Synthesis of Nucleic Acid and Protein in L Cells Infected with the Agent of Meningopneumonitis

ESTHER M. SCHECHTER

Department of Microbiology, The University of Chicago, Chicago, Illinois

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ABSTRACT

SCHECHTER, ESTHER M. (The University of Chicago, Chicago, Ill.). Synthesis of nucleic acid and protein in L cells infected with the agent of meningopneumonitis. *J. Bacteriol.* **91**:2069–2080. 1966.—Synthesis of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and protein in uninfected L cells and in L cells infected with the meningopneumonitis agent was compared by measuring rates of incorporation of H^3 -cytidine and C^{14} -lysine into nuclear, cytoplasmic, and agent fractions in successive 5-hr periods during the meningopneumonitis growth cycle. Synthesis of meningopneumonitis DNA, RNA, and protein was first clearly evident in the labeling period 15 to 20 hr after infection, soon after initiation of agent multiplication. The rates of synthesis of agent DNA, RNA, and protein increased logarithmically for a brief period and then declined. However, rates of isotope incorporation into all three meningopneumonitis macromolecules were sustained at near maximal values throughout the remainder of the meningopneumonitis growth cycle. These data are most readily interpreted in terms of multiplication of the meningopneumonitis agent by binary fission. The L cell response to infection was a decreased rate of DNA and RNA synthesis and an accelerated rate of cell death. Host protein synthesis was unaffected. The inhibition of nucleic acid synthesis in infected L cells probably involved competition between host and parasite for nucleic acid precursors. Different sublines of L cells varied greatly in the degree to which their nucleic acid-synthesizing mechanisms were damaged by infection. The cytoplasm of infected L cells contained newly synthesized DNA and RNA that could not be accounted for as intact meningopneumonitis cells. This nucleic acid probably arose from disintegration of the fragile intracellular forms of the meningopneumonitis agent.

Microorganisms of the psittacosis group contain both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (20, 31, 32), multiply by binary fission within membrane-bound vacuoles in the cytoplasm of their host cells (1, 10), and degrade glucose and pyruvate (37, 38) by enzymes that are qualitatively different from those of the corresponding enzymes of their host (22). These observations, together with many others, as reviewed by Moulder (21), indicate that the growth of a psittacosis agent within a host cell represents an intimate interaction of two organisms, each with its own set of macromolecule-synthesizing enzyme systems.

Schechter, Tribby, and Moulder (28) used P^{32} -orthophosphate to follow the synthesis of nucleic acids in L cells infected with the agent of meningopneumonitis. Cell fractionation methods were employed to distinguish between synthesis of men-

ingopneumonitis nucleic acids in the cytoplasm and host nucleic acids in the nucleus. This paper describes further investigations on macromolecule synthesis in meningopneumonitis-infected L cells in which nucleic acids were labeled with H^3 -cytidine and proteins with C^{14} -lysine. Particular attention was paid to the course of synthesis of meningopneumonitis DNA, RNA, and protein and to the effect of meningopneumonitis multiplication on the synthesis of macromolecules by the L cell.

MATERIALS AND METHODS

Growth of L cells. Strain L cells (6, 27) obtained from H. M. Jenkin (University of Washington, Seattle) were cloned in this laboratory by I. I. E. Tribby, and all the work reported here was done with a single subline, designated clone 5b. L cells were grown routinely in medium 199 (19; Grand Island Biological Co., Grand Island, N.Y.) containing 16%

newborn calf serum (Grand Island Biological Co.), 0.12% sodium bicarbonate, and 200 $\mu\text{g}/\text{ml}$ of streptomycin. This medium will be referred to as 199₈CaSi₁₆. The L cells were maintained at 37 C as monolayer cultures in 4-oz (about 120 ml) prescription bottles in an atmosphere of 95% air–5% carbon dioxide or as suspension cultures in the apparatus described by McLimans et al. (16) with the modifications of Mika and Pirsch (17). Counts of viable cells in suspension cultures were made by the trypan blue exclusion test as performed by McLimans et al. (16). Suspension cultures received complete changes of medium every 2 or 3 days and were divided when the cell density reached 1 million cells per milliliter.

Growth of meningopneumonitis agent in L cells. The Cal 10 strain of the meningopneumonitis agent, adapted to growth in L cells by Tribby (35), was serially propagated in L cell monolayers. These experiments were performed with the 109th to the 163rd L cell passages. Monolayers (2 to 3 days old) were washed twice with medium 199 containing 10% newborn calf serum (199₀CaSi₁₀) and infected with 2 ml of a meningopneumonitis agent concentrate prepared as described below and containing 200 meningopneumonitis cells per L cell (on the assumption that 1 chick embryo LD₅₀ = 10 cells). After the bottles were gently shaken for 1 hr at 37 C, the supernatant fluid was decanted, the monolayers were washed twice with 199₀CaSi₁₀, 10 ml of 199₈CaSi₁₆ was added, and the infected monolayers were incubated for 72 hr at 37 C in an atmosphere of 95% air–5% carbon dioxide. Then the supernatant liquid was decanted from the almost completely destroyed monolayer, centrifuged at 150 $\times g$ for 5 min to remove cell debris, and then centrifuged at 5,000 $\times g$ for 30 min to sediment the meningopneumonitis agent. The resulting agent pellet was resuspended in 4 ml of 199₈CaSi₁₆ and used to infect monolayer or suspension cultures.

