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Studies on the Developmental Cycle of *Chlamydia trachomatis*: Selective Inhibition by Hydroxyurea

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Hydroxyurea, a potent inhibitor of deoxyribonucleic acid synthesis, inhibits the development of trachoma agent when applied at a concentration of 5×10^{-2} M. At a lower concentration, 5×10^{-4} M, hydroxyurea permits the development of the trachoma inclusion bodies and initial bodies, but arrests the formation of elementary bodies, the infectious entity of the agent. The inhibitory effect of 5×10^{-4} M hydroxyurea is reversible and can be used to synchronize the development of the agent. The synthesis of deoxyribonucleic acid, ribonucleic acid, and proteins takes place in the initial bodies after the removal of the inhibitor.

The infectious trachoma elementary body, which contains the deoxyribonucleic acid (DNA) genome (10, 23, 25), develops in the host cell cytoplasm into large inclusion bodies. The molecular events which occur during the initial 18 h of the developmental cycle are still unknown due to a low metabolic activity of the agent which can not be distinguished from the host cell metabolism (1, 8). Later in the developmental cycle of the agent in the infected cells, which were treated with emetine (1) or with anisomycin (unpublished) to inhibit cellular protein metabolism, trachoma agent-specific processes were detected (1, 8). It also became possible to isolate the initial bodies which develop in the trachoma inclusion bodies by zone centrifugation of the homogenized infected cells in sucrose gradients (7). Similar initial bodies were isolated from *Chlamydia psittaci* cells (24). The availability of the techniques to suppress cellular metabolism without affecting the agent's specific processes made possible the study of the role of the trachoma DNA during the agent's developmental cycle. In the present study we used hydroxyurea, a potent inhibitor of DNA viruses (13, 14) and DNA synthesis in prokaryotic (18, 21, 26) and eukaryotic (4, 9, 16) cells to determine at which stages the synthesis of DNA is essential for the development of the agent. It was found that different concentrations of hydroxyurea had different effects on the development of trachoma agent. The inhibitory effect of hydroxyurea (at a concentration of 5×10^{-4} M) is reversible, and treatment of infected cells with the inhibitor was used for the synchronization of the agent's developmental cycle.

MATERIALS AND METHODS

Trachoma agent and cells. The T'ang strain of *Chlamydia trachomatis* was serially propagated in FL cells. Two-day-old FL cells were infected with a suspension of trachoma elementary bodies (EB) (5-10 plaque-forming units [PFU]/cell) to obtain the development of one inclusion body in each cell. The cells were incubated in a synthetic medium (15) at 37 C. The EB infectivity titer was determined by Bernkopf's plaque technique as previously reported (1).

Labeling the cells. The cells were treated with emetine (1 μg/ml), which was added immediately after infection, labeled with ^{3}H-cytidine (2.5 μCi/ml) and ^{14}C-amino acids (0.5 μCi/ml), and incubated until the end of the developmental cycle. When labeled for a shorter period of time, as indicated in the legends to the figures, the infected cells were treated with 50 μg of emetine per ml for 1 h prior to the addition of isotope.

Isolation of initial and elementary bodies by centrifugation in sucrose gradients. The initial and elementary bodies were isolated from the homogenates of infected cells. The cells were scraped and passed through a fine capillary until all cells were damaged and nuclei were released. The samples were layered on top of sucrose gradients (50% to 30% [wt/wt] made in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride [pH 7.2]) and centrifuged in the rotor (SW50.1) of a Beckman preparative ultracentrifuge for 10 min at 22,000 rpm at 5 C. The gradients were collected dropwise, and the radioactivity in each fraction was determined.

Treatment with hydroxyurea. Hydroxyurea was

dissolved in the Tris buffer mentioned previously, and different concentrations of the drug were added to the infected cells at various time periods after infection. To reverse the effect of hydroxyurea, the cells were carefully washed with medium and reincubated in fresh medium.

Electrophoresis of ribonucleic acid (RNA) in acrylamide gels. Infected, radioactively labeled cells were treated with 1% (wt/vol) sodium dodecyl sulfate (SDS) and analyzed by electrophoresis in acrylamide gels as previously reported (8).

Materials. ^{3}H-cytidine (26 Ci/mmol) and ^{14}C-protein hydrolysate (54 mCi of carbon per mA) were obtained from the Radiochemical Centre, Amersham, England. Hydroxyurea was obtained from Sigma Chemical Co., St. Louis, Mo. Acrylamide and *N,N,-N',N'*-tetramethylethylendiamine was purchased from Eastman Chemical Co., Rochester, N.Y.

