Availability of Bases and Nucleosides as Precursors of Nucleic Acids in L Cells and in the Agent of Meningopneumonitis

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ABSTRACT

TRIBBY, ILSE I. E. (University of Chicago, Chicago, Ill.), AND JAMES W. MOULDER. Availability of bases and nucleosides as precursors of nucleic acids in L cells and in the agent of meningopneumonitis. *J. Bacteriol.* **91**:2362-2367. 1966.—Uninfected L cells and the meningopneumonitis agent propagated in L cells utilized exogenous adenine, guanine, and their ribonucleosides and deoxyribonucleosides for synthesis of both deoxyribonucleic acid (DNA) and ribonucleic acid. Cytosine, cytidine, and uridine were also incorporated into the nucleic acids of both host and parasite. L cells and the meningopneumonitis agent incorporated uracil, thymine, and deoxyuridine very poorly. L cells utilized thymidine and deoxycytidine almost exclusively for DNA synthesis, but the meningopneumonitis agent did not incorporate these nucleosides at all. Since the L cell had previously been shown to convert added thymidine to its nucleotides, mainly the triphosphate, it was concluded that the meningopneumonitis agent can utilize neither the thymidine nor the thymidine nucleotides of the L-cell pool, and that it can probably synthesize the thymidine triphosphate needed for DNA synthesis from the uridine of the L-cell pool.

Pelc and Crocker (16) first observed that exogenous thymidine was not incorporated into the deoxyribonucleic acid (DNA) of psittacosis group organisms growing in cell cultures, although it was readily incorporated into the DNA of host cell nuclei. This finding was confirmed in several host cell-psittacosis agent systems by autoradiography (4, 17, 23) and by isolation of the agent from labeled infected cells (21).

The unusual exclusion of exogenous thymidine from the DNA of the psittacosis group was investigated in more detail by determining the availability of the nine nucleosides and five nitrogen bases commonly found in nucleic acids for the synthesis of ribonucleic acid (RNA) and DNA by an agent of the psittacosis group and by its host cell. Each tritium-labeled nucleoside or base was added to L-cell suspensions, uninfected and infected with the agent of meningopneumonitis. After a suitable period of meningopneumonitis multiplication, the incorporation of the labeled precursor into the RNA and DNA of both the L cell and the meningopneumonitis agent was determined.

MATERIALS AND METHODS

L-cell cultures. Strain L cells (6, 20) obtained from H. M. Jenkin (University of Washington, Seattle) were cloned by one of us (I. I. E. T.), and a single subline designated clone 5b was used in all experiments. L cells were propagated as described by Schechter (21) in monolayer and suspension cultures in medium 199 (12) containing 16% newborn calf serum (Grand Island Biological Co., Grand Island, N.Y.) and 100 µg/ml of streptomycin.

Meningopneumonitis agent. The Cal 10 strain of the meningopneumonitis agent adapted to growth in L cells (Tribby, M.S. Thesis, Univ. of Chicago, Chicago, Ill., 1963) was serially propagated in L-cell monolayers, and seed for infection of suspension cultures was prepared according to Schechter (21). At the time of these experiments, the meningopneumonitis agent had been passed more than 100 times in L cells.

Incorporation of labeled precursors into the RNA and DNA of uninfected and infected L-cell suspensions. L-cell suspensions were prepared from 3-day-old monolayer cultures about 1 week prior to use. The growth medium was changed every 48 hr, and the cell population was not permitted to exceed 1.5×10^6 cells per milliliter. The medium was not changed for 48 hr before start of the experiment. Then the cells in 100 ml of a suspension containing 10^6 cells per milli-

liter were sedimented by centrifuging at $150 \times g$ for 10 min, suspended in 15 ml of growth medium, and mixed with 20 chick embryo yolk sac LD_{50} of meningopneumonitis seed for every L cell. The cells were gently shaken at 37 C for 1 hr, washed once in Eagle's minimal essential medium (5) containing 16% newborn calf serum, and then suspended in 100 ml of the same medium. Eagle's medium was used during the period of isotope incorporation to avoid dilution of the labeled precursors with the unlabeled bases of medium 199. Uninfected L-cell suspensions were similarly treated, with the substitution of growth medium for the meningopneumonitis seed. The per cent infected cells was determined by examination of L cells stained 24 hr after infection (21). In most experiments, the per cent infection was 80 to 90%; it was never less than 50%.