Macromolecule synthesis in uninfected and infected L cells was studied in suspension cultures prepared from monolayers about 1 week previously. The growth medium was changed 24 hr before infection to insure log-phase metabolism. The cells were recovered by centrifugation at 150 $\times g$, suspended in 199₈CaSi₁₆, and mixed with enough meningopneumonitis agent to give a multiplicity of infection of 200 and a volume of 20 ml. The suspension was gently shaken for 1 hr at 37 C, centrifuged for 5 min at 150 $\times g$, washed once in 199₈CaSi₁₆, and resuspended to a final volume of 50 ml and a final cell count of approximately 5 $\times 10^5$ cells per milliliter.

To determine the percentage of L cells infected with the meningopneumonitis agent, duplicate Leighton tubes containing flying cover slips were inoculated with 0.5 ml of the infected cell suspension and 0.5 ml of medium. The tubes were incubated at 37 C in 95% air–5% carbon dioxide for 24 hr, at which time the cover slips were fixed in absolute ethyl alcohol and stained with Giemsa; the percentage of infection was determined by examining at least 200 cells for the presence of meningopneumonitis inclusions. Infection in the cultures used in these experiments was 85 to 100%. No corrections for uninfected cells were applied

to any of the results. Most infected L cells contained more than one inclusion.

Fractionation of L cells. The L cells in a 50-ml suspension culture were centrifuged at 150 $\times g$ for 5 min, the growth medium was discarded, and the cells were washed three times with a modification of the balanced salt solution of Hanks and Wallace (9) containing 0.15% methylcellulose (Methocel 15 centipoises, reagent grade, Dow Chemical Co., Midland, Mich.). Addition of the Methocel was required to prevent leakage of L cell constituents during washing. The cells were suspended in 5 ml of cold 20% citric acid and shaken vigorously for 1 hr at 4 C. They were then centrifuged at 150 $\times g$ for 10 min at 0 C, washed once with 5 ml of 20% citric acid, resuspended in 5 ml of 30% citric acid, and shaken again for 30 min. After this treatment, the liberated nuclei were sedimented by centrifugation for 10 min at 150 $\times g$. The supernatant liquid was added to the previous two citric acid supernatant fractions to yield the cytoplasmic fraction, which was clarified by a final centrifugation. The sediment was combined with the main nuclear fraction in a volume of 4 ml. The nuclei in each nuclear fraction were counted in a hemocytometer to an error of less than 5%. The calculations for each experiment were based on the nuclear count rather than the whole cell count.

Of the cytoplasmic fraction, 4 ml was removed for analysis, and the rest was centrifuged at 5,000 $\times g$ for 30 min at 0 C to sediment the meningopneumonitis agent. The pellet was washed twice with balanced salt solution containing Methocel, and was resuspended in 2 ml of the same diluent containing 0.05 M MgSO₄. Ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) and deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.) were added to final concentrations of 0.25 mg/ml each, and the mixture was incubated for 30 min at 37 C. Then the proteinase Pronase (Calbiochem) was added to final concentrations of 0.50 mg/ml, and incubation was continued for another 30 min at 37 C to yield a third fraction, called the cytoplasmic sediment, which consisted almost entirely of intact meningopneumonitis cells. When examined with an electron microscope, a cytoplasmic sediment from L cells infected 24 hr previously contained large, small, and intermediate cell types of the meningopneumonitis agent (Fig. 1). There was no recognizable host cell debris. The meningopneumonitis cells in the cytoplasmic sediment were no longer infectious for chick embryos.

Three milliliters each of the nuclear, cytoplasmic, and cytoplasmic sediment fractions were separated by the Schneider-Schmidt-Thannhauser procedure (36) into acid-soluble, phospholipid, RNA, and DNA fractions and analyzed for radioisotope content as described below.

Evidence that this L cell fractionation method was suitable for the uses to which it was put in these investigations will be presented in Results.

Radioisotopic labeling of L cells. Infected and uninfected L cell suspensions were exposed to radioactively labeled precursors of nucleic acid and protein at successive 5-hr intervals after infection. To measure radioisotope incorporation during a given interval, an

FIG. 1. Electron micrograph of the cytoplasmic sediment of infected L cells fractionated by the citric acid method 24 hr after infection. The preparation was air-dried and chromium-shadowed. See Litwin et al. (15) for a detailed description of sample preparation. The electron micrograph was taken by Robert R. Friis. $\times 13,000$.

L cell suspension culture was divided into four subcultures; two were infected and two were kept as uninfected controls. Eagle's (5) minimal essential medium was used during the 5-hr period of radioisotope labeling, to keep dilution of the labeled precursors to a minimum. However, it could not be used throughout the growth cycle because, although it is a complete medium for L cells, it does not support maximal growth of the meningopneumonitis agent.

