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High Resolution Autoradiography of *Escherichia coli* Cells Infected with Bacteriophage R17

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ABSTRACT

GRANBOULAN, NICOLE (Institute de Recherches sur le Cancer, Villejuif, Seine, France), AND RICHARD M. FRANKLIN. High-resolution autoradiography of *Escherichia coli* cells infected with bacteriophage R17. J. Bacteriol. **91**:849–857. 1966.—The ultrastructural alterations in *Escherichia coli* infected with the RNA bacteriophage R17 were further investigated by means of the technique of high-resolution autoradiography. Tritiated precursors to ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein were employed in separate experiments. A striking inhibition of cellular RNA, DNA, and protein synthesis was noted. Whereas normal RNA synthesis occurs in the nucleoid, in infected cells RNA synthesis is predominantly cytoplasmic, but later in the latent period, and during the stage of active viral growth, the label is localized in a polar region. In the late stages of viral growth, RNA synthesis occurs only around the crystals. Protein synthesis also becomes localized in a polar region, but DNA synthesis remains confined to the nucleoid. Under conditions of chloramphenicol inhibition of viral-coat protein synthesis, RNA label is localized in the paranuclear lesion, providing further indication that RNA forms this fibrillar structure.

In the preceding paper (9*a*), the growth of ribonucleic acid (RNA) bacteriophage R17 in *Escherichia coli* Hfr_1 cells was studied by biological, ultrastructural, and ultrastructural cytochemical means. Observations were also made on infected cells in which virus synthesis was blocked by addition of chloramphenicol at 20 min postinfection. This procedure allows synthesis of viral RNA and, under the conditions described, a fibrillar RNA was found in the cytoplasm. Cytochemical studies suggested that this fibrillar area was rich in RNA.

In the present study, high-resolution autoradiography with the use of tritiated precursors of RNA, deoxyribonucleic acid (DNA), and protein was used to localize synthetic sites in the infected cell, to compare patterns of synthesis in infected and uninfected cells, and to further investigate infected cells which were inhibited by chloramphenicol at 20 min postinfection.

Because of the very small size of bacteria and subcellular components of bacteria, it is not possible to make an unambiguous assignment of exposed silver grains to nuclear or cytoplasmic compartments (*see* 2). Striking qualitative changes in labeling patterns can be noted, and quantitative data on overall grain counts can be obtained as a measure of synthetic activities of individual cells.

MATERIALS AND METHODS

A description of the cells, virus, and general biological and cytological techniques is given in the preceding paper (9*a*).

Media. TCG medium, described by Erikson, Fenwick, and Franklin (8), is a slight modification of the TPG medium of Sinsheimer et al. (20). This medium was used for growth of host cells and virus when isotope incorporation was being studied. For studies on the incorporation of H^3-amino acids, the Casamino Acids were omitted and only L-methionine (100 μg/ml) was added.

Sources of materials. Tritiated uridine, thymidine, L-phenylalanine, L-leucine, and L-tyrosine were all preparations with high specific activity from The Radiochemical Center, Amersham, England.

Chloramphenicol was obtained from Parke, Davis & Co., Detroit, Mich.

The Scientia NUC 307 emulsion was obtained from Agfa-Gevaert Co., Paris, France. The 200-mesh grids were from Fullam, Schenectady, N.Y.

Growth of virus. For high-resolution autoradiography, cells were grown in TCG, harvested during logarithmic growth, and infected as described in the

preceding paper, allowing either 5 or 10 min at room temperature for adsorption. Since the virus stock was in MS broth, the cells were then centrifuged and resuspended in TCG at 37 C to initiate virus multiplication.

Radioisotopic labeling of cells. Noninfected cells in the logarithmic growth phase or infected cells (which had been infected during logarithmic growth) were labeled in DNA, RNA, or protein by use of the appropriate H^3-labeled precursors. H^3-uridine of specific activity greater than 20 c/mmole was utilized for labeling RNA. Since this is labeled in the 5 position, this label should be lost during conversion of uridine to thymidine (through deoxyuridine). Therefore, this compound is ideal for specific labeling of RNA.

DNA was labeled with a 4-min pulse of H^3-thymidine.

To label protein, cells grown in TCG or infected in TCG were centrifuged and incubated in TPG containing L-methionine and no other amino acids. A mixture of tritiated-phenylalanine, leucine, and tyrosine was used for a 5-min labeling period.