RESULTS

Different concentrations of hydroxyurea were added to trachoma-infected FL cell cultures, and the effect of the drug on the development of the inclusion bodies and infective EB was determined. We found that hydroxyurea, at a concentration of 10^{-2} M and 5×10^{-3} M, prevented the appearance of inclusions in the infected cells. No infectious EB were detected in these cells (Table 1). The inhibitory effect of hydroxyurea could not be reversed by removal of the drug from the cultures and reincubation of the cells at 37 C for an additional period of 48 h. At a concentration of 10^{-3} M hydroxyurea, small inclusion bodies appeared in the cytoplasm of the infected cells but a low titer of infectivity was found (Table 1). Addition of 5×10^{-4} M hydroxyurea to the infected cells did not affect the development of the trachoma inclusion bodies, but only about 8% of the infectious EB progeny was formed in these cells. Treatment of infected cells with 10^{-4} M hydroxyurea did not affect the development of the inclusion bodies and the formation of infectious particles (Table 1).

Trachoma agent develops and matures in emetine-treated cells (1, 6, 8) under conditions which reduce the synthesis of host cell RNA and proteins (5). In these cells the synthesis of trachoma RNA can be detected above the background of cellular RNA synthesis (8). These conditions made possible the study on the effect of hydroxyurea on trachoma macromolecular processes. We found (Table 1) that the synthesis of DNA and RNA in the infected cells was markedly inhibited by 10^{-3} M hydroxyurea. The incorporation of radioactive ^{3}H-cytidine into RNA in infected, treated cells was dependent on the concentration of the drug: 5×10^{-4} M hydroxyurea only partially affected RNA synthesis. An increase in the concentration of the drug markedly affected RNA synthesis (Table 1). DNA synthesis was much more sensitive to hydroxyurea than is RNA synthesis; 5×10^{-4} M hydroxyurea inhibited 78% of DNA synthesis. At 10^{-3} M hydroxyurea, DNA synthesis was abolished (Table 1 and Fig. 1). The effect of hydroxyurea on trachoma-specific protein synthesis (Fig. 1) follows the same pattern as the inhibition of DNA synthesis.

These results demonstrate that hydroxyurea at 5×10^{-3} M inhibits the formation of inclusion bodies. It is not known if this inhibitory effect is specific (inhibition of DNA synthesis) or is nonspecific (inhibition of other molecular processes). The second stage in the developmental cycle, the conversion of the initial bodies into the EB by a process which resembles binary fission (6, 23), is dependent on DNA synthesis. This process is inhibited by 5×10^{-4} M hydroxyurea.

Preparations of infected cells labeled with ^{3}H-cytidine and treated with different concentrations of hydroxyurea were centrifuged in sucrose gradients to isolate the initial or elementary bodies which develop in the inclusion bodies (7).

We found (Fig. 2A) that the particles which appear in the inclusion bodies at the end of the developmental cycle are the EB which were labeled in their RNA and DNA. Analysis of infected cells treated with 10^{-4} M hydroxyurea also revealed a distinct band of EB (Fig. 2B).

TABLE 1. *Effect of hydroxyurea on the incorporation of ^{3}H-cytidine into RNA and DNA of trachoma agent-infected FL cells and on the development of infectious progeny*

Hydroxyurea concn (M)	Infectivity		Counts/min in DNA	Inhibition (%)	Counts/min RNA	Inhibition (%)
	PFU/ml	Inhibition (%)				
0	1.5×10^7	0	12,900	0	69,100	0
10^{-4}	1.4×10^7	6	9,616	25.5	61,614	12.7
5×10^{-4}	1.2×10^6	92	2,951	77.8	64,850	6.2
10^{-3}	2.5×10^3	99.9	418	96.7	29,037	58.0
5×10^{-3}	0	100	841	93.4	16,217	76.6
10^{-2}	0	100	256	98.9	11,746	83.1

