THE ROLE OF METHIONINE DEFICIENCY IN POLIOVIRUS REPLICATION IN TISSUE CULTURES*, ‡

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Studies on the interaction of nutrition and infections have been reviewed by Scrimshaw, Taylor, and Gordon (1) and Scrimshaw (2). Some viral infections in combination with certain types of nutritional deficiencies are less severe than they would ordinarily be. This type of suppressive interaction of nutrition and infectious diseases is known as "antagonism" and has been shown to occur with poliovirus in experimental infections (3, 4).

The methods of tissue culture offer the possibility of evaluating the specific effect of single nutrients upon viral replication. Previous studies have shown that it is possible to interfere with intracellular growth of viruses in tissue culture by addition of analogues of certain metabolites (5–8). Inhibition of poliovirus by ethionine in primary cultures of monkey testicular cells has been reported by Brown (5) indicating that the amino acid methionine is essential for the synthesis of poliovirus. Ackerman (6) reported the necessity of methionine for influenza virus and Thompson (7) and Morgan (8) showed the same requirement for vaccinia and psittacosis viruses respectively.

It is the purpose of this study to present information on the interaction between methionine deficiency and poliovirus infection in established and primary cell cultures.

Materials and Methods

Cell cultures.—The role of methionine in the cell susceptibility to poliovirus infection was investigated using the methionine analogue, ethionine in human and monkey cell cultures.

HeLa strain derived from human cervical carcinoma (from Microbiological Associates, Bethesda, Maryland) was maintained in Eagle's medium (9) containing 10% calf serum. For experimental work, cells were planted in screw capped tubes at a concentration of 7 to 8 $\times$ 10^4 cells/ml/tube. The cultures were incubated in a stationary position at 36 to 37°C for 2 days to develop cell monolayers.

Primary monkey (Rhesus) kidney cell cultures were prepared by Microbiological Associates. The cells had been planted in Eagle's basal medium (9) containing 5% calf serum and 1% pooled, inactivated monkey and SV_5 (Simian Virus) antiserum. 10-day-old tube cultures were

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received in this laboratory and were incubated for 2 additional days before they were used for experiments.

All culture media contained 100 units/ml penicillin and 100 μg/ml streptomycin.

Compounds.—*l*-Methionine and its analogue *l*-ethionine (A grade preparations from California Corporation for Biochemical Research, Los Angeles) were dissolved in deionized distilled water. The solutions were then adjusted to pH 7.1–7.2 and were sterilized by filtration.

Virus.—Two strains of poliovirus were used in this study: (*a*) Mahoney (type 1), HeLa adapted strain (from Dr. R. Rustigan, Tufts University, Medical School, Boston); and (*b*) Lansing (type 2) adapted in human amnion cells (from Dr. J. F. Enders, Childrens' Hospital, Boston).

The stock pools of both virus strains were prepared in HeLa cultures and their infectivity titers were determined by the tube titration method, Merchant *et al.* (10). Virus-infected cultures were examined microscopically daily and were scored according to the progressive cytopathogenic changes: none, −; virus-induced morphological changes of less than 25% of the cells, ±; definite morphological change of 25% of the cells, 1+; 25 to 50% cellular degeneration, 2+; 50 to 75% cellular destruction, 3+; and complete cellular destruction, 4+. The highest dilution of virus-producing infection in 50% of the cultures was designated as the $TCID_{50}$ and was calculated by the method of Reed and Muench (11).

The $TCID_{50}$ for the Mahoney strain was $10^{-6.5}$/0.1 ml and for the Lansing strain $10^{-6.2}$/0.1 ml.

Test Procedures.—The effect of *l*-ethionine on the growth of poliovirus, as well as on the growth of the cell cultures, was studied in two differently pretreated cultures:

Ethionine-treated cells: When cell monolayers had developed, the medium was removed and replaced with complete Eagle's medium containing 2.0×10^{-3} moles and 4.0×10^{-3} moles ethionine. The cultures were then reincubated for 24 hr prior to poliovirus inoculation. The medium was removed again and replaced with fresh medium containing the same levels of ethionine and then 0.1 ml of a virus dilution containing 16 to 31 $TCID_{50}$ was added. The cultures were examined microscopically daily for 3 to 4 successive days to determine the cytopathogenicity. Each day, the virus-containing medium was harvested and pooled from identical cultures for titration of viral progeny. After every harvesting procedure, fresh medium containing the same level of the analogue was added to the culture tubes.