For labeling with tritiated uridine or thymidine, the cells were kept in TCG, exposed to labeled compound for the appropriate length of time, and then fixed with 10% (v/v) of 1% OsO_4 in the acetate-Veronal buffer of Michaelis (11).

For labeling with tritiated amino acids, the procedure was more complicated, since the bacteria grew very poorly in TPG supplemented only with methionine. The cells were grown in TCG and, in the case of infected cells, infection progressed in TCG. Prior to labeling, a given volume of cells was centrifuged, resuspended in the same volume of prewarmed TPG plus L-methionine, and incubated for 5 min before being exposed to tritiated amino acids. After a given labeling time, the cells were fixed in OsO_4 as described above.

Labeled precursor compounds. The following compounds were used: L-thymidine, one lot at 12.6 c/mmole, used at 25 or 50 μc/ml; L-uridine, one lot at 24.4 c/mmole (used for the 30-sec pulse) and one lot at 22.2 c/mmole (used for the 10-sec pulse), used at concentrations of 25 to 100 μc/ml; L-phenylalanine, one lot at 7.95 c/mmole; L-leucine, one lot at 8.20 c/mmole; L-tyrosine, one lot at 39.6 c/mmole. The last three amino acids were used simultaneously, each at 33.3 μc/ml.

Electron autoradiography. Since the details of the techniques for quantitative autoradiography are available (10*a*), only an outline is presented here.

Bacteria were embedded in HPMA as described in the preceding paper. Sections of equal thickness were put on glass slides covered by a collodion membrane. One section of infected cells and one of uninfected cells were placed on the same glass slide. The emulsion (Scientia NUC 307, Gevaert), with a crystal size of 700 A, was diluted 1:4 and was layered by the dipping procedure. During the drying process, all slides were arranged at the same angle of inclination and left for the same time in a humid atmosphere. Under these conditions, a uniform, continuous, monogranular layer of emulsion was regularly obtained over the sections. The specimens to be compared were developed in the same bath of D19 for 5 min at 18 C. After fixing, rinsing, and drying, the collodion membrane was floated off, and a 200-mesh grid was placed under each section.

Only samples embedded in HPMA were used. The sections were cut either with a type MT2 Porter-Blum microtome or with an LKB ultramicrotome. (Uniformity of section-thickness is still the main problem in quantitative studies.) The results obtained with the two types of ultramicrotomes were both satisfactory. Some unstained sections were treated with H_2O_2 and enzymes before staining and autoradiography. A carbon layer was always deposited over the sections and slides before layering the emulsion (1, 18).

Grain counts were carried out directly on the screen of the electron microscope at a magnification of 10,000 times. The background was never greater than 0.3 grain per 100 μ^2. The mean number of reduced silver grains per labeled bacterium and per total number of bacteria and the percentage of unlabeled bacteria were calculated. No corrections for background were necessary.

RESULTS

Localization of synthetic processes in uninfected bacteria. When bacteria were labeled for 10 sec with H^3-uridine, incorporation into RNA was limited to the nucleoid (Fig. 1a; *see also* Franklin and Granboulan, J. Mol. Biol., *in press*). Cytoplasmic labeling increased with increased pulse time. Over the period of 10 to 150 sec, there was a linear incorporation of label into RNA, as shown by a linear relationship between the number of reduced silver grains per bacterium and the pulse time (Franklin and Granboulan, *in press*). When bacteria were labeled with H^3-thymidine for 4 min, incorporation into DNA was limited to the nucleoid (Fig. 3a) (3). When the bacteria were labeled with three H^3-amino acids simultaneously, only the cytoplasm was labeled (Fig. 2a). Therefore, the localization of synthetic processes obtained with electron autoradiography appeared to be satisfactory.

Localization of synthetic processes in infected bacteria. The site of RNA synthesis shifted from the nucleoid in uninfected bacteria to the cytoplasm in infected bacteria at 30 min postinfection (Fig. 1b). The cytoplasmic label was not localized at this time. At 45 min postinfection the H^3-uridine label was also located in the cytoplasm, but was less dispersed than at 30 min postinfection (Fig. 1c). It generally was located at one pole of the bacterium. This labeling pattern was also seen at later times of infection (60 and 120 min). At 120 min, there was indication of a small amount of incorporation around the paracrystals (Fig. 1d). There was a strong depression of incorporation of labeled amino acids into protein, even at 25 min postinfection (Fig. 2b). The residual incorpora-