H^3 -cytidine. At 5-hr intervals after infection, 50-ml suspension cultures were centrifuged at $150 \times g$ for 5 min, and the cells were resuspended in 50 ml of minimal essential medium with 16% newborn calf serum; $167 \mu c$ of H^3 -cytidine with a specific activity of $1.0 c/mmole$ (Schwarz Bio Research Inc., Orangeburg, N.Y.) was added to each suspension culture. Exactly 5 hr later, the cells were collected by centrifugation, washed with balanced salt solution-Methocel containing $10^{-4} M$ cytidine, and fractionated. Samples (1 ml) of the Schneider-Schmidt-Thannhauser fractions of nucleus, cytoplasm, and cytoplasmic sediment were mixed with 9 ml of Bray's (2) scintillation mixture, and were counted in a Packard model 3324

Tri-Carb liquid spectrometer at a gain setting of 50%. The counting efficiency varied from 9 to 16%, depending on the nature of the sample.

C^{14} -lysine. The same general procedure was followed as for H^3 -cytidine labeling; $5 \mu c$ of L-lysine- C^{14} , with a specific activity of $180 mc/mmole$ (Schwarz Bio Research Inc.), was added to each suspension culture. The wash solution contained $10^{-4} M$ L-lysine. Chemical fractionation was carried through the separation of trichloroacetic acid-soluble and -insoluble fractions. Insoluble fractions were dissolved in 1.5 ml of a 1 M solution of the hydroxide of Hyamine (Packard Instrument Co., LaGrange, Ill.) at 4 C overnight. Samples were counted at a gain setting of 9%; the counting efficiency was only 15% because of the considerable quenching effect of the Hyamine solution.

P^{32} -orthophosphate. Carrier-free sodium orthophosphate- P^{32} (Volk Radiochemical Co., Skokie, Ill.) was used to prepare labeled suspensions of the meningopneumonitis agent. To 100-ml L cell suspensions containing 10^6 cells per milliliter, 2.5 mc of P^{32} -orthophosphate was added at the time of infection, and labeled meningopneumonitis agent was prepared

as described in Results. P^{32} -containing samples were counted in Bray's scintillation mixture at a gain setting of 2.9% and an efficiency of 73%.

No corrections for quenching were made. Since corresponding fractions from infected and uninfected cultures seemed to be quenched to the same degree, the data were interpreted in direct comparison of the observed counts. An external standardization system has become available since these data were collected. Its use with similar samples confirms the conclusion that quenching is significant only in samples dissolved in Hyamine and that it is equal in all corresponding fractions of infected and uninfected cells. Samples were counted long enough to insure that the counting error would not exceed 0.9% in 96% of the observations.

RESULTS

Growth of the meningopneumonitis agent in clone 5b L cells. Figure 2 shows the growth curve obtained by infecting suspensions of clone 5b L cells with enough L cell-adapted meningopneumonitis agent to infect virtually 100% of the L cells. The titer of both intracellular and extracellular infections began to rise 10 hr after infection, and reached its peak at 40 hr. Growth of the meningopneumonitis agent in the cloned L cells differed from that in the uncloned L cells used previously (28) in that multiplication continued for about 10 hr longer, reached a terminal titer more than 1 log higher, and resulted in a slower rate of killing of the host cells.

H^3 -cytidine incorporation. Cytidine was a good precursor of both RNA and DNA in L cells and in the meningopneumonitis agent. Figure 3 is a graphic representation of cytidine incorporation into the RNA and DNA of the cytoplasmic sediment of infected L cells (see also Table 1). In Fig. 3A, incorporation in each individual 5-hr labeling period was plotted on an arithmetic scale. Uptake of cytidine into meningopneumonitis nucleic acids was first clearly evident 15 to 20 hr after infection, reached its maximum at 25 to 30 hr, and declined slightly after 30 to 35 hr. The rates of incorporation of labeled cytidine into meningopneumonitis RNA and DNA rose and fell in unison. In Fig. 3B, cumulative cytidine incorporation into meningopneumonitis nucleic acids was plotted on a logarithmic scale. Cytidine uptake into RNA and DNA increased logarithmically from 15 to 30 hr after infection, but significantly declined in rate thereafter, indicating that the infected L cell can support a maximum rate of synthesis of meningopneumonitis RNA and DNA for only a brief period.

Incorporation of labeled cytidine into the nuclear RNA and DNA of infected L cells proceeded at only half the uninfected rate from the 20 to 25 hr labeling period onward (Table 1). In the earlier labeling periods, host DNA synthesis was inhibited

FIG. 2. Growth of the meningopneumonitis agent in a suspension of clone 5b L cells. The intracellular LD_{50} was obtained by centrifuging down the cells in 1 ml of suspension, resuspending them in 1 ml of 2% ammonium acetate, and incubating at 4 C for 30 min to lyse the L cells and release the meningopneumonitis agent (14). The lysate was then diluted at least 10-fold and immediately inoculated via the yolk sac into groups of 15 6-day old embryos. The LD_{50} was calculated by the single-dilution method of Golub (8). The supernatant fluid from each cell sample used for intracellular titration was employed to obtain the extracellular LD_{50} . Each value is the mean of four independent observations.

slightly, whereas host RNA synthesis was unaffected. Incorporation of cytidine into phospholipids of infected L cells was at no time inhibited. The effect of meningopneumonitis infection on cytidine uptake into nonsedimentable cytoplasmic fractions of the L cell will be discussed in a later section.

