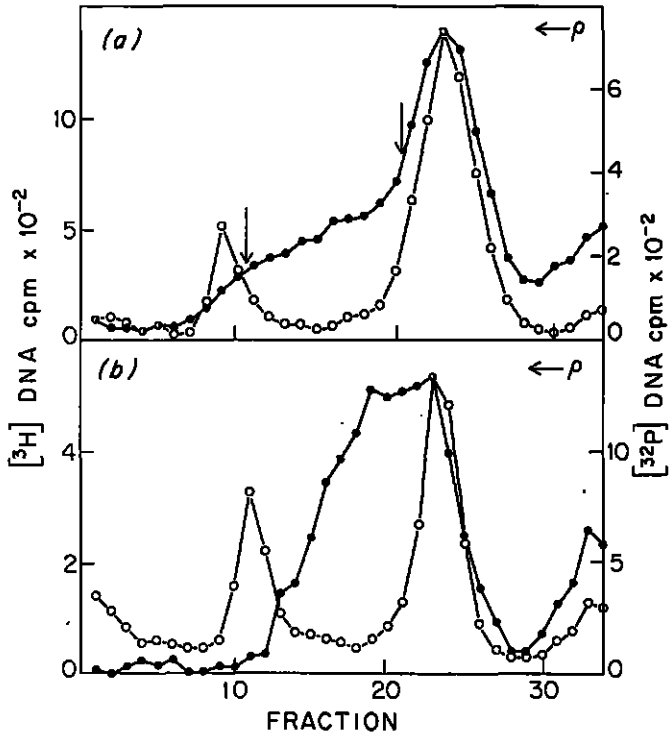

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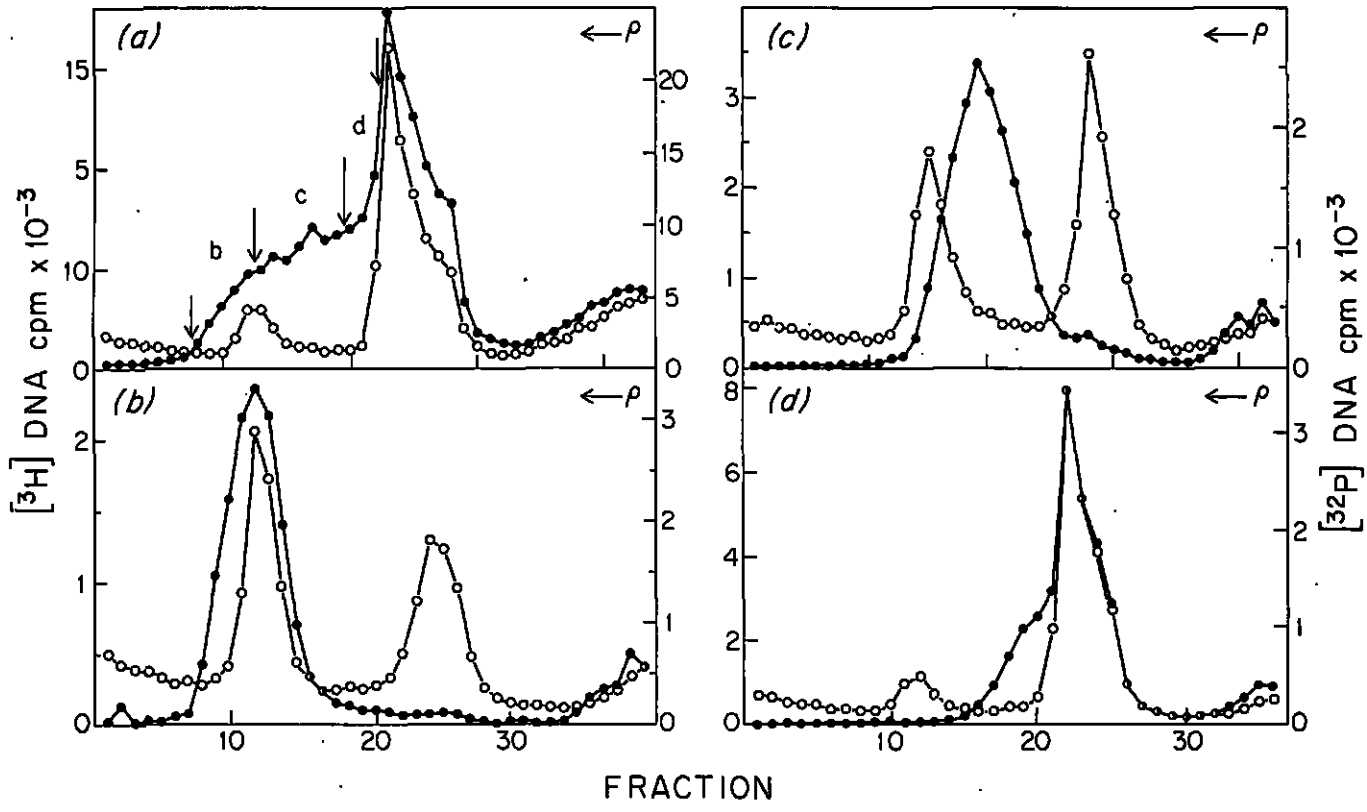