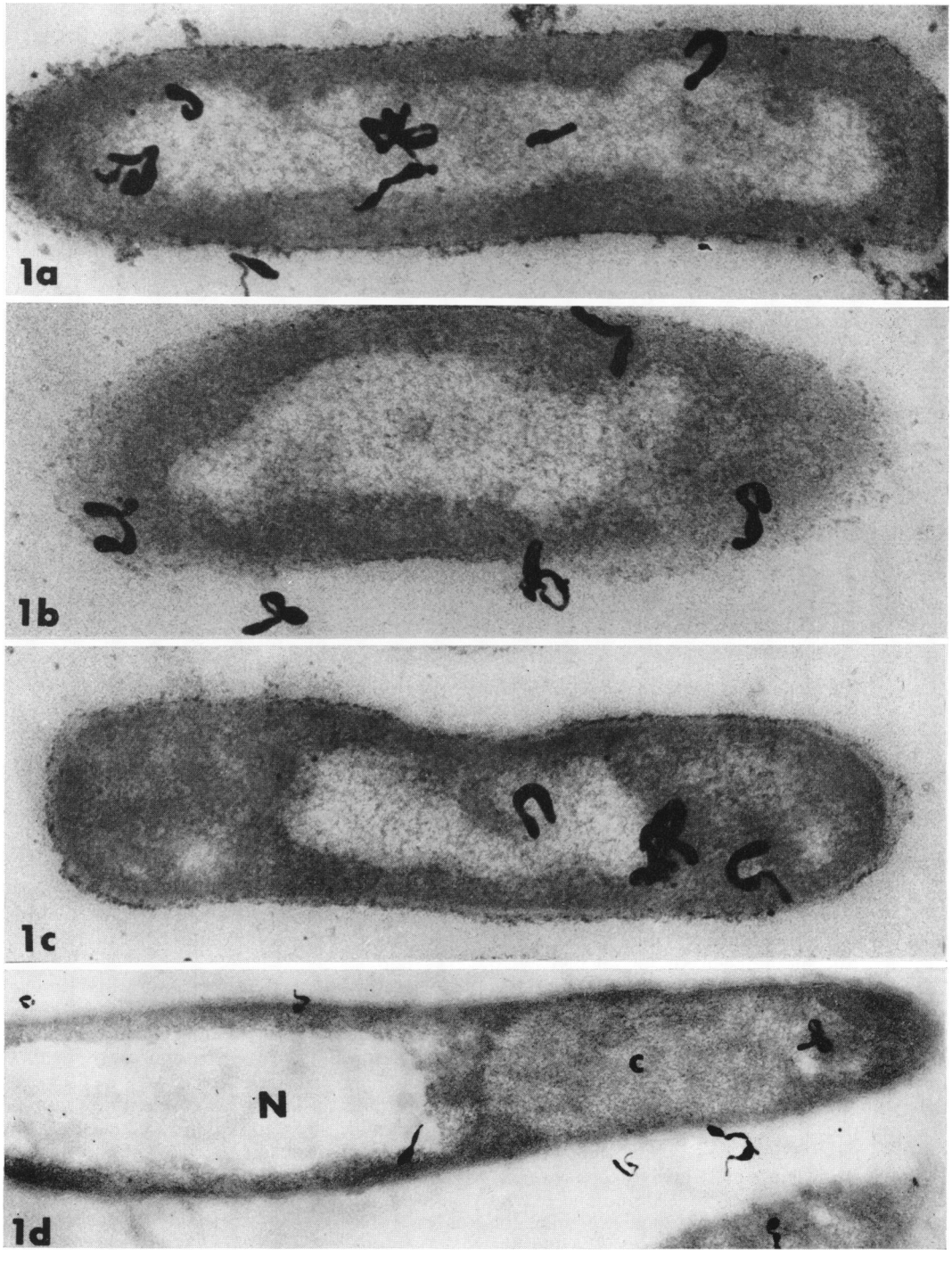

FIG. 1. (*a*, *b*, *c*) *H^3-uridine for 10 sec.* (*a*) *Uninfected bacterium, nuclear incorporation.* × *58,700.* (*b*) *Infected by R17 for 30 min, cytoplasmic incorporation without preferential localization.* × *58,700.* (*c*) *Infected by R17 for 45 min, cytoplasmic incorporation with preferential polar localization.* × *58,700.* (*d*) *Infected by R17 for 120 min, H^3-uridine for 30 sec, incorporation around paracrystalline area* (*c*) *containing viral particles* (*N* = *nucleus*). × *36,700.*