The L-cell suspensions were incubated at 37 C, and 15 hr later 500 μ c of a tritium-labeled nucleoside or base was added to each 100-ml suspension. The concentration of the labeled bases was 2×10^{-6} M, and that of the labeled nucleosides was 2×10^{-5} M. Approximately 44 hr after infection, at the completion of the meningopneumonitis developmental cycle (21), the L cells were sedimented by centrifuging at $150 \times g$ for 10 min. The supernatant fluids from infected cultures were used for purification of the meningopneumonitis agent; those from uninfected cultures were discarded. The L cells were washed twice in 0.15 M NaCl containing 10^{-4} M unlabeled nucleoside or base as carrier. Then each cell population was resuspended in 10 ml of 0.15 M NaCl and used for determination of incorporation of the isotope into whole cells and DNA and RNA for uninfected cells, and into whole cells only for infected ones.

Purification of labeled meningopneumonitis agent. Supernatant fluids of 44-hr infected L-cell cultures contained 10^7 to 10^8 chick embryo yolk sac LD_{50} of the meningopneumonitis agent as determined by the single dilution method of Golub (7). They were centrifuged for 30 min at $12,000 \times g$ and 0 C. The sedimented agent was suspended in 20 ml of 2% ammonium acetate containing carrier nucleoside or base, centrifuged down, and resuspended in 20 ml of 2% ammonium acetate-carrier solution. This washing was repeated twice. Then the agent was suspended in 10 ml of 2% ammonium acetate containing 0.5 mg/ml of crystalline trypsin (Worthington Biochemical Corp., Freehold, N.J.) and incubated for 30 min at 37 C. The trypsin-treated agent was washed once in 20 ml of 2% ammonium acetate and then suspended in 10 ml of 2% ammonium acetate containing 0.25 mg/ml each of crystalline pancreatic ribonuclease and deoxyribonuclease (Worthington Biochemical Corp.) and 0.05 M $MgSO_4$. After 30 min at 37 C, the meningopneumonitis agent was sedimented and washed once as already described. The purified agent was finally suspended in 5 ml of 2% ammonium acetate. Such suspensions contained $10^{7.5}$ to $10^{8.5}$ LD_{50} /ml and had protein contents (10) of 45 to 150 μ g/ml. An electron micrograph of a typical purified meningopneumonitis suspension is shown in Fig. 1. Such suspensions con-

sisted almost entirely of the small, mature cell type. Host-cell debris was not visible.

Analytical methods. Uninfected and infected L cells and purified meningopneumonitis agent were separated into RNA and DNA fractions by the method of Schmidt and Thannhauser as described by Volkin and Cohn (25). Samples (1-ml) of whole L cells or RNA and DNA fractions were mixed with 15 ml of Bray's (1) scintillation mixture and counted in a Packard Tri-Carb liquid spectrometer at a gain setting of 50% and with an efficiency of 15%. Use of an external standardization system indicated that quenching was negligible in all samples. The counting errors were less than 5% except in samples of minimal isotopic content.

Tritium-labeled bases and nucleosides were obtained from Volk Radiochemical Co., Skokie, Ill., Schwarz Bio Research Inc., Orangeburg, N.Y., and New England Nuclear Corp., Boston, Mass.

RESULTS

Table 1 shows the availability of H^3 -uridine as a precursor of the nucleic acids of uninfected and infected L cells and of the meningopneumonitis agent. Its behavior was typical of the nucleosides and bases that were readily incorporated into the DNA and RNA of both L cells and meningopneumonitis agent. To compare the metabolism of 14 potential nucleic acid precursors in many different cell cultures, the incorporation of H^3 -cytidine was arbitrarily set at 100%, and the incorporation of all precursors was expressed as percentages of the cytidine uptake. Cytidine was chosen as the standard of comparison because it was known to be a good DNA and RNA label in both L cells and the meningopneumonitis agent (21). Table 2 compares the relative efficiency with which the nine nucleosides and five bases commonly found in nucleic acids were incorporated into L-cell and meningopneumonitis DNA and RNA. From the information given in the footnotes of Table 2, the data for incorporation of uridine given in Table 1 may be converted into the relative uridine incorporation shown in Table 2. Values for the relative incorporation of all other precursors were calculated in a similar way. In using this method of comparing potential precursors of nucleic acids, we shall ignore many small differences in relative incorporation and shall base our interpretation of the pathways of nucleoside and base metabolism in L cells and the meningopneumonitis agent on a few large differences in behavior among the various labeled intermediates studied.