The good agreement among uninfected L cell populations used as control for the different periods of isotopic labeling is evidence of the reproducibility of the cell culture and fractionation methods (Table 1). These cell samples should theoretically be identical log-phase populations. The incorporation into the nuclear DNA in the 5 to 10 hr labeling period was unaccountably low. Incorporation of cytidine into the nuclear RNA of infected L cells declined somewhat as the uptake into cytoplasmic RNA increased fourfold, indicating that the nuclear fraction obtained by citric acid fractionation was relatively free of cytoplasmic contamination. The cytoplasmic sediment of uninfected L cells contained only traces of incorporated cytidine. These small amounts of radioactivity were in forms resistant to hydrolysis by

FIG. 3. Incorporation of H^3 -cytidine into DNA and RNA and of C^{14} -lysine into protein of the cytoplasmic sediment of infected L cells. (A) Incorporation in each 5-hr labeling period (arithmetic scale). (B) Cumulative incorporation (logarithmic scale). The counts per minute for lysine incorporation were multiplied by a factor of 10 for ease of plotting.

either ribonuclease or deoxyribonuclease. This is further evidence for concluding that this fraction of infected L cells consisted almost entirely of meningopneumonitis cells.

H^3 -cytidine labeled the phospholipids of uninfected and infected L cell cytoplasm and nucleus and the cytoplasmic sediment of infected L cells (Table 1). This observation suggests that both the L cell and the meningopneumonitis agent contain cytidine diphosphate choline or other similar compounds (13). Incorporation of the cytidine label into the meningopneumonitis agent phospholipid roughly paralleled its incorporation into RNA and DNA.

C^{14} -lysine incorporation. L-Lysine- C^{14} was used as the labeled precursor of L cell and meningopneumonitis proteins because it is an essential amino acid for the L cell (4) and a major constituent of meningopneumonitis protein (23). Table 2 shows that lysine was incorporated into the trichloroacetic acid-insoluble fractions (assumed to be protein) of both L cells and the meningopneumonitis agent. Uptake of lysine into the proteins of the uninfected L cell populations used as controls for the successive 5-hr labeling periods did not vary significantly in the different

cell samples. The amount of label incorporated into the cytoplasmic sediment of uninfected L cells was very small.

Incorporation of C^{14} -lysine into the cytoplasmic sediment (meningopneumonitis agent) of infected L cells was first detected in the 15 to 20 hr period and reached a maximum 30 to 35 hr after infection, 5 hr later than the period of maximal incorporation into nucleic acid (Table 2, Fig. 3). With this exception, the rate of lysine incorporation into the cytoplasmic sediment of infected L cells closely paralleled the rate of cytidine uptake into the RNA and DNA of these fractions (Fig. 3A, 3B). The failure of Starr et al. (30) to find specific psittacosis group antigen with fluorescent labeled antibody until late in the growth cycle may have been due to the relative insensitivity of this method.

In contrast to the reduced rate of nucleic acid turnover observed in infected L cells (Table 1), there was no inhibition of lysine incorporation into the proteins of the cytoplasmic and nuclear fractions of the host cells at any time in the growth cycle of the meningopneumonitis agent (Table 2).

Nature of the nonsedimentable "extra" DNA and RNA in the cytoplasmic fractions of infected L

TABLE 1. Incorporation of H^3 -cytidine in uninfected *L* cells and in *L* cells infected with the meningopneumonitis agent

Period of labeling	Uninfected cells									Infected cells								
	Nucleus			Cytoplasm			Cytoplasmic sediment			Nucleus			Cytoplasm			Cytoplasmic sediment		
	PL*	RNA	DNA	PL	RNA	DNA	PL	RNA	DNA	PL	RNA	DNA	PL	RNA	DNA	PL	RNA	DNA
<i>hr</i>																		
5-10	677†	26,700	2,020	508	3,060	37	3	8	<1	447	25,100	2,080	2,150	4,490	118	6	51	10
10-15	1,320	23,600	6,630	1,110	3,380	40	4	10	<1	1,420	22,900	4,340	4,920	4,780	357	8	22	12
15-20	688	23,400	6,540	837	5,890	53	4	16	<1	1,130	22,500	5,690	1,380	7,370	738	6	80	57
20-25	738	28,400	8,380	1,130	6,730	94	2	20	<1	1,030	19,100	3,680	1,300	19,700	4,260	507	809	1,150
25-30	478	30,700	7,550	840	7,390	101	2	25	<1	577	17,900	3,600	1,490	16,700	3,710	141	1,300	1,740
30-35	278	22,700	5,050	611	3,640	34	5	24	<1	319	15,400	2,370	2,700	10,600	3,070	73	1,210	1,160

* Phospholipid.

† Counts per minute per 10^6 *L* cells. Each value is the mean of two cultures.