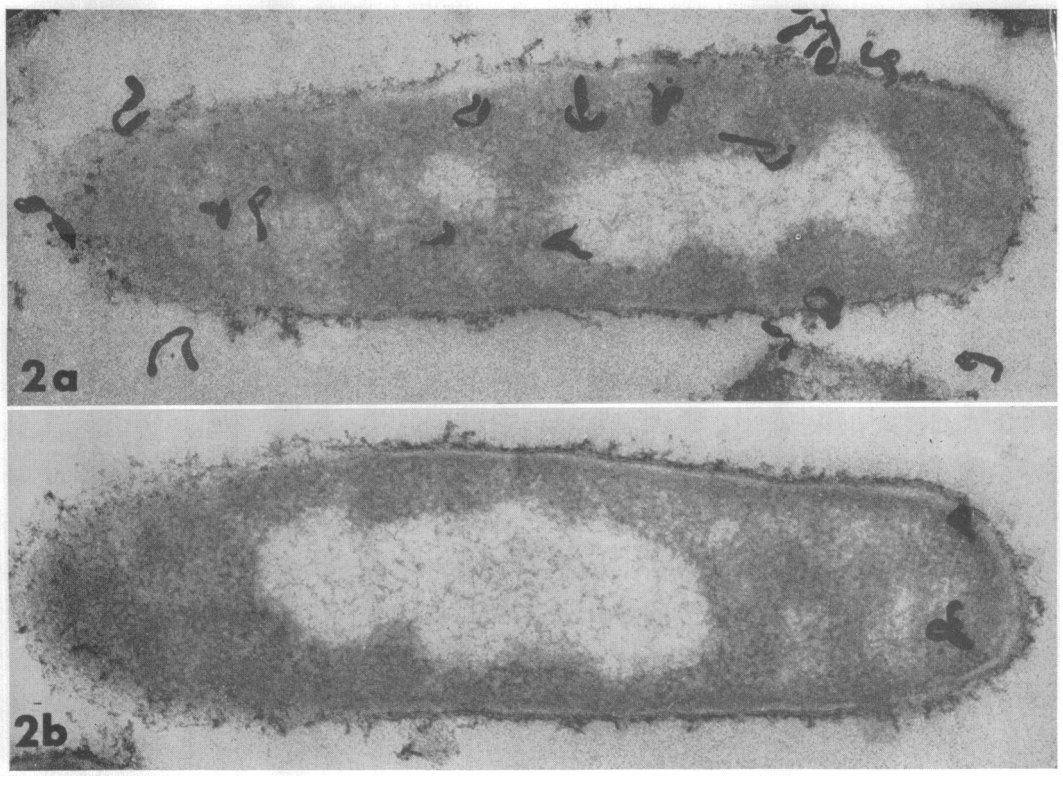

FIG. 2. *H^3-L-leucine, H^3-L-phenylalanine, H^3-L-tyrosine, given simultaneously for 5 min. (a) Uninfected bacterium, heavy cytoplasmic incorporation. × 40,000. (b) Infected by R17 for 25 min, incorporation strongly depressed. × 40,000.*

tion was localized in a polar region. There was also inhibition of incorporation of H^3-thymidine into DNA, almost complete by 45 min postinfection (Fig. 3b).

Localization of synthetic processes in bacteria treated with chloramphenicol. Uninfected bacteria treated with chloramphenicol for 40 min at 50 μg/ml showed no change in RNA labeling pattern when compared with untreated, uninfected cells (Fig. 4a). In bacteria infected by R17 for 60 min and treated with chloramphenicol from 20 to 60 min, the RNA labeling pattern was localized in the cytoplasm in a paranuclear area (Fig. 4b).

In uninfected bacteria treated with chloramphenicol for 100 min, the RNA labeling was still in the nucleoid but was strongly depressed (Fig. 4c). In bacteria infected for 120 min and treated with chloramphenicol from 20 to 120 min, the incorporation of H^3-uridine was solely within the fibrillar area (Fig. 4d). Sections of this sample treated with H_2O_2 for 10 min, followed by ribonuclease for 1 hr, had no residual grains, whereas sections treated with H_2O_2 followed by 1 hr in distilled water had the same pattern and number of grains as did untreated sections.