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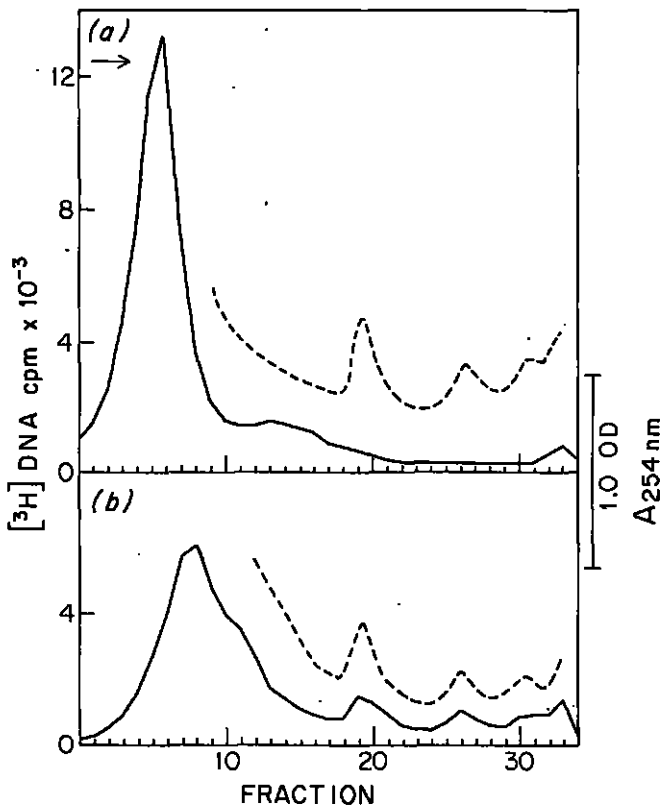

Fig. 4. Band sedimentation profiles in alkaline sucrose gradients of sonicated *L. tarentolae* kDNA isolated from cells pulse-labeled with [^{3}H]thymidine and chased in unlabeled medium. (a) 130 ml of log-phase cells (98 × 10^6 cells/ml) were pulse-labeled for 30 min at 27 C with 38 μc/ml [^{3}H]thymidine in NeoYE-199+ medium, and were then mixed with 370 ml of unlabeled culture. KDNA was isolated, sonicated and centrifuged in alkaline sucrose gradients as described in Methods. The optical density at 254 nm was used to mark the position of the closed monomeric circles and to normalize the graphs. (b) Cells were pulse-labeled for 30 min as in (a) and were then resuspended in unlabeled medium for 4 hr. KDNA was isolated, sonicated and centrifuged as in (a).