TABLE 2. Incorporation of C^{14} -lysine into trichloroacetic acid-insoluble fractions of uninfected L cells and L cells infected with the agent of meningopneumonitis

Period of labeling	Uninfected cells			Infected cells		
	Nucleus	Cyto-plasm	Cyto-plasmic sediment	Nucleus	Cyto-plasm	Cyto-plasmic sediment
hr						
5-10	141*	434	2	155	343	1
10-15	121	286	1	138	264	2
15-20	118	358	2	138	338	17
20-25	130	398	2	119	318	49
25-30	121	370	1	127	373	74
30-35	143	374	2	130	292	90
35-40	95	234	2	88	414	67

* Counts per minute per 10^8 L cells. Each value is the mean of two cultures.

cells. Infection with the agent of meningopneumonitis caused an increased incorporation of H^3 -cytidine into the cytoplasmic fractions of L cells that was greater than could be accounted for by the presence of meningopneumonitis agent in the corresponding cytoplasmic sediments (Tables 1 and 3). Cytoplasmic fractions from infected L cells contained newly labeled DNA not sedimented by centrifugation at $5,000 \times g$ for 30 min (sufficient to sediment more than 99.9% of intact meningopneumonitis cells) and partially sensitive to deoxyribonuclease (intact cells are completely resistant). This extra DNA could have been synthesized by either the L cell or the meningopneumonitis agent. The cytoplasmic fractions of infected L cells also contained increased amounts of nonsedimentable and partially ribonuclease-sensitive RNA that could not be accounted for by the cytoplasmic sediments derived from them. This extra RNA became particularly prominent at the 20 to 25 hr labeling period. It could also have been made by either the L cell or the meningopneumonitis agent. No increase in nonsedimentable protein in infected L cell cytoplasm was noted (Table 2). It may have been obscured by the high level of protein synthesis in the L cell ribosomes.

The citric acid fractionation procedure was first suspected as the source of these discrepancies. However, when infected L cells were separated into cytoplasmic and nuclear fractions by an entirely different procedure (7), which uses Tween 80 to disrupt the L cells, essentially the same amount of extra DNA and RNA was found as with the citric acid method.

It was possible that intact meningopneumonitis cells were not being centrifuged down into the

TABLE 3. Nonsedimentable meningopneumonitis nucleic acids in the cytoplasmic fractions of infected L cells

Period of labeling	H^3 -cytidine incorporated into DNA		H^3 -cytidine incorporated into RNA		RNA-DNA ratio	
	Sed.*	Nonsed.†	Sed.	Nonsed.	Sed.	Nonsed.
hr						
5-10	10‡	72	51	1,390		
10-15	12	306	22	1,390		
15-20	57	629	80	1,420		
20-25	1,150	3,020	809	12,180	0.70	4.21
25-30	1,740	1,870	1,300	8,130	0.75	4.35
30-35	1,160	1,870	1,210	5,770	1.03	3.06

* Sedimentable DNA or RNA. These values were taken from the cytoplasmic sediment columns of Table 1.

† Nonsedimentable DNA or RNA. These values were calculated from those in Table 1 by subtracting the sum of the incorporation into uninfected cytoplasm and infected cytoplasmic sediment from that of the infected cytoplasm. The high RNA values for the early labeling periods probably reflect the errors inherent in such an approximation. For this reason RNA-DNA ratios were calculated only during the intervals when the amount of agent present was appreciable.

‡ Counts per minute per 10^8 L cells.

cytoplasmic sediment but were remaining in the cytoplasmic supernatant fluid. Therefore, infected and cytidine-labeled cytoplasmic fractions were centrifuged for 60 min at $35,000 \times g$ before and after dilution to reduce the density of the citric acid solution in which they were contained. Such centrifugation left just as much extra labeled DNA in the cytoplasmic fraction as did the usual centrifugation at $5,000 \times g$ for 30 min.

The extra nucleic acid could also have been newly synthesized host cell DNA and RNA that had leaked out of L cell nuclei damaged in the course of infection or of cell fractionation. This hypothesis was tested by using the autoradiographic observation of Pelc and Crocker (24) that H^3 -thymidine was incorporated into host cell DNA but not into the DNA of psittacosis group agents. A 50-ml suspension of infected L cells was exposed to $250 \mu c$ of H^3 -thymidine (specific activity, 0.36 c/mmole; Schwarz BioResearch Inc.) during the 20 to 25 hr period after infection, and was fractionated as in the experiments with H^3 -cytidine (Table 4). Thymidine was incorporated into the DNA of both infected and uninfected L cell nuclei, although the inhibitory effect of infection on DNA synthesis was readily apparent. However, the thymidine label did not appear in either the whole cytoplasmic fraction or in the

TABLE 4. Incorporation of H^3 -thymidine into the nucleic acids and phospholipids of uninfected L cells and of L cells infected with the agent of meningopneumonitis*

Fraction	DNA		RNA		Phospholipid	
	Uninfected	Infected	Uninfected	Infected	Uninfected	Infected
Nucleus	8,900†	3,920	109	39	149	73
Cytoplasm	20	40	11	14	8	3
Cytoplasmic sediment	1	13	1	9	1	1

* The experiment was carried out in exactly the same way as those with H^3 -cytidine. The labeling period was 20 to 25 hr after infection.

† Counts per minute per 10^6 L cells. Each value is the mean of two cultures.

cytoplasmic sediment, thus confirming the observations of Pelc and Crocker and indicating that the extra nonsedimentable DNA in the cytoplasmic fractions of infected L cells was not made in the L cell nucleus. This experiment also showed that cytoplasmic fractions obtained by the citric acid method were not contaminated with host DNA.