Quantitative measurements. Studies on the reliability of the grain counting procedure were carried out. In several cases, two sections from the same block were counted, and the resulting numbers of grains per bacterium were similar. Sections of the same sample were kept for a variable number of days (8 to 16) before development of the latent image. A linear relationship between number of reduced silver grains and exposure time was obtained. This relationship was used to correct for different times of exposure for a group of samples which were to be compared. A statistical analysis was made on the data obtained from counting 4,253 uninfected bacteria. This analysis showed that the data were significant at the 5% level (Table 1).

Synthetic processes in R17-infected bacteria. RNA synthesis was markedly inhibited by 15 min postinfection (Table 2). The number of nonlabeled bacteria was higher than in the uninfected population (40% versus 16%). Although RNA

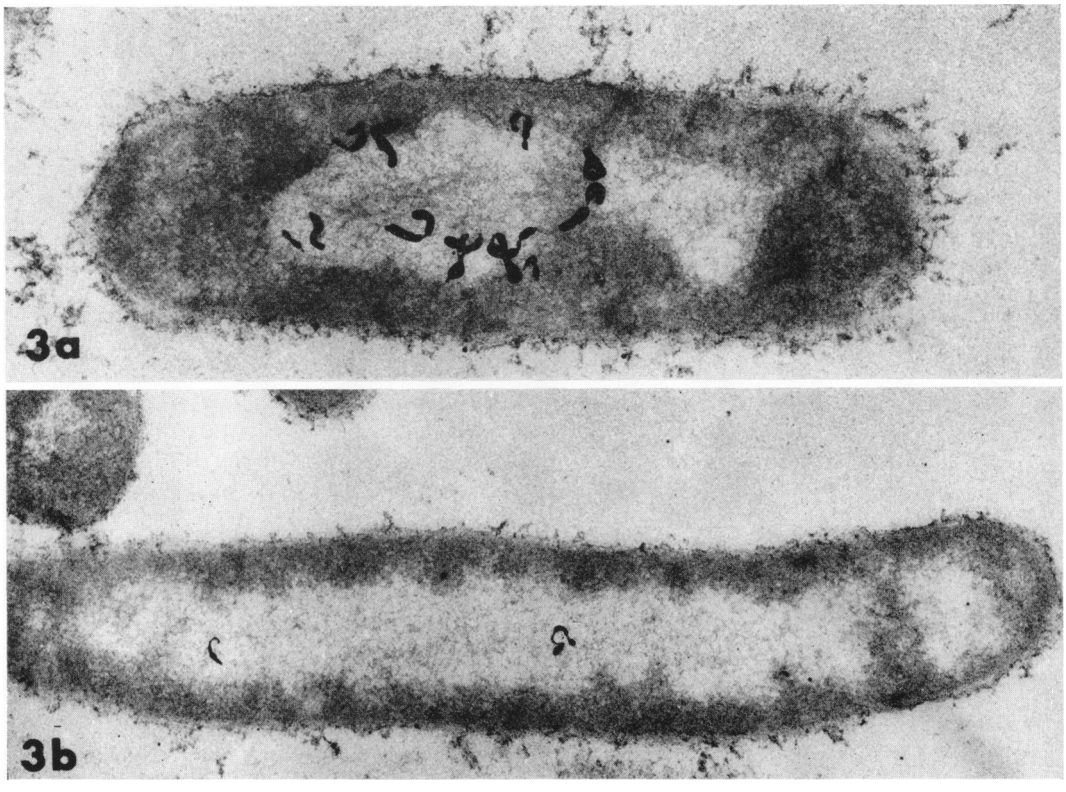

FIG. 3. *H^3-thymidine for 4 min. (a) Uninfected bacterium, incorporation within the nucleus. × 40,000. (b) Infected with R17 for 45 min, incorporation inhibited. × 30,000.*

synthesis increased at 30 and 45 min postinfection, when cytoplasmic incorporation of H^3-uridine was seen (Fig. 1b, c), there was still less incorporation than in uninfected bacteria (Table 1). H^3-uridine incorporation decreased again from 45 to 60 min postinfection and became very low at 120 min (Table 1). At this stage, the number of nonlabeled bacteria was very high in the infected population (66% versus 16% in the noninfected population).

DNA synthesis was strongly inhibited at 45 and 90 min postinfection (Table 1). The incorporation of H^3-thymidine was less per labeled infected bacterium than per labeled uninfected cell, and the number of nonlabeled infected bacteria was very much higher (70% versus 38% in the uninfected population).