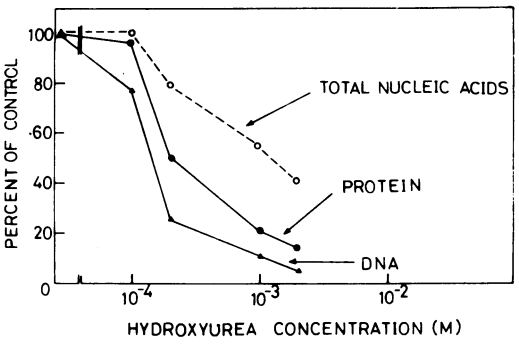

FIG. 1. *Effect of hydroxyurea on the synthesis of trachoma agent macromolecules. Trachoma-infected FL cells were treated with increasing concentrations of hydroxyurea. The cells were labeled with ^{3}H-cytidine (2.5 μCi/ml) and ^{14}C-amino acids (0.5 μCi/ml) and incubated at 37 C until the end of the developmental cycle (48 h postinfection). The untreated and hydroxyurea-treated cells were washed with a solution of 8% (wt/wt) sucrose prepared in 0.01 M Tris-hydrochloride, pH 7.2. The cells were scraped into the buffer, and two samples were removed from each preparation: one was treated with trichloroacetic acid to determine the radioactivity in the nucleic acids (DNA + RNA) and the other was treated overnight with alkali (0.33 N KOH) to determine the alkali-resistant radioactivity (DNA). The radioactivity in the untreated infected cells was taken as 100%. The samples from cell cultures labeled with the radioactive amino acids were treated with trichloroacetic acid to determine the radioactive proteins. The samples were collected on filters, and the radioactivity was determined in a Packard scintillation counter. Symbols: O- - - - O, total nucleic acids; ●——●, protein; ▲——▲, DNA.*

However, the inclusion bodies in infected cells which were treated with 5×10^{-4} M hydroxyurea (Fig. 2C) contained mainly initial bodies which contain labeled RNA and not labeled DNA (8). A small band of EB was also detected in these cells as can be seen from the presence of a DNA band in the gradient in the position of the EB. Treatment of infected cells with 5×10^{-3} M hydroxyurea (Fig. 2D) completely inhibited the formation of DNA and the development of trachoma agent. Similar inhibitory effects of hydroxyurea were found when trachoma-infected cells were labeled with radioactive amino acids in the presence of emetine to suppress the synthesis of cellular proteins. As shown in Fig. 3, EB were formed in the inclusion bodies in the absence of the inhibitor or in the presence of 10^{-4} M hydroxyurea. A small amount of radioactive initial bodies was also detected at that concentration of the inhibitor. In the presence of 5×10^{-4} M hydroxyurea only initial bodies were formed, whereas in the presence of 5×10^{-3} M hydroxyurea the synthesis of trachoma agent proteins was completely inhibited. These results indicate that (i) hydroxyurea inhibits the initiation of trachoma agent development at a high concentration of

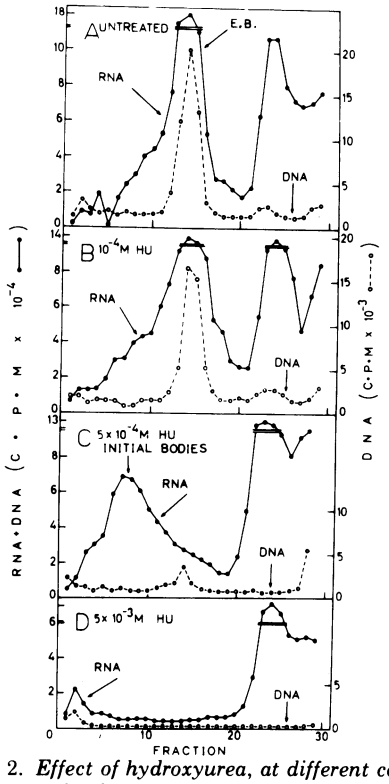

FIG. 2. *Effect of hydroxyurea, at different concentrations, on the development of trachoma initial and elementary bodies. Trachoma-infected cells were incubated at 37 C in the presence of emetine (1 μg/ml) and the different amounts of hydroxyurea. At 24 h postinfection, the medium, containing emetine, hydroxyurea, and ^{3}H-cytidine, was added to the cultures which were incubated for an additional period of 24 h and then harvested. The cells were washed twice with a solution of 8% (wt/vol) sucrose in Tris buffer (0.01 M, pH 7.2). The cells were scraped off the glass, and the glass and the homogenates were centrifuged in sucrose gradients (30–50%, wt/wt) on top of 65% (wt/wt) sucrose cushion. The gradients were centrifuged for 10 min at 22,000 rpm in an SW50.1 rotor of a Beckman preparative ultracentrifuge. The gradients were collected and divided into two portions; one was treated overnight with alkali to determine the radioactive DNA, and the other was precipitated to determine total nucleic acids. The radioactivity in either the DNA or RNA was plotted in the figure. The radioactivity in the different fractions of the gradient was determined after trichloroacetic acid treatment and collection of the precipitate on filters. A, Untreated infected cells; B, infected cells treated with 10^{-4} M hydroxyurea; C, infected cells treated with 5×10^{-4} M hydroxyurea; D, infected cells treated with 5×10^{-3} M hydroxyurea. Symbols: ●——●, RNA; O- - - -O, DNA.*