Depleted cells: To deplete the cells of their intracellular amino acids and other nutrients, cell monolayers were washed twice with Hanks' balanced salt solution and then exposed to 1 ml of Hanks' salt solution at 36 to 37°C for 6 hr (12). After the depletion the salt solution was removed and replaced with medium 199 (13) without serum containing 2.0×10^{-3} moles ethionine. At the same time the cultures were infected with 0.1 ml of a virus dilution containing 60 $TCID_{50}$. Cultures were observed in the manner described above. For the titration of the viral progeny, the medium was pooled at 6, 12, 24, and 48 hr after virus inoculation. Also, at each time interval, cultures received fresh medium containing the same level of ethionine. All viral pools were titrated in HeLa cells.

Cytotoxicity Tests.—Parallel to the virus experiments, sets of identical cultures were incubated without virus to study the effect of ethionine on cell growth during the same incubation periods. The cytotoxicity was evaluated by changes in cell morphology and by growth inhibition. The growth was expressed as increase of cell protein per culture. To measure protein, cell monolayers were washed twice with balanced salt solution to remove traces of protein present in the test medium. The washed cells were then dissolved in Lowry's solution and the protein content was determined with the Folin-Ciocalteau reagent according to the method of Oyama and Eagle (14).

RESULTS

The Cytotoxicity of Ethionine.—When *l*-ethionine was added in graded concentrations to HeLa and monkey kidney cultures, the growth of cells was inhibited. The growth inhibition of both cultures was proportional to the test

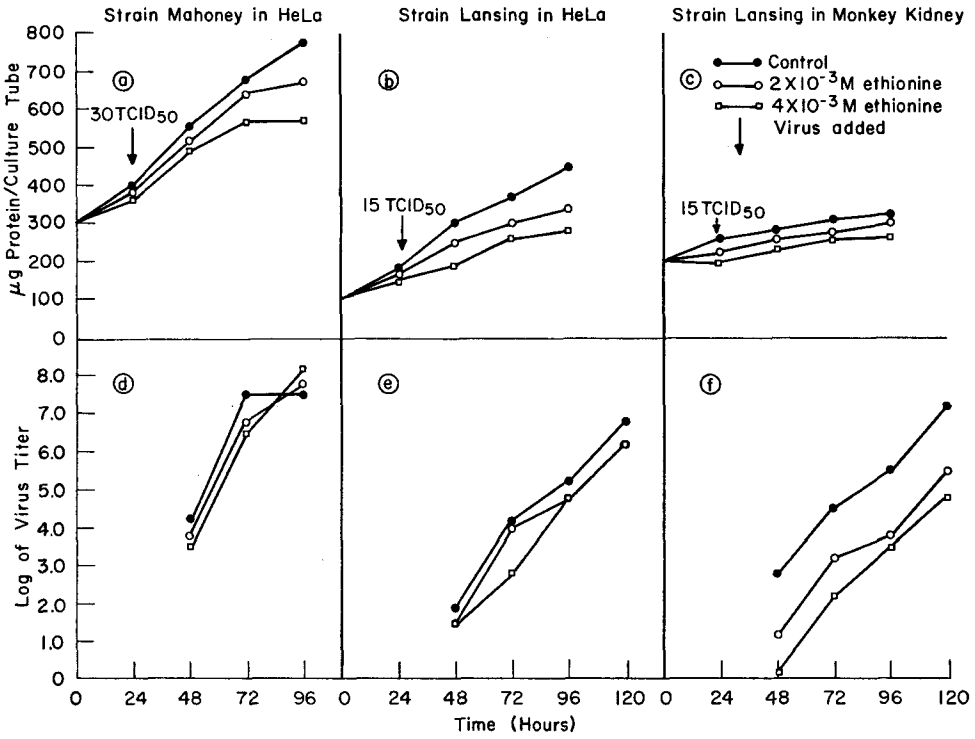

FIG. 1. Effect of *l*-ethionine on the growth of HeLa cells (*a*) and (*b*), and monkey kidney cells (*c*). Cells were exposed to ethionine for a period of 4 to 5 days. In the same figure, (*d*), (*e*), and (*f*) show the effect of ethionine on poliovirus synthesis in the corresponding cell cultures.

concentrations and the length of incubation. The results are illustrated with growth curves of cultures, shown in Figs. 1 *a* to 1 *c*.

In the presence of 2.0×10^{-3} moles ethionine for 96 hr, both cell cultures remained normal morphologically, but the growth of HeLa cells was reduced 10 to 20%, and monkey kidney cells 20 to 30%. A more pronounced inhibition of growth was evident at a concentration of 4.0×10^{-3} moles ethionine, which induced also a slight granulation of the cells and a growth depression of ap-

proximately 30 to 40% in HeLa (Figs. 1 *a* and 1 *b*) and 40 to 50% in monkey kidney cultures (Fig. 1 *c* and Fig. 3 *a*).