The rate of accumulation of extra nonsedimentable RNA and DNA in infected L cell cytoplasmic fractions (Table 3) closely paralleled meningopneumonitis multiplication and maturation, as judged by infectivity titers (Fig. 2) and appearance of labeled RNA and DNA in cytoplasmic sediments (Fig. 3). Accumulation of extra cytoplasmic DNA and RNA reached its highest rate during the 20 to 25 hr labeling period, a time when the meningopneumonitis population consisted chiefly of the large and fragile immature cell type, and subsequently declined in rate as the large cell matured into the more resistant small cell type [see Moulder (21) for discussion of psittacosis group cell types]. This correlation suggested that the large meningopneumonitis cells were being disrupted during L cell fractionation and were releasing their DNA and RNA into the nonsedimentable portion of infected cytoplasmic fractions.

To test this hypothesis, two experiments were performed with P^{32} -labeled meningopneumonitis agent. In the first, infected L cells were allowed to incorporate P^{32} -orthophosphate for 25 hr, and then were lysed by shaking in 2% ammonium acetate at 0°C for 60 min (14). This released the intracellular meningopneumonitis agent which, at 25 hr after infection, consisted mainly of the immature large cell type. L cell debris was removed by centrifuging for 10 min at $150 \times g$. The meningopneumonitis agent was sedimented by centri-

fugation at $5,000 \times g$ for 30 min, and was washed twice in balanced salt solution-Methocel; a portion was then removed to test its initial sensitivity to ribonuclease and deoxyribonuclease. The remaining crude labeled meningopneumonitis agent was mixed with unlabeled L cells, and was carried through the usual citric acid cell fractionation. Portions of whole cytoplasmic and cytoplasmic supernatant fractions were dialyzed against phosphate-buffered saline for 48 hr to remove citric acid, and half of each preparation was then treated with the two nucleases. The cytoplasmic sediment was also divided in two portions, and one was treated with nuclease and one not. Nuclease digestions were carried out, and sensitivity to ribonuclease and deoxyribonuclease was estimated by comparing the P^{32} content of RNA and DNA fractions obtained with and without nuclease digestion. An uninfected L cell culture was carried through the same procedure, so that the P^{32} counts obtained for each infected sample could be corrected for the contribution of any labeled L cell debris that might be present. Such corrections were less than 13% of the infected cell RNA values, and less than 5% of the infected cell DNA values.

In the second experiment, mature extracellular agent, consisting chiefly of the small mature meningopneumonitis cell type, was obtained from the supernatant fraction of an infected L cell suspension that had been allowed to incorporate P^{32} -orthophosphate for 44 hr. The supernatant fraction was clarified by centrifugation at $150 \times g$ for 10 min, and the meningopneumonitis cells were sedimented at $5,000 \times g$ for 30 min. They were resuspended in balanced salt solution-Methocel, taken through another cycle of high- and low-speed centrifugation, and treated in the same manner as the 25-hr meningopneumonitis preparation.

The mature extracellular agent was insensitive to both ribonuclease and deoxyribonuclease before and after citric acid treatment (Table 5). It was almost quantitatively recovered in the cytoplasmic sediment. The immature intracellular meningopneumonitis population was initially resistant to deoxyribonuclease but partially sensitive to ribonuclease. After citric acid fractionation, a significant amount of the P^{32} originally associated with the immature meningopneumonitis cells appeared in the cytoplasmic supernatant fraction, and the labeled DNA and RNA in the cytoplasmic sediment had become partially susceptible to the respective nucleases. These experiments suggest that the extra nonsedimentable RNA and DNA in the infected cytoplasmic fractions may have come from the breakdown of the large immature forms of the meningopneumonitis agent.

TABLE 5. *Stability of the meningopneumonitis agent during fractionation of L cells*

Fraction	Kind of P ³² -labeled meningopneumonitis agent added to unlabeled L cells	
	Intra-cellular, 25 hr	Extra-cellular, 44 hr
Labeled agent before cell fractionation	%	%
Sensitivity to deoxyribonuclease.....	0	0
Sensitivity to ribonuclease.....	46	0
Cytoplasmic fraction after cell fractionation		
Nucleic acid in sediment		
DNA recovered.....	85	93
Sensitivity to deoxyribonuclease.....	70	0
RNA recovered.....	77	94
Sensitivity to ribonuclease.....	33	0
Nucleic acid in supernatant fluid		
DNA recovered.....	15	7
Sensitivity to deoxyribonuclease.....	42	0
RNA recovered.....	33	6
Sensitivity to ribonuclease.....	40	0

DISCUSSION

Macromolecular synthesis in the meningopneumonitis agent in relation to its growth cycle. The results of Higashi, Tamura, and Iwanaga (11) and of Tamura (31) showed that absolute increases in the amount of RNA and DNA in various fractions of L cells infected with the meningopneumonitis agent cannot be demonstrated until relatively late in the growth cycle, after extensive multiplication of the agent has occurred. The techniques used in the present investigation allow the synthesis of new macromolecules in the meningopneumonitis agent to be detected much earlier, but they in turn suffer from the disadvantages of measuring only relative rates of synthesis, not absolute amounts, and of failing to distinguish between net synthesis and turnover. However, in the discussion to follow it will be assumed that incorporation of isotopic labels was the result of net synthesis and that the observed relative rates of incorporation are valid measures of the rates of synthesis of the macromolecules of uninfected and infected L cells.