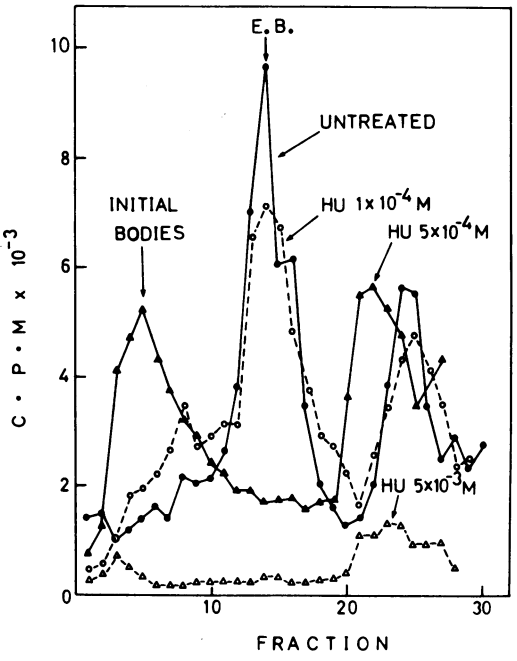

FIG. 3. *Effect of hydroxyurea on the incorporation of radioactive amino acids into trachoma agent proteins in initial and elementary bodies. Trachoma-infected, emetine-treated cells were incubated with different concentrations of hydroxyurea. The cells were labeled with ^{14}C-protein hydrolysate, which was added to the cultures at 24 h postinfection. The cultures were reincubated at 37 C until the end of the agent's growth cycle (48 h postinfection). The cells were treated as indicated in Fig. 1, and the homogenates were centrifuged in sucrose gradients. The radioactivity in the trachoma agent elementary and initial bodies was calculated by determining the radioactivity in each fraction of the different gradients. The result of four different gradients are plotted in the figure. Symbols: ●——●, untreated infected cells; ○ - - - - ○, infected cells treated with 1 × 10^{-4} M hydroxyurea; ▲——▲, infected cells treated with 5 × 10^{-4} M hydroxyurea; △——△, infected cells treated with 5 × 10^{-3} M hydroxyurea.*

the drug (10^{-3} M and higher concentrations), (ii) the initial bodies are formed in the presence of 5 × 10^{-4} M hydroxyurea, and (iii) the transition of the initial bodies to EB was prevented in the presence of 5 × 10^{-4} M hydroxyurea. The effect of different concentrations of hydroxyurea on the incorporation of ^{3}H-cytidine into initial and elementary bodies is summarized in Fig. 4. It can be seen (Fig. 4A) that the largest amount of initial bodies is synthesized and accumulates in the inclusion bodies in the presence of 5 × 10^{-4} M hydroxyurea. Lower and higher concentrations of hydroxyurea resulted in the inhibition of initial bodies formation. The formation of EB was almost completely prevented by 5 × 10^{-4} M hydroxyurea and higher concentrations (Fig. 4B).

Addition of 10^{-3} M hydroxyurea to infected cultures completely prevented the synthesis of EB when added prior to 30 h postinfection (Fig. 5). Addition of the inhibitor later than 30 h had only a partial inhibitory effect.