These experiments were not designed to compare the rates of nucleic acid synthesis in host and parasite. Only a portion of the terminal meningopneumonitis population was represented in

FIG. 1. Electron micrograph of meningopneumonitis agent purified from supernatant fluids of infected L cells. The final suspension in 2% ammonium acetate was deposited on Formvar- and carbon-coated grids in a Sharp particle counting cell (Ivan Sorvall, Inc., Norwich, Conn.), dried overnight in vacuo, and shadowed with chromium at an angle of 15°. $\times 4,100$.

the purified preparation analyzed, and the size of the meningopneumonitis population was constantly increasing during most of the labeling period. From a different kind of experiment, it has been estimated that, during the period of most active parasite multiplication, the amounts of nucleic acid synthesized by the infected L cell and the meningopneumonitis agent within it are approximately equal (21).

Incorporation of purines and purine nucleosides. Adenine, guanine, and their ribo- and deoxyribonucleosides were all good precursors of both DNA and RNA in the uninfected L cell and in the meningopneumonitis agent. Adenine and its nucleosides were more efficiently utilized by the L cell than were guanine and its nucleosides. Differences in utilization by the meningopneumonitis agent were of doubtful significance. With

the exception of adenosine, which contributed relatively more label to L-cell and meningopneumonitis DNA than any other precursor tested, these intermediates consistently contributed two to three times as much label to RNA as to DNA in both L cells and the meningopneumonitis agent. L cells have more than twice as much RNA as DNA (22), whereas meningopneumonitis preparations such as those used here contain equal amounts of the two nucleic acids (24).

Incorporation of pyrimidines and pyrimidine ribonucleosides. Cytidine and uridine behaved almost identically. Both nucleosides were good nucleic acid precursors in the L cell and in the meningopneumonitis agent, and both labeled RNA two to three times more heavily than DNA. Cytosine labeled nucleic acid less efficiently than cytidine. Uracil and thymine were very poor

TABLE 1. *Incorporation of H³-uridine into the nucleic acids of uninfected and infected L cells and the agent of meningopneumonitis*

Measurement	L cells		Meningopneumonitis agent
	Uninfected	Infected	
L cells in 100 ml of culture, total number.....	1.2×10^8	0.95×10^8	230
Meningopneumonitis protein recovered, μg			
H ³ -uridine added, counts per min.....	1.1×10^8	9.1×10^7	2.4×10^5
Incorporation of uridine label, counts per min.....			
Whole cells.....	2.9×10^7	3.4×10^7	0
Schmidt-Thannhauser fractions			
Acid-soluble.....	0.6×10^7		0.5×10^5
DNA.....	0.8×10^7		
RNA.....	1.8×10^7		1.6×10^5

TABLE 2. *Availability of exogenous bases and nucleosides as precursors of nucleic acids in uninfected and infected L cells and in the agent of meningopneumonitis*

Labeled intermediate added	Uninfected L cells			Infected L cells		Meningopneumonitis agent	
	Total incorporation	Relative incorporation into		Total incorporation	Total incorporation	Relative incorporation into	
		DNA	RNA			DNA	RNA
	%	%	%	%	%	%	%
Cytidine.....	100*	27	73	100†	100‡	34	66
Deoxycytidine.....	29	97	3	26	0.9	—	—
Cytosine.....	5.5	33	66	8.6	6.8	34	66
Uridine.....	79	30	70	138	75	24	76
Deoxyuridine.....	1.3	—	—	1.0	1.1	—	—
Uracil.....	2.3	—	—	0.6	0.8	—	—
Thymidine.....	23	98	2	15	0.9	—	—
Thymine.....	0.5	—	—	0.2	0.9	—	—
Adenosine.....	90	70	30	31	34	50	50
Deoxyadenosine.....	340	35	65	244	51	38	62
Adenine.....	450	35	65	316	95	25	75
Guanosine.....	31	23	77	30	30	30	70
Deoxyguanosine.....	115	34	66	26	32	38	64
Guanine.....	23	34	66	15	28	23	77

* This L cell culture contained a total of 10^8 cells and incorporated 28% of the added H³-cytidine. The per cent incorporation of other intermediates in other L-cell cultures was calculated, adjusted for slight differences in L-cell count, and expressed as the percentages of the H³-cytidine incorporation.