In a previous study of meningopneumonitis agent growth in uncloned L cells, RNA and DNA synthesis was first observed 10 to 15 hr after infec-

tion; in this investigation on the same agent growing in a cloned L cell line, nucleic acid synthesis was first seen 15 to 20 hr after infection. Moore (18), working with the same host-parasite system employed here, and Crocker et al. (3), using an ornithosis agent in HeLa cells, made similar observations by means of autoradiography. On the basis of thin-section electron microscopy, Higashi (10) concluded that members of the psittacosis group begin to multiply by binary fission no later than 10 hr after infection of their host cells. This is the time when intracellular infectivity began to rise in L cell suspensions infected with the meningopneumonitis agent (Fig. 1). Podolyan et al. (25) used phase-contrast microcinematography to establish the generation time of psittacosis group agents as 2 to 3 hr. Thus, when DNA synthesis in the meningopneumonitis agent was first unmistakably evident, approximately 15 hr after infection, each invading meningopneumonitis cell had probably undergone only two or three divisions. As measured by increase in the intracellular infectivity titer, the meningopneumonitis agent continued to multiply until 40 hr after infection. Synthesis of DNA, RNA, protein, and phospholipid was maintained throughout this period, although the rates of incorporation of labeled precursors decreased in the later labeling periods. The autoradiographic studies of Crocker et al. (3) and Moore (18) also showed that the DNA of the ornithosis and meningopneumonitis agents was synthesized throughout the growth cycle.

The nearly simultaneous appearance of new intracellular infectivity and of newly synthesized meningopneumonitis DNA, RNA, protein, and phospholipid, the ensuing period of logarithmic increase in macromolecules, and their sustained synthesis throughout the growth cycle are all consistent with the concept of multiplication by binary fission. Growth of the meningopneumonitis agent in the L cell may be analogized to the bacterial growth cycle. The lag phase is the time required for reorganization of the invading small cells into the large intracellular ones; the logarithmic phase is the period during which the large cells undergo binary fission at a constant rate; the decreased logarithmic phase is the time when the concentration of agent precursors in the L cell becomes limiting, and the large cells divide at decreased rates and begin to reorganize into small cells; and the stationary phase coincides with the termination of the growth cycle when the L cell can no longer support meningopneumonitis multiplication, and mature small cells are released into the medium.

Because 5'-fluorodeoxyuridine (34), aminopterin (26), and 5'-fluorodeoxycytidine (29) in-

hibit multiplication of psittacosis group agents when added to infected cells early in the growth cycle but are unable to do so when added later than 15 to 20 hr after infection, it was concluded that most of the DNA of these organisms is made early in the growth cycle and that there is a lag between synthesis of DNA and appearance of new infectious units. The results of these investigations, together with those of Crocker et al. (3) and Moore (18), show that this interpretation cannot be correct. DNA synthesis in psittacosis group agents continues throughout the growth cycle and is most rapid at a time when their multiplication is no longer affected by these drugs. Another explanation must be sought for this interesting change in drug susceptibility during the course of the growth cycle. Such a change is not limited to inhibitors of DNA synthesis. Meningopneumonitis multiplication in L cells is first susceptible and then resistant to inhibition by actinomycin D, which prevents DNA-directed RNA synthesis (33), and psittacosis multiplication in McCoy cells is similarly affected by *p*-fluorophenylalanine, an inhibitor of protein synthesis (26). It is possible that all these observations have a common explanation.

Significance of nonsedimentable forms of meningopneumonitis DNA and RNA in the cytoplasmic fractions of infected L cells. The experiment with H^3 -thymidine, which labels L cell DNA but not meningopneumonitis DNA (24), conclusively demonstrated that the nonsedimentable DNA was made by the meningopneumonitis agent and not by the L cell. No such specific label was available for either L cell or meningopneumonitis RNA, and so the origin of the extra cytoplasmic RNA could not be settled with certainty. However, according to Tamura and Iwanaga (33), the RNA made in L cells 10 to 20 hr after infection with the meningopneumonitis agent was localized in the agent inclusions and had the base composition of agent RNA. Therefore, it is highly probable that the extra cytoplasmic RNA was also made by the meningopneumonitis agent.

These nonsedimentable nucleic acids of meningopneumonitis origin could have come from the destruction of intact meningopneumonitis cells during L cell fractionation, or they could have originated by a viruslike reproductive mechanism and never have been a part of the intact meningopneumonitis cell. The experiments in which crude preparations of P^{32} -labeled meningopneumonitis agent were added to unlabeled L cells and the mixtures fractionated as usual favor the first possibility but do not rule out the second. However, in light of present knowledge of the nature of microorganisms of the psittacosis group, the

possibility of the meningopneumonitis agent reproducing in the form of its naked nucleic acids seems unlikely.