Treatment of trachoma-infected cells with 5 × 10^{-4} M hydroxyurea resulted in a marked inhibition of the formation of infectious EB (Tables 1 and 2). Removal of hydroxyurea from the treated cultures triggered the synthesis of trachoma EB in the inclusion bodies. At 12 h after the removal of the inhibitor, the yield of

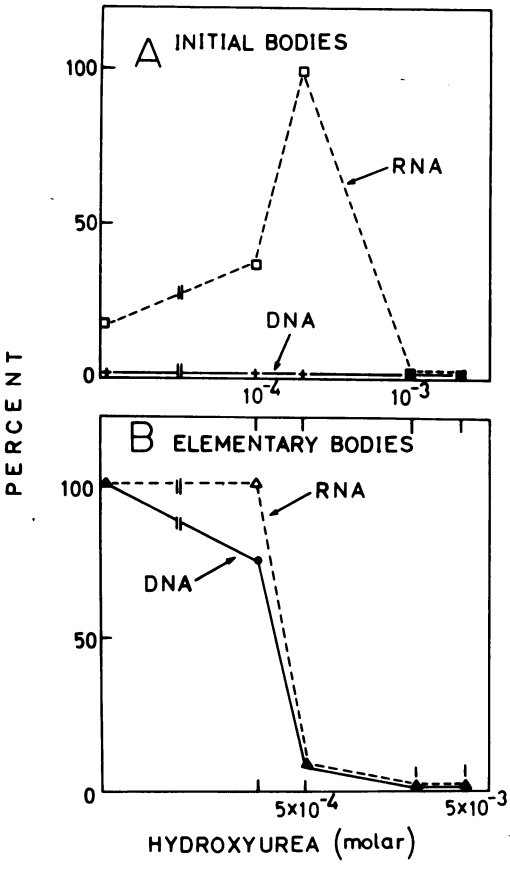

FIG. 4. *Effect of hydroxyurea on the development of trachoma elementary and initial bodies. The experiment was carried out as described in Fig. 2 and 3. The radioactivity present in the bands of elementary and initial bodies, isolated from trachoma-infected, hydroxyurea-treated cells, was determined and plotted in the figure. The radioactivity in the elementary bodies of untreated, infected cells was taken as 100%. The radioactivity in the initial bodies obtained from infected cells treated with 5 × 10^{-4} M hydroxyurea was taken as 100%. A, Radioactivity in initial bodies; B, radioactivity in elementary bodies.*

infectious progeny reached the infectivity level which is present in infected, untreated cells at 48 h postinfection (Table 2). It is of interest that, 24 h after the removal of hydroxyurea, the infectious progeny of EB was almost 10-fold higher than in the infected, untreated cells. This result might indicate that, during the normal course of trachoma development, not all the initial bodies develop into EB, whereas after the removal of hydroxyurea the process of EB maturation is more efficient.

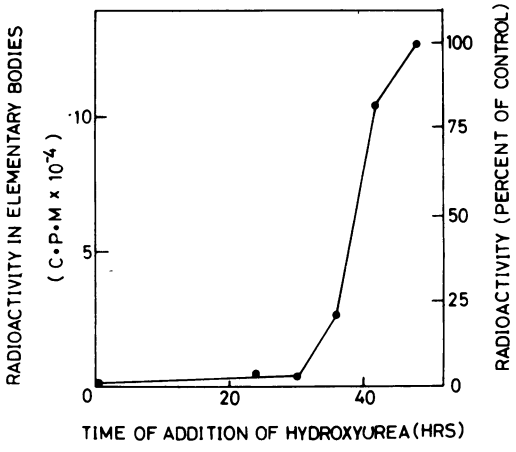

FIG. 5. *Effect of hydroxyurea on the formation of elementary bodies when added at different stages of the growth cycle. To a series of trachoma-infected cells, ^{3}H-cytidine was added immediately after infection. To different cultures, hydroxyurea (10^{-3} M) was added immediately after infection and at different times thereafter. The cells were incubated until 48 h postinfection, and the elementary bodies were isolated by centrifugation of the infected cells homogenates in sucrose gradients. The total radioactivity in each band of elementary bodies was determined. The yield of radioactive elementary bodies present in untreated, trachoma-infected cells was taken as 100%.*