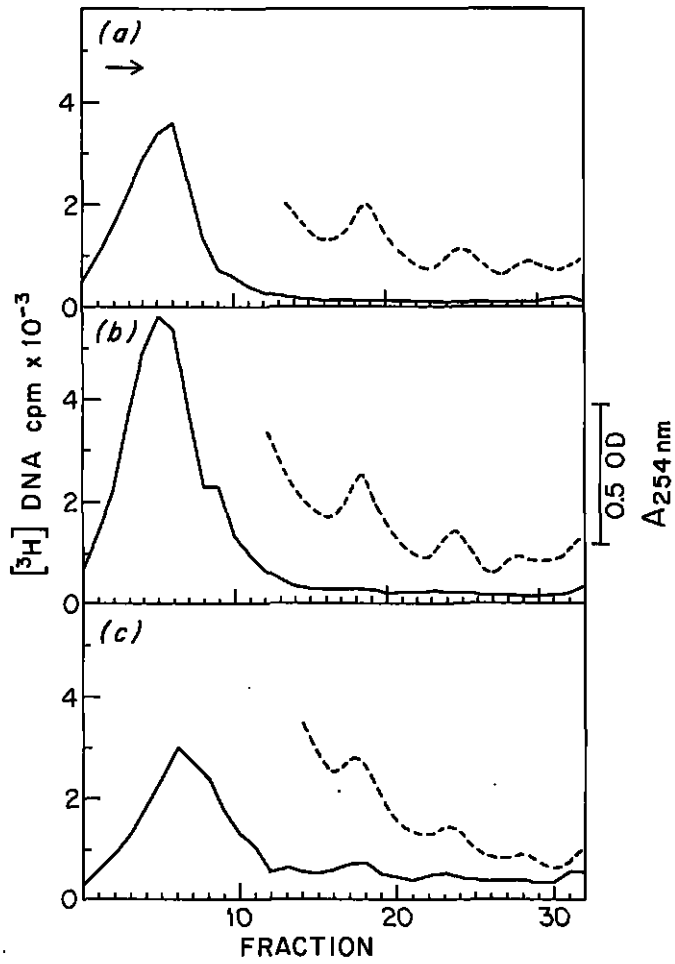

Fig. 5. Band sedimentation profiles in alkaline sucrose gradients of sonicated *L. tarentolae* kDNA isolated from cells pulse-labeled with [^{3}H]thymidine and chased in unlabeled medium. Three hundred ml of log-phase cells (29 × 10^6 cells/ml) were pulse-labeled for 30 min at 27 C with 10 mc [^{3}H]thymidine. The cells were then pelleted and washed by centrifugation and mixed with 1200 ml of unlabeled culture (29 × 10^6 cells/ml). At each time point, 400 ml of culture were harvested for isolation of kDNA. (a) Immediately after the 30 min pulse. (b) After 1 hr of chase. (c) After 3 hr of chase.

play a structural role in the composition of the pulse-labeled intermediate band.

In order to examine the rate of appearance of the [^{3}H]thymidine pulse label in covalently closed minicircles, we performed a pulse-chase experiment with log-phase *L. tarentolae* cells. This species was selected due to the availability of effective methods to isolate closed monomeric minicircles from networks (7, 10). Cells were pulse-labeled with [^{3}H]thymidine for 30 min and were then washed by centrifugation and resuspended in unlabeled medium at 27 C for varying periods. Equal aliquots of the culture were removed onto filter discs and the acid-insoluble radioactivity was measured to confirm the existence of a true chase. The radioactivity/unit vol was constant throughout the chase. Although this method only provides a measurement of total cell incorporation, the kDNA represents such a large portion of the total cell DNA that continued incorporation into kDNA from a sequestered isotope pool would have been clearly apparent.

To measure the specific activity of covalently closed monomeric minicircles, at each chase time an aliquot of cells was removed and kDNA networks were isolated, sonicated, and subjected to band sedimentation in alkaline sucrose gradients. Both the optical density at 254 nm and the acid-insoluble radioactivity were measured. The results of 2 separate experiments are shown in Figs. 4 and 5. It is clear that the specific activity of closed minicircles does not increase significantly until after 3 to 4 hr of chase. This is a striking delay, since Brack et al. (1) calculated that only a few seconds are required for the complete replication of the larger 0.45 μm minicircles from *Trypanosoma cruzi*. This time delay for the appearance of radioactivity in closed minicircles in *L. tarentolae* after an *in vivo* pulse with [^{3}H]thymidine implies the existence of intermediate events between replication and final closure of the circular molecules. It is entirely consistent with our working hypothesis that extensive recombination occurs after replication but, of course, does not prove this hypothesis.

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