In previously depleted HeLa cells 2.0×10^{-3} moles ethionine inhibited the growth significantly more than it did in the normal, nondepleted cells. The results are shown in Fig. 2 *a*. It is evident that during the first 24 hr of incubation the growth was inhibited completely; however, at the end of 48 hr there was a slight growth, but 80% less than in the normal culture (nondepleted without ethionine).

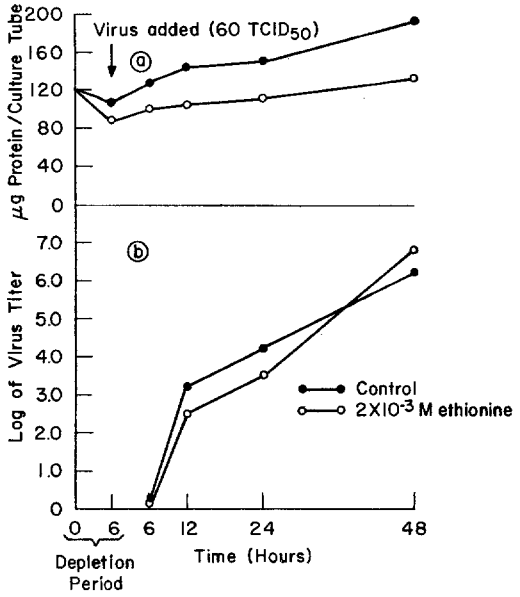

FIG. 2. Mahoney strain in depleted HeLa cells. Effect of *l*-ethionine on the growth of depleted HeLa cells and on the synthesis of poliovirus in medium 199.

Effect of Ethionine on Poliovirus Infection.—(*a*) Cell susceptibility to the lethal action of virus is shown in Table I. The extent of cytopathogenicity in the ethionine-treated HeLa cells did not differ significantly from the control cells; however, in the monkey kidney cultures, cells degenerated slower than in the controls. 96 hr after infection, the ethionine-treated monkey kidney cell cultures showed a very slight degree of cytopathogenicity, whereas the corresponding control cultures were completely destroyed.

(*b*) Biosynthesis of poliovirus was investigated by titration of the viral progeny harvested from the ethionine-treated and the corresponding control cultures 24, 48, and 96 hr after infection. The results are expressed as the logarithms of the virus titers and are summarized in Figs. 1 *d*, to 1 *f*. It is evident that the virus yields of both polio strains, Mahoney and Lansing, were

reduced in the presence of ethionine. Generally, the reduction of virus yield was proportional to the applied concentration of ethionine and the length of exposure. In HeLa cells ethionine inhibited Mahoney and Lansing strain viruses very slightly, the differences in the $TCID_{50}$ titers of the ethionine-treated cultures and those of the control cultures did not exceed one logarithm. Also, the results from depleted HeLa cells infected with the Mahoney strain

TABLE I

Cytopathogenic Effect of Poliovirus in l-Ethionine Pretreated HeLa and Monkey Kidney Cultures

Cell culture and virus	After infection	Concentration of *l*-ethionine in medium		
		–	2.0×10^{-3}	4.0×10^{-3}
	hr		M	M
Mahoney strain in HeLa	24	±	–	–
	48	++	++	+
	72	++++	++++	++++
Lansing strain in HeLa	24	–	–	–
	48	±	–	–
	72	+	++	+
	96	+++	++++	++++
Lansing strain in monkey kidney	24	–	–	–
	48	±	–	–
	72	++	±	±
	96	++++	+	+

Degree of cytopathogenicity:
±, Slight morphological changes.
+, Definite morphological changes in 25% of cells.
++, 25 to 50% cellular degeneration.
+++, 50 to 75% cellular degeneration.
++++, Complete cellular destruction.

in the presence of 2.0×10^{-3} moles ethionine showed no significant inhibition of viral biosynthesis, as illustrated in Fig. 2.

In contrast to the results obtained with HeLa cells, the primary monkey kidney cells showed a marked inhibition of viral biosynthesis in the presence of ethionine. The results with poliovirus Lansing strain are summarized in Fig. 1 *f*. In the presence of 4.0×10^{-3} moles ethionine the virus titer was reduced by 2 to 2.5 log and at 2.0×10^{-3} moles ethionine, by 1.0 to 1.5 log. The presented results of reduced viral synthesis correlated well with the results of the cytopathogenicity which also indicated a reduced rate of cell degeneration.