To study the time course of EB formation in trachoma inclusion bodies treated with 5×10^{-4} M hydroxyurea, the infected cells were labeled with ^{3}H-cytidine during the development of the inclusion bodies. The isotope and hydroxyurea were removed at 48 h postinfection by extensive washing of the cells which were reincubated in fresh medium. Samples were removed at different time intervals, and the trachoma initial and elementary bodies were isolated by centrifugation in sucrose gradients. It was found (Fig. 6A, 0 h) that a distinct band of initial bodies was present in the inclusion bodies at the time of hydroxyurea removal. The radioactivity present at the top of the gradient was partly cellular RNA and partly RNA from disrupted initial bodies. At 3 h after the removal of the inhibitor, an increase in the amount of radioactive particles which banded in the position of elementary bodies was noted (Fig. 6A, 3 h), indicating an increase in the number of EB. Analysis of the hydroxyurea-treated cells at 12 h after removal of the inhibitor revealed that most of the radioactivity originally present in the initial bodies was present in the EB (Fig. 6B, 12 h). At 24 h after the removal of the inhibitor (Fig. 6B, 24 h) all the radioactivity was present in a narrow band of EB. These results are in agreement with the gradual increase of infectious EB yields (Table 2). It can be concluded that treatment of infected cells with 5×10^{-4} M hydroxyurea markedly inhibits the development of the EB and leads to the accumulation

TABLE 2. *Development of infectious elementary bodies in hydroxyurea-treated cells after the removal of the inhibitor*

Treatment	Time	Elementary bodies progeny			
		Expt 1		Expt 2	
		PFU	Inhibition (%)	PFU	Inhibition (%)
Untreated	48 h PI[a]	4×10^7	0	2×10^7	0
HU treated (5×10^{-4} M)	48 h PI zero time of reversion	5.5×10^6	86	2.5×10^5	99
HU treated + reversion	2 h reversion	1.0×10^7	75	2×10^5	99
HU treated + reversion	4 h reversion	1.4×10^7	65	6×10^5	97
HU treated + reversion	6 h reversion	1.6×10^7	60	ND	ND
HU treated + reversion	8 h reversion	ND[b]	ND	2×10^6	90
HU treated + reversion	12 h reversion	ND	ND	2×10^7	0
HU treated + reversion	24 h reversion	2.4×10^8[c]	0	1.2×10^8[c]	0

[a] PI, Postinfection.
[b] ND, Not done.
[c] Titer of elementary bodies yield higher than the yield in untreated, infected cells.

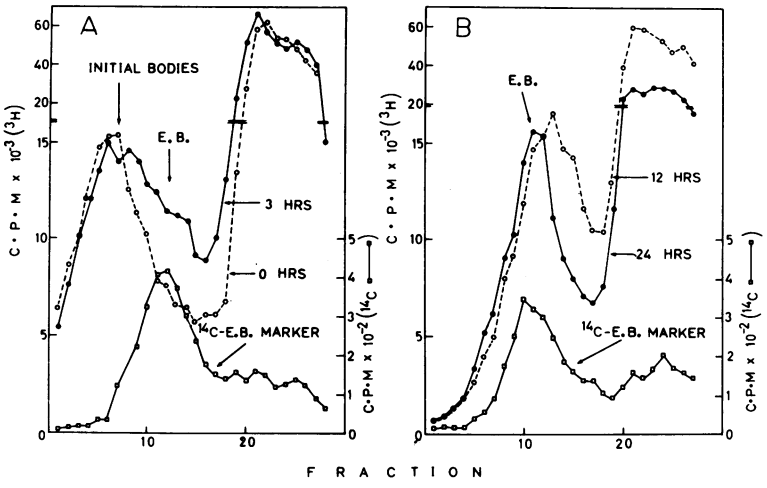

FIG. 6. *Reversibility of hydroxyurea inhibitory effect on the trachoma initial bodies. Infected, emetine-treated cells were incubated for 48 h in the presence of 5 × 10⁻⁴ M hydroxyurea and ³H-cytidine. The cells were then washed carefully to remove the inhibitor and the isotope and were reincubated in fresh medium in the absence of the inhibitor. At different time intervals thereafter, cultures were harvested and the cytoplasmic fractions were centrifuged in sucrose gradients to isolate initial or elementary bodies. Purified elementary bodies labeled with ¹⁴C-cytidine were prepared and added to each sample to serve as a marker. The gradients of cells which were removed at 0 time immediately after the removal of the inhibitors (●——●) and at 3 h after reversion (O- - - -O) as well as a ¹⁴C-EB marker (□——□) were plotted in A. The sucrose gradients of cells harvested at 12 h (O- - - -O) and 24 h (●——●) after the removal of the inhibitor and the ¹⁴C-EB marker (□——□) were plotted in B.*

of initial bodies. The reversibility of the hydroxyurea inhibitory effect provides a technique for the synchronization of the development of trachoma initial bodies. The latter develop into EB upon the removal of the drug.