† This L-cell culture also contained a total of 10^8 cells and incorporated 28% of the added H³-cytidine (the exact agreement between the two cytidine-containing cultures was fortuitous). Incorporation of other intermediates was expressed as for uninfected cultures.

‡ The infected L-cell culture labeled with H³-cytidine yielded a preparation of purified meningopneumonitis agent which contained 720 μg of protein and 1.1% of the added label. The per cent incorporation of other intermediates into the meningopneumonitis agent was calculated by correcting for differences in amount of agent protein isolated and expressing the incorporation as the percentage of that obtained with H³-cytidine.

nucleic acid precursors in both L cells and meningopneumonitis agent.

Incorporation of pyrimidine deoxyribonucleosides. Thymidine and deoxycytidine were incorporated almost exclusively into the DNA of the L cell. Neither deoxyribonucleoside was signifi-

cantly incorporated into either the DNA or the RNA of the meningopneumonitis agent. This was the only clear-cut difference in the way the meningopneumonitis agent and its L cell host handled exogenous nucleosides and bases. Deoxyuridine was a very inefficient precursor of

nucleic acids in both the L cell and the meningopneumonitis agent. Failure of each pyrimidine deoxyribonucleoside to be incorporated into meningopneumonitis nucleic acids was observed in at least two independent experiments.

DISCUSSION

Adenine, guanine, and their ribo- and deoxyribonucleosides were freely available for the synthesis of both DNA and RNA in the L cell and in the meningopneumonitis agent, as were cytidine and uridine. Of the free pyrimidines, only cytosine was significantly incorporated into the nucleic acids of either host or parasite. The L cell utilized thymidine and deoxycytidine almost exclusively for the synthesis of DNA. All of these observations are in accord with many previous investigations on the precursors of nucleic acids (2, 8).

Neither the L cell nor the meningopneumonitis agent utilized exogenous deoxyuridine for synthesis of either the DNA or RNA. Information on the fate of added deoxyuridine in other cells is meager. In rats, this nucleoside was incorporated into the thymine of DNA (19). It was taken up into the intracellular phase of Ehrlich ascites cells (9), but its incorporation into nucleic acid was not measured. Two different groups of investigators have reported that infection of L cells with pleuropneumonia-like organisms resulted in a complete inhibition of the incorporation of labeled thymidine and uridine (15, 18). The L cells used in these investigations efficiently incorporated these nucleosides, but the possibility that their failure to utilize deoxyuridine was due to contamination with pleuropneumonia-like organisms cannot be ruled out.

The one major difference between the L cell and the meningopneumonitis agent was in their handling of thymidine and deoxycytidine. The L cell, like many other cells and intact animals already studied, used these nucleosides almost exclusively for the synthesis of DNA. The meningopneumonitis agent did not use them at all. Thus, the previously observed (4, 16, 21, 23) failure of thymidine to incorporate into agent DNA appears to have been a reflection of the inability of members of the psittacosis group to utilize any exogenously supplied pyrimidine deoxyribonucleoside. Why they lack this capacity is unknown. Perhaps they do not have the appropriate nucleoside kinases.

Since L cells rapidly convert added thymidine to nucleotides, mainly thymidine triphosphate (3), the meningopneumonitis agent must also be unable to use the thymidine nucleotides made by

its host as well as the exogenously supplied thymidine itself. If this is so, then the thymidine triphosphate for synthesis of meningopneumonitis DNA is probably made by the following series of reactions, all of which occur within the meningopneumonitis cell itself:

Deoxycytidine triphosphate would be synthesized in a comparable series of reactions. This hypothesis requires that the meningopneumonitis cell be impermeable to the triphosphates of thymidine and deoxycytidine and yet able to pirate from its host the adenosine triphosphate (ATP) required for phosphorylation of the nucleosides to the nucleoside triphosphates. This may be because the concentration of ATP in intracellular pools is usually many times higher than that of the deoxynucleoside triphosphates (11). The present data allow no conclusions as to the availability of other nucleotides of the host pool for synthesis of nucleic acids in the meningopneumonitis agent. However, they are sufficient to make untenable the hypothesis (13, 14) that members of the psittacosis group make their nucleic acids from the nucleoside triphosphates of their hosts.