Protein synthesis was also markedly inhibited in infected bacteria at 25 min postinfection. Although it increased at 45 min, it was still less than that in the uninfected population (Table 1). The incorporation of H^3-amino acids decreased again at 90 min postinfection.

Synthetic processes in chloramphenicol-treated bacteria. There was no decrease in the rate of RNA synthesis in uninfected bacteria treated with chloramphenicol for 40 min (Table 3). The inhibition of RNA synthesis in the infected population treated with chloramphenicol from 20 to 60 min postinfection was no greater than the inhibition at 60 min postinfection in the absence of chloramphenicol (Table 2). The incorporation of H^3-uridine was strongly inhibited in uninfected bacteria treated with chloramphenicol for 100 min and was about the same in the infected population treated with chloramphenicol from 20 to 120 min postinfection (Fig. 4c and d, and Table 2).

As expected, protein synthesis was inhibited to the same extent in uninfected and infected populations treated for 70 min (Table 2). About 90% of the bacteria were not labeled. Bacteria were also infected for 15 min, then labeled with H^3-amino acids from 15 to 20 min postinfection, and then blocked with chloramphenicol from 20 to 70 min postinfection. In this case, the amount of

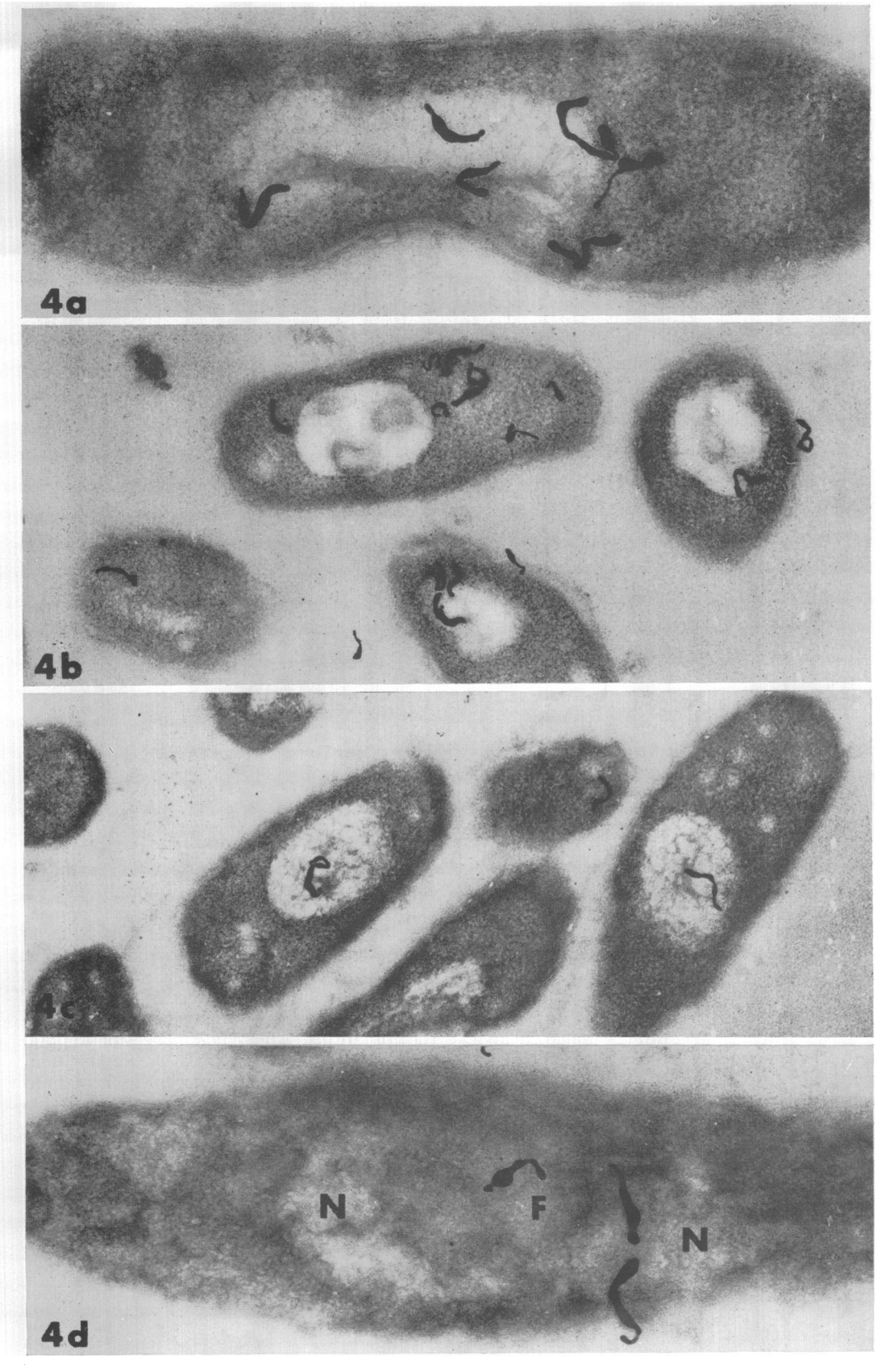

FIG. 4

H^3-amino acids incorporated was about the same as in the infected population which was not treated with chloramphenicol (Table 2), suggesting stability of the protein labeled in the early stages of infection. The label was localized in a polar region, but not in as striking a fashion as in cells infected for 25 min in the absence of chloramphenicol (Fig. 2b).

TABLE 1. *Statistical analysis of the reliability of grain counts on replicate samples of labeled bacteria**

Sample no.	No. of bacteria	No. of grains	Mean no. of grains/ bacterium
1	659	1,777	2.7
2	897	2,430	2.7
3	325	878	2.7
4	805	2,141	2.6
5	594	1,692	2.8
6	405	1,317	3.2
7	568	1,458	2.6
Total	4,253	11,693	2.7

* Samples of uninfected bacteria were exposed to H^3-uridine for 30 sec. $\chi^2 = 12.27$; $P \sim 5\%$.

TABLE 2. *Quantitative autoradiography on control and infected bacteria after labeling of RNA, DNA, or protein**

Time postinfection	Precursor					
	H^3-uridine, 30-sec labeling		H^3-thymidine, 4-min labeling		H^3-amino acids, 5-min labeling	
	Grains/ labeled bacterium	Grains/ bacterium	Grains/ labeled bacterium	Grains/ bacterium	Grains/ labeled bacterium	Grains/ bacterium
min						
Control	3.2	2.7	3.2	2.0	3.2	2.6
15	2.0	1.2				
25					1.7	1.0
45	2.7	2.1	2.4	1.0	2.2	1.4
60	2.5	1.7				
90			2.2	0.6	2.4	1.2
120	2.6	0.9				