To study the molecular processes which take place during the transformation of the initial bodies to EB, trachoma-infected, hydroxyurea (5×10^{-4} M)-treated cells were labeled with ^{3}H-cytidine at 48 h after infection, immediately after the removal of the inhibitor. The incorporation of ^{3}H-cytidine into RNA and DNA was determined.

The synthesis of RNA is initiated after the removal of the inhibitor. The amount of radioactive RNA gradually increased with time (Fig. 7A). Synthesis of RNA also takes place in uninfected cells but to a lesser extent than in the infected cells. DNA synthesis started immediately after the removal of the inhibitor and continued during the period of EB formation. All the newly synthesized DNA is trachoma specific, as DNA synthesis was not noted in uninfected cells (Fig. 7B). The incorporation of ^{3}H-cytidine into DNA is 2.5% of the label which was incorporated into the RNA molecules. Proteins were also synthesized in parallel to the synthesis of trachoma RNA (Fig. 7C). These results indicate that the sythesis of macromolecules in the initial bodies accompanies the development of the initial bodies to EB after the removal of the inhibitor.

Analysis of the RNA species synthesized in the trachoma initial bodies after the removal of the hydroxyurea is presented in Fig. 8. Ribosomal RNA molecules (23*S*, 16*S*, and 17.5*S*, the precursor of the 16*S*) as well as other RNA species were found to be synthesized at 2 and 4 h after the removal of the inhibitor. The nature of the nonribosomal RNA species is under investigation.

DISCUSSION

The major finding of this study is the demonstration that hydroxyurea, a potent inhibitor of DNA synthesis (4, 9, 13, 14, 16–22, 26), inhibits the developmental cycle of trachoma agent at two different stages in the agent's growth cycle. A high concentration of hydroxyurea (5×10^{-3} M) inhibits the development of the elementary bodies into inclusion bodies, whereas at a lower concentration (5×10^{-4} M) the inhibitor does not interfere with the development of the inclusion bodies and the formation of the initial bodies inside them, but inhibits the transformation of the initial bodies to EB. This process, which occurs by a mechanism resembling binary fission in prokaryotic cells (7, 11), is

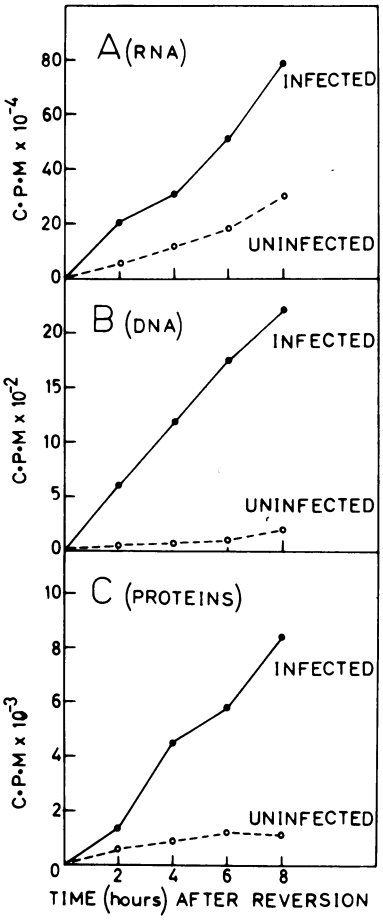

FIG. 7. *Kinetics of RNA, DNA, and protein synthesis in the inhibited trachoma initial bodies after the removal of hydroxyurea. Trachoma-infected cells were treated with hydroxyurea (5×10^{-4} M) which was added to the culture medium immediately after infection, at 48 h postinfection. The medium was replaced with medium containing ^{3}H-cytidine or ^{14}C-protein hydrolysate and 0.5 μg of emetine per ml. The cultures were reincubated at 37 C, and samples were removed at different time intervals. The radioactivity in the RNA (A), DNA (B), and proteins (C) was determined.*

prevented by hydroxyurea. These findings demonstrate that DNA synthesis occurs during the formation of elementary bodies. It is not known if 5×10^{-3} M hydroxyurea inhibits the development of the inclusion bodies by inhibiting the agent's DNA synthesis or by affecting other molecular processes. Little is known as yet on the processes which occur in the elementary body after their entry into the host cell cytoplasm. The main reason for that is the low level of molecular processes which take place in the developing elementary bodies. The latter are overshadowed by the cellular processes of the host cell, even when inhibited by emetine (1).