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LITERATURE CITED

1. BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* **1**:279-285.
2. BROWN, G. B., AND P. M. ROLL. 1955. Biosynthesis of nucleic acids, p. 341-392. *In* E. Chargaff and J. N. Davidson [ed.], *The nucleic acids*, vol. 2. Academic Press, Inc., New York.
3. CLEAVER, J. E., AND R. M. HOLFORD. 1965. Investigations into the incorporation of H^3 thymidine into DNA in L-strain cells and the formation of a pool of phosphorylated derivatives during pulse labeling. *Biochim. Biophys. Acta* **103**:654-671.
4. CROCKER, T. T., S. R. PELC, B. I. NIELSEN, J. M. EASTWOOD, AND J. BANKS. 1965. Population dynamics and deoxyribonucleic acid synthesis in HeLa cells infected with an ornithosis agent. *J. Infect. Diseases* **115**:105-122.

5. EAGLE, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science* **130**:432-437.
6. EARLE, W. R. 1943. Production of malignancy *in vitro*. IV. The mouse fibroblast cultures and changes seen in the living cell. *J. Natl. Cancer Inst.* **4**:165-212.
7. GOLUB, O. J. 1948. A single dilution method for estimation of LD₅₀ titers of the psittacosis-LGV group of viruses in chick embryos. *J. Immunol.* **59**:71-82.
8. HARTMAN, S. C., AND J. M. BUCHANAN. 1959. Nucleic acid, purines, pyrimidines (biosynthesis). *Ann. Rev. Biochem.* **28**:365-410.
9. JACQUEZ, J. A. 1962. Transport and enzymic splitting of pyrimidine nucleosides in Ehrlich cells. *Biochim. Biophys. Acta* **61**:265-277.
10. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
11. MANDEL, P. 1964. Free nucleotides in animal tissues, p. 299-334. *In* J. N. Davidson and W. E. Cohn [ed.], *Progress in nucleic acid research and molecular biology*, vol. 3. Academic Press, Inc., New York.
12. MORGAN, J. F., H. L. MORTON, AND R. C. PARKER. 1950. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. *Proc. Soc. Exptl. Biol. Med.* **73**:1-8.
13. MOULDER, J. W. 1962. The biochemistry of intracellular parasitism. Univ. of Chicago Press, Chicago.
14. MOULDER, J. W. 1964. The psittacosis group as bacteria. John Wiley & Sons, Inc., New York.
15. NARDONE, R. M., T. TODD, P. GONZALEZ, AND E. V. GAFFNEY. 1965. Lability of host cell DNA in growing cell cultures due to mycoplasma. *Science* **149**:1098-1099.
16. PELC, S. R., AND T. T. CROCKER. 1961. Differences in the utilization of labeled precursors for the synthesis of deoxyribonucleic acid in cell nuclei and in psittacosis virus. *Biochem. J.* **78**:20P.
17. POLLARD, M., AND N. SHARON. 1963. Induction of prolonged latency in psittacosis-infected cells by aminopterin. *Proc. Soc. Exptl. Biol. Med.* **112**:51-54.
18. RANDALL, C. C., L. G. GAFFORD, G. A. GENTRY, AND L. A. LAWSON. 1965. Nucleoside incorporation into strain L cells: inhibition by pleuropneumonia-like organisms. *Science* **149**:1100-1101.
19. REICHARD, P. 1955. Utilization of deoxyuridine and 5-methyl uridine for the biosynthesis of thymine by the rat. *Acta Chem. Scand.* **9**:1275-1285.
20. SANFORD, K. K., W. R. EARLE, AND G. D. LIKELY. 1948. The growth *in vitro* of single isolated cells. *J. Natl. Cancer Inst.* **9**:229-246.
21. SCHECHTER, E. M. 1966. Synthesis of nucleic acid and protein in L cells infected with the agent of meningopneumonitis. *J. Bacteriol.* **91**:2069-2080.
22. SIMINOVITCH, L., A. F. GRAHAM, S. M. LESLEY, AND A. NEVILL. 1957. Propagation of L strain mouse cells in suspension. *Exptl. Cell Res.* **12**:299-308.
23. STARR, T. J., AND N. SHARON. 1963. Autoradiography with the agents of psittacosis and trachoma. *Proc. Soc. Exptl. Biol. Med.* **113**:912-914.
24. TAMURA, A., AND N. HIGASHI. 1963. Purification and chemical composition of meningopneumonitis virus. *Virology* **20**:596-604.
25. VOLKIN, E., AND W. E. COHN. 1954. Estimation of nucleic acid. *Methods Biochem. Anal.* **1**:287-305.