* Results are given as the mean number of grains.

TABLE 3. *Quantitative autoradiography on control and infected bacteria treated with chloramphenicol (CM) after labeling of RNA or protein**

Treatment of sample	Precursor			
	H^3-uridine, 30-sec labeling		H^3-amino acids, 5-min labeling	
	Grains/ labeled bacterium	Grains/ bacterium	Grains/ labeled bacterium	Grains/ bacterium
Control	3.0	2.5	3.1	2.6
CM, 40 min	3.1	2.2		
R17, 60 min; CM, 20 to 60 min postinfection	2.6	1.7		
CM, 70 min			1.1	0.1
R17, 90 min; CM, 20 to 90 min postinfection			1.2	0.2
CM, 100 min	1.7	0.4		
R17, 120 min; CM, 20 to 120 min postinfection	1.8	0.7		
R17, 15 min; H^3-amino acids 15 to 20 min postinfection; CM, 20 to 90 min postinfection			2.1	1.4

* Results are given as the mean number of grains.

DISCUSSION

In the uninfected cell H^3-thymidine is incorporated into the nucleoid as has been reported by Caro and van Tubergen (2). This is to be expected, since the nucleoid is believed to contain all of the DNA of the cell because of its fibrillar structure (11, 17), and because it probably corresponds to the centrally located Feulgen-positive "nuclear" body (*see* 15). The extensions of the fibers to the periphery of the cell can account for only a small fraction of the total fibrillar material (i.e., DNA) in the cell (16). RNA synthesis appears to occur in the nucleoid (Franklin and Granboulan, *in press*), and this is expected on the basis of the

FIG. 4. *H^3-uridine for 30 sec. (a) Uninfected bacterium treated for 40 min with chloramphenicol, incorporation within the nuclear area. × 60,000. (b) Bacterium infected by R17 for 60 min and treated with chloramphenicol from 20 to 60 min, cytoplasmic incorporation preferentially located at one pole of the bacterium. × 30,000. (c) Uninfected bacteria treated with chloramphenicol for 100 min, depressed nuclear incorporation. × 30,000. (d) Infected with R17 for 120 min and treated with chloramphenicol from 20 to 120 min, incorporation located only in fibrillar area close to the nucleus (N). × 60,000.*

template function of DNA in controlling RNA synthesis (Franklin and Granboulan, *in press*). Protein synthesis seems to be localized in the cytoplasm, again as expected on the basis of the cytoplasmic concentration of ribosomes and polyribosomic configurations (9*a*, 19). Thus, high-resolution autoradiography of normal bacteria results in labeling patterns predictable on the basis of our current general knowledge of macromolecular syntheses. This means that altered patterns of incorporation in bacteriophage-infected bacteria can be interpreted in terms of new metabolic patterns and new sites of macromolecular syntheses.