The next step in the developmental cycle of trachoma agent requires DNA synthesis for the duplication of the agent's DNA genome in the initial bodies. The requirement of this metabolic process was demonstrated by the inhibitory effect of 5×10^{-4} M hydroxyurea. In the presence of the inhibitor the development of the inclusion and the initial bodies was unaffected, but the formation of the EB was prevented. The synthesis of DNA in the initial bodies accompanies the formation of EB. This is in agreement with the observation of Kramer and Gordon (11) that initial bodies divide by a process resembling binary fission in bacteria (2, 3).

The inhibitory effect of hydroxyurea on the development of initial bodies provides us with a tool to synchronize the developmental cycle of the agent. In the presence of 5×10^{-4} M hydroxyurea, most of the initial bodies remain as such while only a small percentage (about 5%) of the initial bodies escape the inhibitory effect and develop into EB. Upon removal of the inhibitor, the synthesis of DNA in initial bodies is stimulated and within 12 h the infectious EB progeny is formed. The synthesis of RNA and proteins accompanies DNA replication. The synthesis of ribosomal RNA and other RNA species, not yet characterized, was found to occur during the period of transition of initial bodies to EB. Further studies on the molecular processes and the macromolecules synthesized during the formation of the EB are in progress.

Hydroxyurea is a potent inhibitor of DNA synthesis in prokaryotic and eukaryotic cells (4, 9, 13, 14, 16-22, 26), but its mechanism of action is not fully known. The effect of hydroxyurea on the trachoma agent initial bodies suggests that DNA duplication is inhibited by hydroxyurea, possibly at the site of DNA replication on the initial body's membrane. Further studies are in progress to elucidate the mechanism by which hydroxyurea inhibits trachoma agent.

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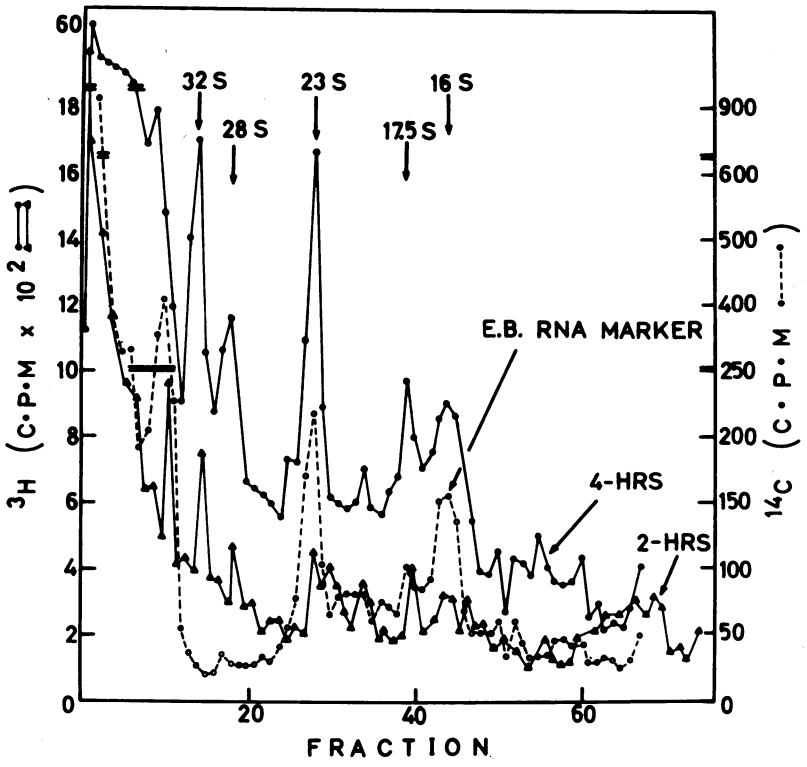

FIG. 8. *Characterization of the RNA species synthesized in trachoma initial bodies after the removal of hydroxyurea. Infected, hydroxyurea-treated cells were carefully washed to remove the inhibitor and were reincubated in a fresh medium containing ^3H-cytidine. At 2 and 4 h after the removal of the inhibitor, samples of infected cells were removed and analyzed by electrophoresis in acrylamide gels. RNA from EB labeled with ^{14}C-cytidine was used as a marker. Symbols:* O- - - -O, *^{14}C-EB RNA marker;* ▲——▲, *cells labeled for 2 h;* ●——●, *cells labeled for 4 h.*

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