(*c*) To prove that the inhibition of cell growth and poliovirus syntheses by *l*-ethionine was due to its interference with the utilization of methionine, at-

tempts were made to reverse the inhibitory effects of *l*-ethionine by addition of graded concentrations of *l*-methionine.

The results are shown in Figs. 3 *a* and 3 *b*, and indicate that 6.0×10^{-3} moles methionine reversed partly the inhibitory effects of 4.0×10^{-3} moles ethionine.

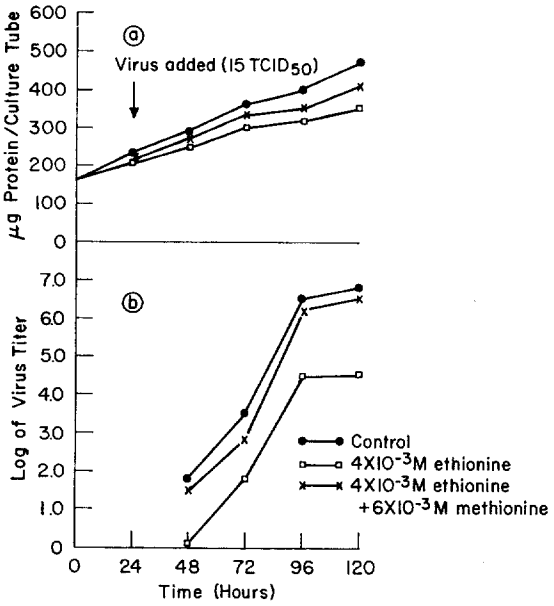

FIG. 3. Lansing strain in monkey kidney cells. Reversal effect of methionine upon the inhibitory effect of *l*-ethionine, on the growth inhibition of monkey kidney cells (*a*) and on the inhibition of poliovirus synthesis (*b*).

DISCUSSION

The results of this study have demonstrated that *l*-ethionine inhibits the growth of HeLa and primary monkey kidney cells. Since the inhibitory effect on cell growth was reversed by addition of an excess of methionine, this indicates that ethionine interferes with the utilization of methionine, an amino acid essential for the growth of cell cultures (12, 15, 16). The fact that cultures containing ethionine continued to grow at a reduced rate appears to indicate that the analogue of methionine at the concentration used in the experiments induced some nutritional deficiencies.

The inhibitory effect of *l*-ethionine upon poliovirus replication was found to be different in HeLa and monkey kidney cell cultures. A marked inhibition of viral biosynthesis was observed in monkey kidney cultures, whereas under the same conditions, no significant inhibitory effect was seen in HeLa cultures.

Previous studies have shown that the amino acid pool of HeLa cells is essential for the propagation of poliovirus (17, 18) and, accordingly, the differences in the capacity of the cells to synthesize virus may be due to differences in the magnitude of their intracellular amino acid pool. The amino acid pool is sufficient to support viral replication provided glutamine and glucose are present, as reported by Darnell and Eagle (19). In agreement with their results, our studies also showed that depleted cells in the presence of ethionine can support the replication of poliovirus.

The observed interference of ethionine with poliovirus biosynthesis in moneky kidney cells and the reversal of the inhibition by addition of methionine is in agreement with the findings of Brown (5) who also demonstrated the necessity of methionine for the synthesis of poliovirus in primary monkey testicular cell cultures.

Under optimal nutritional conditions, the poliovirus infectivity titers obtained in HeLa and monkey kidney cell cultures are approximately the same. Thus, the observed differences between the responses of the two cultures with the nutritional deficiency imposed by depletion of intracellular amino acids and by the presence of ethionine may be due to: (*a*) the difference in the size of their intracellular amino acid pool, which appears to be essential for poliovirus synthesis; or (*b*) to the differences in the metabolic pathway of methionine in each type of cell. More experimental evidence is needed to evaluate these two possibilities.

SUMMARY

The role of methionine in poliovirus infection in HeLa and monkey kidney cells was investigated by using the methionine analogue *l*-ethionine. In the presence of 2.0×10^{-3} and 4.0×10^{-3} moles ethionine, the growth of HeLa and monkey kidney cells was significantly inhibited. Under the same experimental conditions, ethionine had no significant effect on the biosynthesis of two strains of poliovirus (Mahoney and Lansing) in HeLa cells, whereas in primary monkey kidney cells, it markedly inhibited the biosynthesis of the Lansing strain of poliovirus. HeLa cells partly depleted of their intracellular amino acids did not change the rate of viral biosynthesis.

The inhibitory effect of ethionine on cell growth and viral biosynthesis was reversed by addition of an excess of *l*-methionine.

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