During the latent period in R17-infected cells (*see* Fig. 1 of the preceding paper), there are striking alterations in the patterns of RNA, DNA, and protein synthesis. RNA synthesis shifts from a nuclear site to a cytoplasmic site, with a corresponding decrease in rate of incorporation as measured by relative numbers of grains. Just at the end of the latent period, the cytoplasmic incorporation is not localized, but later, during the period of rapid viral synthesis, there is a definite polar concentration of grains, probably in the lesions described in the preceding paper (9*a*). This incorporation continues in the late stages of infection, even when paracrystalline arrays of virus are present; then the grains are localized around the crystals.

Since incorporation of uracil-C^{14} into ribosomal RNA is already strongly inhibited by 30 min postinfection (7), it is most likely that the cytoplasmic incorporation seen at 30 min and later represents viral RNA synthesis. If so, then there may initially be multiple sites of viral RNA synthesis followed later by coalescence to foci where viral components are made.

Protein synthesis is strongly depressed during infection, even during the latent period. Accompanying this inhibition is a shift from generalized cytoplasmic incorporation to a polar focus. Since only one type of lesion has been observed in cells infected with R17 (*see* preceding paper), it is most likely that these lesions are the site of both viral RNA and viral protein synthesis. Since ribosomal arrays are observed to be concentrated at the periphery of the lesions, it is possible that RNA synthesis takes place in the central portion and protein synthesis in the peripheral portion.

DNA synthesis is also markedly inhibited, and there is no shift in the site of residual DNA synthesis. It is most likely that DNA plays no role whatsoever in the synthesis of the small RNA viruses (*see* 4, 9).

Quantitative grain counts show that there is not only a decrease in the number of grains per bacterium, but there is also a drastic decrease in the number of cells showing any label at all late in infection. These are presumably cells in which viral synthesis has stopped and which could lyse soon thereafter. This pattern is similar to that found during the growth of mengovirus in L cells (9, 10). It is not possible to say, however, whether the inhibition of normal synthetic processes in R17-infected *E. coli* is due to some direct effect of virus-induced inhibitors or due to secondary effects of diversion of precursors to viral synthetic pathways. Whereas R17 does block normal RNA synthesis (7), this does not seem to be true for a related RNA phage f2, where there is no decrease in the overall rate of RNA synthesis during infection (13).

In the presence of chloramphenicol, there is a turnover of both ribosomal and transfer RNA with net synthesis being favored, at least during the early stages of chloramphenicol inhibition of protein synthesis (6, 12). The lack of inhibition of normal RNA synthesis and the correspondence of localization of RNA synthesis in control and chloramphenicol-inhibited uninfected cells is in agreement with these findings. With higher doses of chloramphenicol (100 μg/ml), there is eventually inhibition of normal RNA synthesis (12), which is seen in the autoradiography of uninfected cells exposed to chloramphenicol for 100 min. There is more incorporation in infected cells exposed to chloramphenicol from 20 to 120 min postinfection than in uninfected cells exposed to chloramphenicol for 100 min, which agrees with the reports on continued synthesis of viral RNA in cells blocked with chloramphenicol at either 15 or 20 min postinfection (5, 14). The most important observation on infected cells treated with chloramphenicol at 20 min postinfection is the striking localization of grains in the paranuclear fibrillar area, already tentatively identified as a "pool" of RNA (9*a*). The present finding provides further support for this contention.

In conclusion, there are no obvious morphological changes in cells during most of the latent period, although there is a marked inhibition of RNA, DNA, and protein synthesis at this time. By a comparison of high-resolution autoradiographs with sections of Epon-embedded cells, the fibrillar lesions seen during the period of rapid virus synthesis may be foci of synthesis of viral RNA and protein. By use of chloramphenicol to block viral protein synthesis and thus maturation of viral RNA, it is possible to demonstrate a pool of viral RNA.

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