The ratio of RNA to DNA is 1:1 in preparations of small mature meningopneumonitis cells (32) and 4:1 in large immature cells (31). Table 3 compares the RNA-DNA ratio in cytoplasmic sediments and supernatant fractions of infected L cells. These ratios suggest that the meningopneumonitis agents isolated in cytoplasmic sediments represented mainly mature or nearly mature cells, whereas the nonsedimentable nucleic acids of the cytoplasmic supernatant fractions represented the yield from destruction of large immature cells during the isolation procedure. If this is true, then the sum of the incorporation of labeled precursor into the sedimentable and nonsedimentable fractions is an estimate of the true extent of synthesis of meningopneumonitis macromolecules. However, changes in the rate of this combined incorporation closely paralleled changes in the rate of incorporation into the cytoplasmic sediment alone, and its use as a measure of the rate of synthesis of meningopneumonitis macromolecules would not change significantly any of the interpretations of the relation of macromolecular synthesis to the agent's growth cycle offered in the proceeding section.

Effect of meningopneumonitis infection on macromolecular synthesis in the L cell host. Table 6 compares the effect of meningopneumonitis infection on incorporation of labeled precursors into the RNA and DNA of the nuclei of uncloned and cloned L cells. The data on uncloned cells were obtained with a P^{32} -orthophosphate label (28), but more recent use of a H^3 -cytidine label has yielded identical results. The effect of infection on nucleic acid synthesis in the two closely related L cell lines differed greatly. DNA synthesis in the uncloned cells was almost completely stopped 10 to 15 hr after infection, but was never reduced by more than 56% in the cloned cells. RNA synthesis was similarly more severely inhibited in the uncloned cells. These differences are in accord with the observation that meningopneumonitis multiplication is less rapidly lethal and is longer sustained in the cloned L cell line.

Thus, the profound inhibition of nuclear DNA synthesis and rapid death of the host cell, first found in the uncloned L cells by Schechter, Tribby, and Moulder (28), has turned out to be an extreme case of the effect of infection on the host cell. I. I. E. Tribby (*personal communication*) has isolated from the original L cell line received in this laboratory several clones differing in susceptibility to infection with the meningopneumonitis agent, generation time, and pattern of

TABLE 6. *Inhibition of synthesis of DNA and RNA in nuclear fractions of L cells infected with the meningopneumonitis agent*

Period of labeling	Inhibition of uncloned cells, P^{32} -orthophosphate as label*			Inhibition of clone 5b cells, H^3 -cytidine as label		
	DNA	RNA	Dead cells	DNA	RNA	Dead cells
hr	%	%	%	%	%	%
5-10	14	0	6	0	6	4
10-15	93	32	10	35	3	5
15-20	93	16	16	15	4	7
20-25	89	76	30	56	33	15
25-30	99	45	52	52	42	24
30-35				53	53	43

* Data from Schechter, Tribby, and Moulder (28).

growth in suspension cultures. DNA synthesis in one of these clones is as severely inhibited by meningopneumonitis infection as in the uncloned population. Apparently, the dominant cell type at the time the uncloned L cells were studied was one whose nucleic acid synthesis was highly susceptible to inhibition by meningopneumonitis growth.

In their autoradiographic studies, Crocker et al. (3) noted a moderate inhibition of DNA synthesis in HeLa cells infected with an ornithosis agent. By use of the same technique, a partial inhibition of DNA synthesis in cloned 5b L cells infected with the meningopneumonitis agent has also been observed in this laboratory (18). Crocker et al. (3) gave two possible explanations for this inhibition: (i) production during cytoplasmic growth of the psittacosis agent of a factor that blocks DNA synthesis in the host cell nucleus, and (ii) a competition between host cell nucleus and psittacosis agent for cytoplasmic energy sources and precursors needed for DNA synthesis. They favor the latter explanation and suggest that the competition between host and parasite is for nucleoside triphosphates, which Moulder (20) postulated as being required for nucleic acid synthesis in psittacosis group agents. However, recent work in this laboratory (I. I. E. Tribby and J. W. Moulder, *personal communication*) suggests that the meningopneumonitis agent can utilize certain host cell nucleosides for DNA synthesis but that it cannot use the host nucleotides for this purpose. Therefore, the postulated competition may be for nucleoside precursors or for the energy required to phosphorylate them to the nucleoside triphosphates.

In clone 5b L cells infected with the meningopneumonitis agent, it may be calculated that the sum of the host and agent DNA and RNA syn-

thesized in any 5-hr labeling period was equal to that made in uninfected cells. This suggests that, in this system, resources for nucleic acid synthesis were limiting and that the second explanation of Crocker et al. was correct. However, it is unlikely that a simple competition for DNA precursors can explain the profound early inhibition of DNA synthesis observed in uncloned L cells by Schechter, Tribby, and Moulder (68). The amount of meningopneumonitis agent present 10 to 15 hr after infection was much too small for it to rob the host nucleus of DNA precursors. In addition, the total DNA synthesized by the infected uncloned cells fell far below that made by the uninfected cells, indicating that there was no shortage of synthetic resources. Here, the first explanation of Crocker et al. seems more plausible, and the unlikely possibility that the meningopneumonitis agent synthesizes an inhibitory histone such as the one produced in cells infected with ME virus (12) cannot be completely disregarded. It may be significant that the meningopneumonitis agent contains the basic amino acids lysine, arginine, and histidine in nonprotein peptide form (23).

The absence of any detectable inhibition of protein synthesis in infected cells at any time during the meningopneumonitis growth cycle suggests that the observed inhibitions of nucleic acid synthesis were relatively specific and not due to generalized depression of host cell metabolism.

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